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T H È S E

Pour obtenir le grade de **DOCTORAT d'AIX-MARSEILLE UNIVERSITÉ**

Spécialité : Oncologie, Pharmacologie et Thérapeutique

CARACTERISATION MOLECULAIRE DES SYNDROMES MYELOPROLIFERATIFS NON LEUCEMIE MYELOIDE CHRONIQUE

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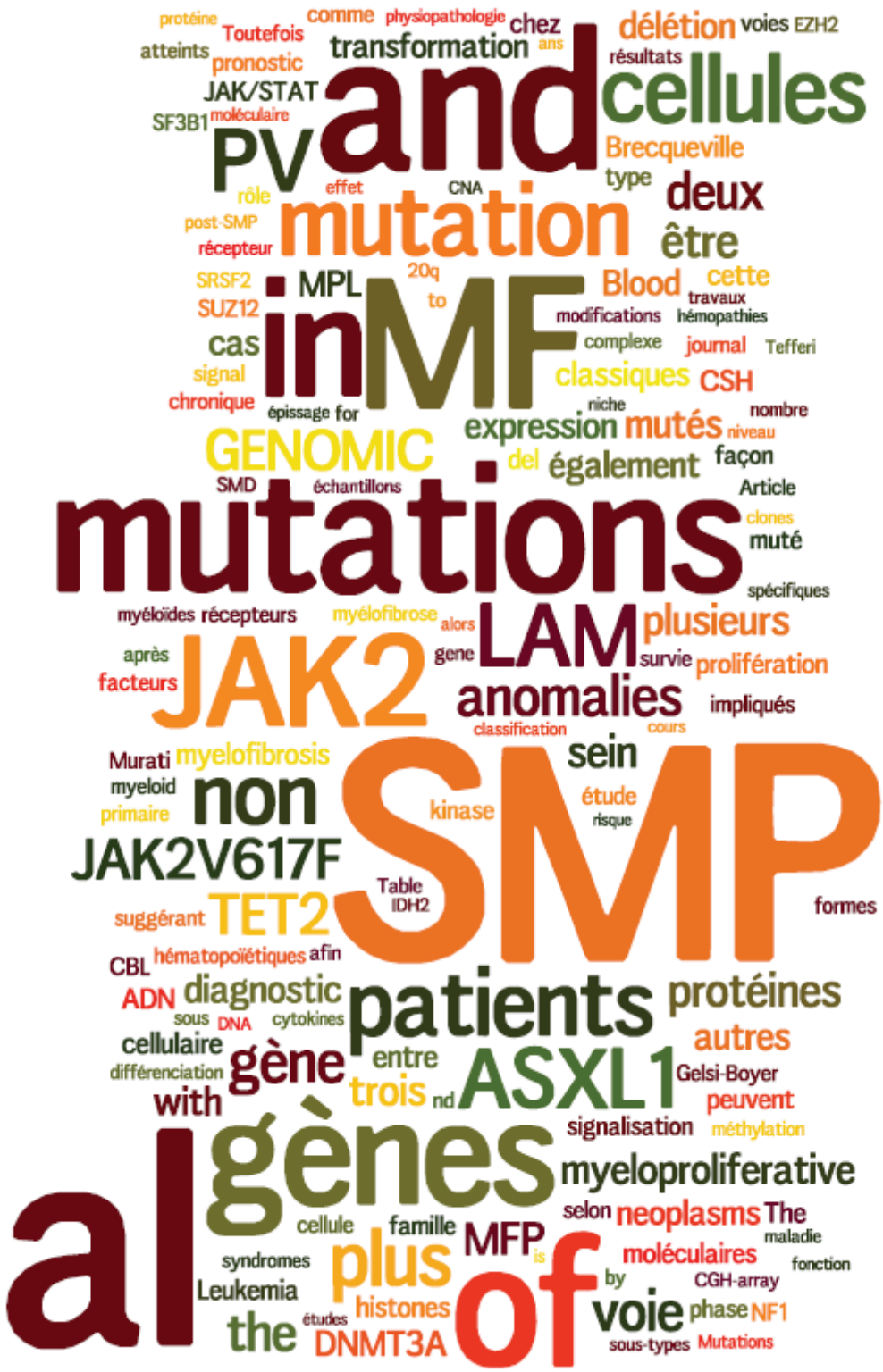

Wordle fait à partir de ce manuscrit

A mes proches qui m'ont toujours soutenue

et que j'aime de tout mon cœur

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Avant-propos

Ce travail de thèse a été dirigé dans l'équipe d'Oncologie Moléculaire sous la direction conjointe des Drs Anne Murati et Daniel Birnbaum.

Les syndromes myéloprolifératifs (SMP) sont des hémopathies myéloïdes clonales affectant la cellule souche hématopoïétique (CSH). Les SMP classiques non leucémie myéloïde chronique (LMC) regroupent la polyglobulie de Vaquez (PV), la thrombocytémie essentielle (ET) et la myélofibrose (MF). Leur diagnostic a longtemps été difficile, en raison des nombreuses similitudes entre ces trois pathologies et de l'absence de marqueurs de clonalité. En 2005/2006, celui-ci a été révolutionné par la découverte des mutations des gènes *JAK2* (2005) et *MPL* (2006) dans les SMP classiques non LMC. La recherche de la mutation *JAK2* (exon 14 (V617F) est un critère pour le diagnostic de SMP non LMC, puisque la mutation est présente dans 97 % des PV et environ la moitié des TE et MF. De même, la recherche des mutations du gène *MPL* peut également être recherchée pour les patients au diagnostic difficile (JAK2V617F négatif), puisque la mutation est présente dans 1 à 5% des TE et 5 à 10% des MF. Bien que ces découvertes aient amélioré le diagnostic, une partie non négligeable des patients atteints de SMP, environ la moitié des patients TE et MF, n'ont pas de mutation pour ces deux gènes.

Afin d'améliorer le diagnostic et le pronostic des SMP, la poursuite de la caractérisation moléculaire continue d'être indispensable. Une meilleure connaissance de ces anomalies moléculaires devrait permettre d'évoluer vers une nouvelle classification et de développer des thérapies ciblées.

Le travail présenté dans cette thèse de **« caractérisation moléculaire des syndromes myéloprolifératifs non leucémie myéloïde chronique »** a débuté en 2009 dès mon stage de master 1, s'est poursuivi en master 2 puis en thèse.

L'**introduction** sera consacrée à la description générale de l'hématopoïèse et des acteurs impliqués. Je présenterai les SMP selon la classification OMS actuelle, puis je m'attarderai davantage sur le diagnostic des SMP classiques non LMC.

Dans la partie intitulée **présentation des travaux**, je ferai un état des lieux des connaissances moléculaires des SMP en 2009 (début de mes travaux). J'exposerai mes objectifs et les démarches associées.

Puis dans la partie **résultats**, je présenterai mes publications et les commentaires associés. Les articles 1-5 concerneront les trois sous-types : PV, TE et MF. L'article 6 sera consacré à l'étude des myélofibroses primaires et secondaires (MF post-PV/TE). Je présenterai également les derniers résultats obtenus mais non publiés sur les leucémies aiguës myéloïdes secondaires aux SMP, ainsi que l'étude préliminaire des profils d'expression génique des PV, TE et MF.

Je terminerai par une **discussion générale,** suivi des **perspectives**.

En fin, les articles 7-10 dans lesquels j'ai participé, mais ne faisant pas partie de mes travaux sur « la caractérisation moléculaire des SMP » seront placés en **Annexes**.

Liste des abréviations

aa : acides aminés
ALK : Activines receptor Like Kinase
ASXL1 : Additional Sex comb Like 1
aUPD : Disomie Uniparentale acquise
BAC : Bac Artificial Chromosome
BCR : Breakpoint Cluster Region
BFU-E : Burst Forming Unit-Erythroid
BMP : Bone Morphogenetic Protein
CBL : Casitas B-lineage lymphoma
CFU-E : Colony Forming Unit-Erythroid
CSF : Colony Stimulating Factor
CSH : Cellule Souche Hématopoïétique
CSL : Cellule Souche Leucémique
DNMT3A : DNA methyltransferase 3 alpha
EPO : Erythropoïétine
EZH2 : Enhancer of Zeste Homolog 2
FC : Facteurs de Croissance
FLT3 : Fms-related tyrosine kinase 3
FT : Facteur de Transcription
G-CSF : Granulocyte-CSF
GDF : Growths Differenciation Factors
GM-CSF : Granulocyte Macrophage-CSF
Hb : Hémoglobine
Ht : Hématocrite
IDH : Isocitrate Deshydrogénase
IFN : Interferon
IL : Interleukine
JAK : Janus kinase
JH : JAK homology
KO : Knock-out
LAM : Leucémie Aiguë Myéloïde
LMC : Leucémie Myéloïde Chronique
LMMC : Leucémie myélo-monocytaire chronique
LMMJ : Leucémie myélo-monocytaire juvénile
LNK : Linker of T-cell receptor pathways
LOH : Loss Of Heterozygosity
MAPK : Mitogen-Activated Protein Kinases
MF : Myélofibrose
MFP : Myélofibrose Primaire
MPL : Myeloproliferative Leukemia
NF1 : Neurofibromin 1
OMS : Organisation Mondiale de la Santé
PPP1R16B : Protein Phosphatase 1, Regulatory subunit 16B
PRV1 : Polycythemia rubra vera
PTPN11 : Protein Tyrosine Phosphatase, non-receptor type 11
PV : Polyglobulie de Vaquez
RCK : Récepteur à Cytokines
RTK : Récepteur Tyrosine Kinase
SCF : Stem Cell Factor
SF3B1 : Splicing Factor 3b, subunit 1
SMD : Syndrome Myélodysplasique
SMP : Syndrome Myéloprolifératif
SOCS : Suppressor of cytokine signaling
SRSF2 : Serine/arginine-rich Splicing Factor 2
STAT : Signal Transducer and activator of Transcription
SUZ12 : Suppressor of Zeste 12
TE : Thrombocytémie Essentielle
TET2 : Ten-Eleven Translocation 2
TK : Tyrosine Kinase
TGFβ : Transforming Growth Factor β
TNFα : Tumour Necrosis factor
TPO : Thrombopoïétine
TRPS1 : Trichorhinophalangeal Syndrome

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Introduction

A- L'HEMATOPOÏESE

1. GENERALITES

Les cellules sanguines matures ont une capacité de prolifération nulle et des durées de vie limitées (120 jours pour les érythrocytes, 7 jours pour les plaquettes et 24 heures pour les polynucléaires), pourtant leur nombre reste constant. L'hématopoïèse est l'ensemble des phénomènes qui concourent à la fabrication et au remplacement continu et régulé des cellules sanguines (production quotidienne de 10^{11} à 10^{12} cellules matures par jour). Ce sont les cellules souches hématopoïétique (CSH) qui vont être capable de donner naissance à tous les types de cellules sanguines (**Figure 1**). Ce renouvellement est assuré par plusieurs facteurs intervenant dans la signalisation, l'épigénétique et l'épissage. Chez l'homme adulte, l'hématopoïèse n'a lieu que dans la moelle osseuse des os courts et des os plats, lieu de la niche hématopoïétique.

Plusieurs étapes sont nécessaires à l'hématopoïèse (de la CSH aux cellules matures) :

i) **Les CSH**, multipotentes, ont des propriétés d'auto-renouvellement et de différenciation vers toutes les lignées hématopoïétiques, qui sous l'action de facteurs de transcription (FT) et facteurs de croissance (FC) se transforment en progéniteurs.

ii) **Les progéniteurs**, sont des cellules indifférenciées avec un haut pouvoir mitotique qui s'engagent dans une voie de différenciation et perdent peu à peu leur pouvoir mitotique.

iii) **Les précurseurs** sont morphologiquement identifiables au microscope, dépourvus de capacités d'auto-renouvellement. En cours de maturation, ils se divisent puis acquièrent les marqueurs spécifiques d'une lignée pour devenir cellules matures.

iv) **Les cellules matures** sont la finalité de l'hématopoïèse et passent dans le sang.

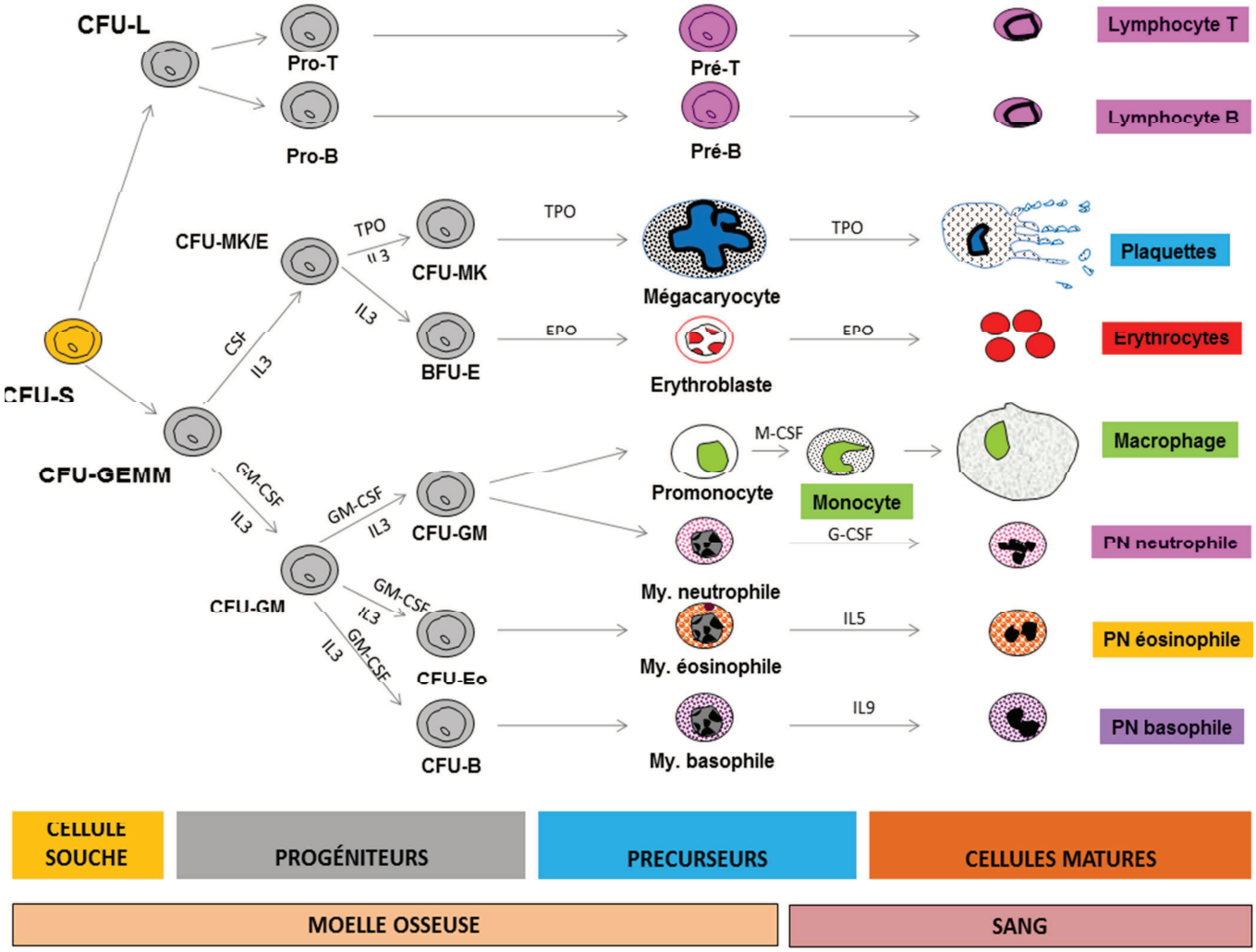

Figure 1 : Schéma général de l'hématopoïèse

2. TRANSCRIPTION et REGULATION DE L'HEMATOPOIESE

Les FT agissent sur l'activité de gènes régulateurs des lignées hématopoïétiques. Les FT peuvent réguler de façon négative ou positive la différenciation cellulaire. Les principaux facteurs de transcription de la lignée myéloïde sont GATA1, GATA2, TAL1, FOG1, GFI1, GFI1B, EKLF, MYB, SP1, le complexe NF-E2, PU.1, CEBPα/ε, MAF, RUNX1, FLi1, $P45^{NF\text{-}E2}$. Les principaux FT de la lignée lymphoïde sont GATA3, Ikaros (IKZF1), PU.1, E2A, EBF, PAX5.

3. SIGNALISATION et REGULATION DE L'HEMATOPOIESE

3.1. Les facteurs de croissance

Les facteurs de croissance (FC) sont des glycoprotéines solubles agissant localement à très faible dose. Leur rôle est de favoriser ou d'inhiber la croissance cellulaire. Ils sont produits *de novo* par le stroma cellulaire, les lymphocytes et les monocytes à l'exception de l'érythropoïétine (EPO) et de la thrombopoïétine (TPO) produites par le rein et le foie. Schématiquement, les FC à régulation positive sont de trois types car agissent à différents temps au cours de l'hématopoïèse :

i) **Non spécifiques d'une lignée, du temps :** *IL3* (interleukine) et le *GM-CSF* (Granulocyte Macrophage-Colony Stimulating Factor) préparent les cellules à recevoir un deuxième signal de prolifération plus spécifique (*G-CSF*, *M-CSF*, *EPO*, *TPO*, *IL5*).

ii) **Précoces :** FLT3L, SCF (Stem cell factor), les cytokines de la famille de l'IL6 (IL6, LIF, IL11, oncostatine-M, cardiotrophine-1), IL12, IL1 et IL4 (facteurs synergiques)

iii) **Tardifs :** G-CSF, M-CSF, EPO, TPO et IL5

A noter que la TPO et le G-CSF peuvent agir précocement; l'IL6 et le SCF peuvent agir tardivement (sur les lignées mégacaryocytaire et mastocytaire, respectivement). Les FC à régulation négative bloquent la prolifération par inhibition de la transition G1/S au niveau du cycle cellulaire. Les principaux FC sont : les interférons (IFN) qui ont un effet inhibiteur sur la croissance des CFU-GM (Colony Forming Unit-Granulo-Macrophagique), BFU-E (Burst Forming Unit-erythroid) et CFU-GEMM (Granuleuse, Erythrocytaire, Macrophage et Mégacaryocytaire) ; le TGFβ (Transforming Growth Factor β) dont l'action varie en fonction de l'état de différenciation des cellules cibles et de l'interaction avec d'autres cytokines ; le TNFα (Tumor Necrosis Factor α) ; la MIP1α (Macrophage Inflammatory Protein 1 α) ; les peptides hémorégulateurs : le tétrapeptide AcSDKP et le pentapeptide pEEDCK (AcSDKP inhibe la mise en cycle de progéniteurs BFU-E et CFU-GM) ; le PF4 (platelet factor) qui inhibe la mégacaryocytopoïèse précoce.

3.2. Les récepteurs des facteurs de croissance et cytokines

Les récepteurs membranaires de nombreuses cytokines et de FC hématopoïétiques regroupent les récepteurs hormonaux (GH, PRL, CNTF), les récepteurs du GM-CSF, du G-CSF, du LIF, de l'EPO, de l'oncostatine M et les récepteurs de FC hématopoïétiques (IL2, IL3, IL9…). Chaque FC possède son récepteur et chaque cellule compte en moyenne 1000 récepteurs de plusieurs types. Il existe deux familles de récepteurs : les récepteurs à activité tyrosine kinase (RTK) et les récepteurs des cytokines (RCK) dépourvus d'activité tyrosine kinase.

3.2.1. Les récepteurs à activité tyrosine kinase

Les RTK ont la propriété de s'homo-dimériser ou de s'hétéro-dimériser, entrainant la phosphorylation de tyrosines situées sur la partie intracellulaire des sous-unités et sur des seconds messagers. Les RTK sont répartis en 20 familles basées sur la séquence du domaine kinase. Les RTK exprimés dans les cellules hématopoïétiques sont les RTK de classes I, II, III, IV, V, VII, XI et XIV (**Figure 2**).

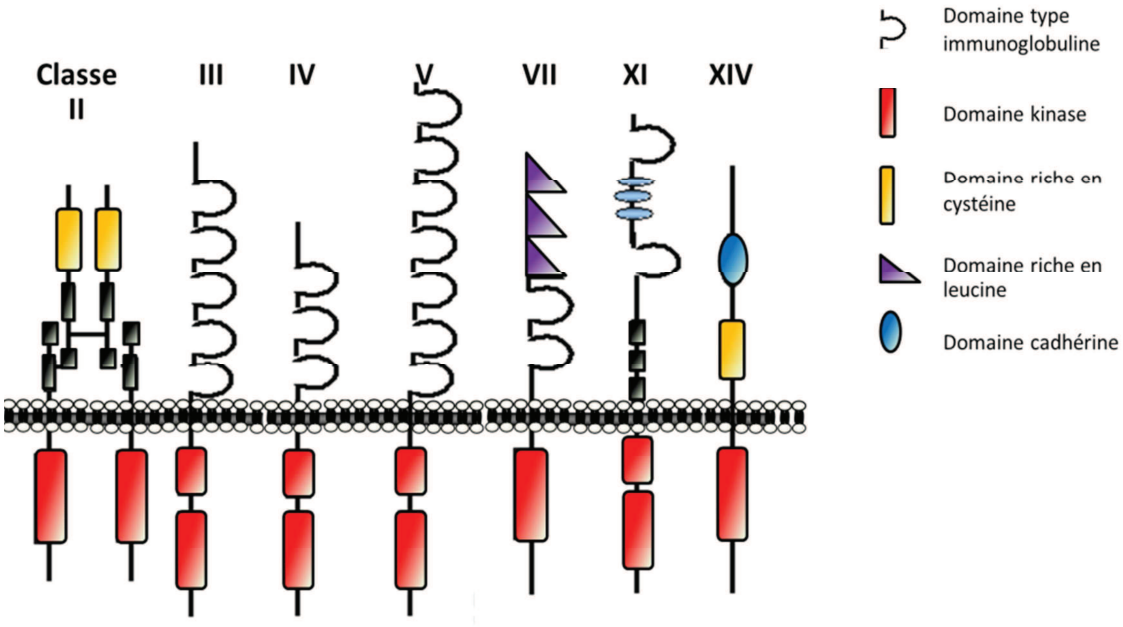

Figure 2 : Schéma des récepteurs tyrosine kinase à la surface des cellules hématopoïétiques

Les RTK de classes I, II, III, IV, V, VII, XI et XIV sont composés d'un domaine extracellulaire généralement glycosylé (avec un domaine de liaison au ligand), d'un domaine transmembranaire (une simple hélice), d'un domaine cytosolique d'environ 100 acides aminés possédant une activité protéine tyrosine kinase intrinsèque.

Les principaux RTK sont : le récepteur à l'insuline (type II) ; PDGFRA/B (Platelet-derivated Growth Factor) (type III) ; KIT, récepteur du SCF (type III) ; FMS : récepteur du M-CSF (Macrophage-CSF) (type III) ; FLT3 ou CD135 : récepteur de FLT3L (Fms-like tyrosine kinase 3-Ligand) (type III) ; FGFR1/2/3/4 : récepteurs des FGF (Fibloblast Growth Factor Receptor) (type IV) ; VEGFR1/2/3 (Vascular Endothelial Growth Factor) (type V) ; NTRK1/2/3 (neutrophic tyrosine kinase) (type VII) ; TIE et TEK : récepteur TIE (tyrosine kinase with Ig and EGF homology domain) (type XI) ; RET (rearranged during transformation) (type XIV).

3.2.2. Les récepteurs des cytokines

Les RCK n'ont pas d'activité catalytique intrinsèque et la plupart transduisent le signal via les protéines JAK. Les RCK sont composés d'un domaine extracellulaire (environ 200 acides aminés)(aa)) comportant 4 résidus cystéine conservés et un motif WSXWS (tryptophane-sérine-X-tryptophane-sérine), d'un domaine transmembranaire (environ 20 aa) et d'un domaine intracellulaire comportant les domaines BOX1-2 responsable de la transduction du signal (**Figure 3**). Les RCK peuvent être homodimériques (ex : EPO-R, TPO-R/MPL, G-CSF-R) ou hétérodimériques (ex : GM-CSF-R, IL3, IL5, IL6). A noter que les récepteurs aux IFN et à l'IL10 ne possèdent pas le motif WSXWS.

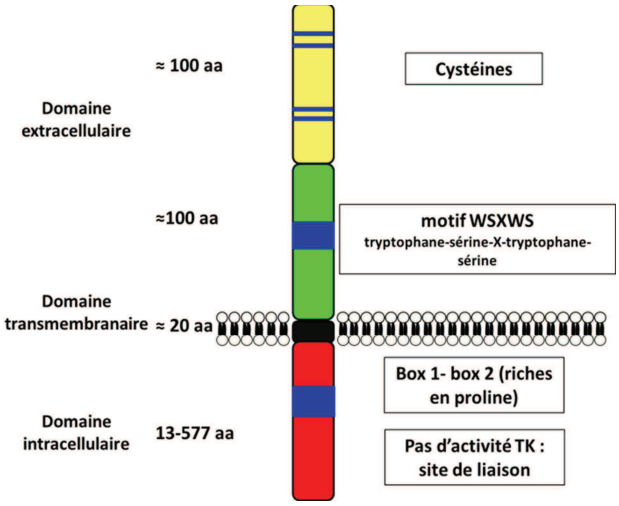

Figure 3 : Schéma de la structure commune aux récepteurs des cytokines

Les RCK sont composés d'un domaine extracellulaire (environ 200 acides aminés)(aa)) comportant 4 résidus cystéine conservés et un motif WSXWS (tryptophane-sérine-X-tryptophane-sérine), d'un domaine transmembranaire (environ 20 aa) et d'un domaine intracellulaire comportant les domaines BOX1-2 responsable de la transduction du signal.

3.2.3. *Cas particulier du récepteur de la thrombopoïétine : MPL*

MPL est le récepteur de la TPO. C'est un homodimère transmembranaire appartenant à la famille des RCK. Ce récepteur est exprimé par les progéniteurs hématopoïétiques, au long de la différenciation mégacaryocytaire (Debili *et al.* 1995). Il est exprimé par les mastocytes, les cellules dendritiques et les cellules endothéliales (Migliaccio *et al.* 2007). Le récepteur MPL et son ligand TPO ont un rôle important dans la survie et la prolifération puisque la fixation de la TPO active les voies RAS/MAPK, PI3K/AKT et JAK/STAT (Majka *et al.* 2002).

3.3. Les voies de signalisation JAK/STAT, RAS/MAPK et SMAD

3.3.1. *Transduction du signal par la voie JAK/STAT*

La transmission du signal JAK/STAT repose sur l'activation, à partir de la membrane cellulaire, des protéines STAT présentes sous leur forme inactive dans le cytoplasme (**Figure 4**). Cette activation permet aux protéines STAT de migrer dans le noyau où elles jouent le rôle de protéines activatrices de la transcription. Cette voie est largement employée par les membres de la famille des cytokines (ex : G-CSF, EPO, TPO, IFN et autres IL) au travers des PTK cytoplasmiques telles que les membres de la famille des protéines JAK.

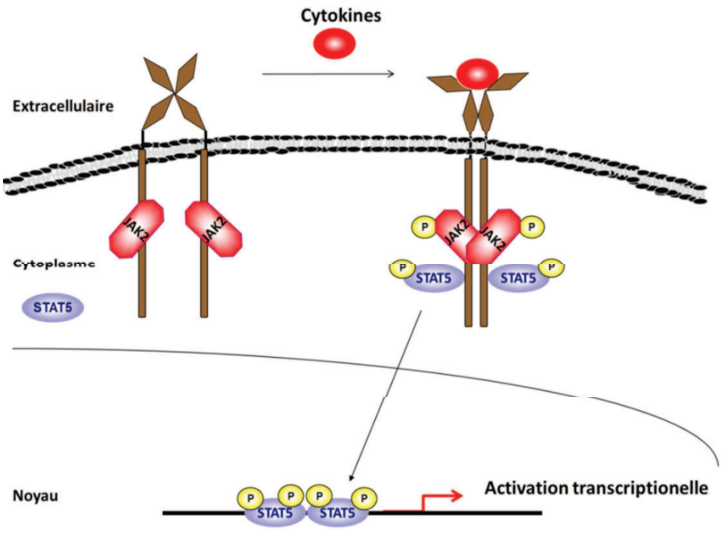

Figure 4 : Transduction du signal par la voie JAK/STAT en réponse aux cytokines

D'après (James, Ugo, Casadevall, *et al.* 2005). La voie JAK/STAT est activée par la liaison d'une cytokine à un récepteur membranaire tel que EPO-R, MPL responsable de l'homodimérisation du récepteur. La phosphorylation des protéines JAK2 et STAT5 participent à la transcription des gènes impliqués dans la survie et dans la différenciation cellulaire.

3.3.1.1. Les protéines JAK

La famille des protéines JAK (Janus kinase ou Just Another kinase) est composée de 4 membres : JAK1, JAK2, JAK3 et TYK2. *Le terme de « Janus kinase » fait référence au dieu romain JANUS représenté par un visage à deux faces qui était le dieu des portes. Les deux faces du Dieu Janus pouvant faire allusion aux deux domaines de phosphorylation de la protéine JAK2. Le terme de « Just Another kinase » est un jeu de mots britannique qui signifie «simplement une autre kinase ».*

L'expression de JAK3 est restreinte aux cellules hématopoïétiques (cellules NK, thymocytes, cellules lymphoïdes T et B, cellules myéloïdes) alors que celles de JAK1, JAK2 et TYK2 est ubiquitaire (Gurniak and Berg 1996). Entre les espèces, sept domaines structuraux sont conservés : les domaines JH (Jak Homology). Le domaine JH1 est le seul à posséder une activité kinase, le domaine JH2 a une activité d'auto-inhibition ou pseudo-kinase, JH3 et JH4 ont des structures très proches de celle des domaines SH2, les domaines JH5-JH7 (domaine FERM) sont responsables de l'association de la partie intra-cytoplasmiques des récepteurs hématopoïétiques, des cytokines et des FC (**Figure 5**).

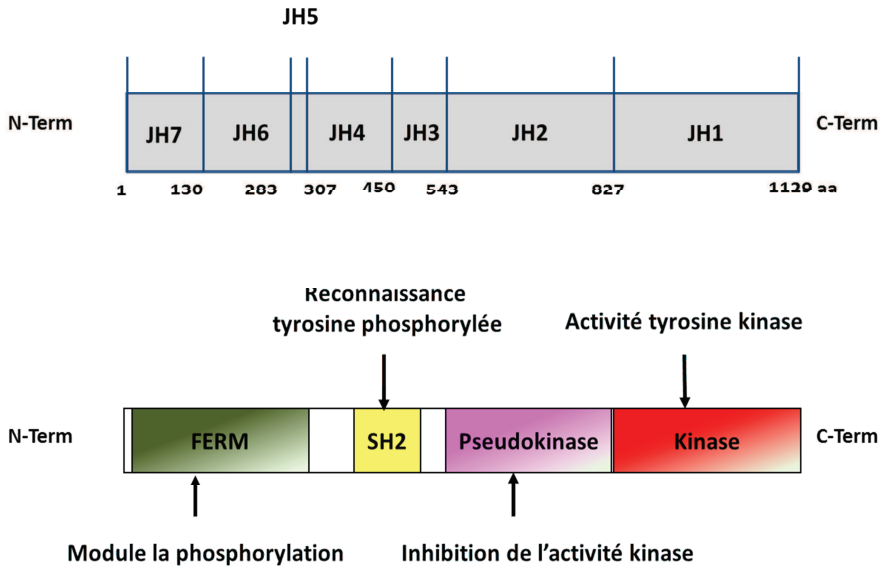

Figure 5 : Structure de la protéine JAK2

D'après (James, Ugo, Casadevall, *et al.* 2005). La protéine JAK2 possède 4 domaines fonctionnels : le domaine FERM, le domaine SH2, le domaine pseudo-kinase, et le domaine kinase à activité catalytique. Le domaine FERM module la phosphorylation, le domaine SH2 est le site de reconnaissance des tyrosines phosphorylées.

3.3.1.2. Les protéines STAT

Les protéines STAT (signal transducers and activators of transcription) comprennent sept membres (1, 2, 3, 4, 5A, 5B, 6) et sont à la fois des transmetteurs du signal et des activateurs transcriptionnels (**Figure 6**). A l'arrivée du signal d'une cytokine à la membrane plasmique, les protéines STAT au repos dans le cytoplasme sont phosphorylées par les tyrosine kinases JAK. Elles se rassemblent sous forme de dimères et migrent dans le noyau où elles stimulent la transcription de gènes cibles spécifiques. Leur phosphorylation sur des résidus sérine/thréonine serait un facteur supplémentaire d'optimalisation de leur fonction activatrice de la transcription. Contrairement au facteur STAT1 dont la translocation nucléaire n'intervient qu'après phosphorylation sur tyrosine, le facteur STAT2 non-activé peut aussi être nucléaire. Le facteur STAT3 semble entrer et sortir du noyau de manière constitutive et indépendamment de son statut de phosphorylation (Liu *et al.* 2005). Après sa phosphorylation, STAT5B s'accumule dans le noyau selon le même mécanisme que le facteur STAT1 (Zeng *et al.* 2002). A l'instar de la protéine STAT3, le facteur STAT5B non-activé navigue constitutivement entre le noyau et le cytoplasme.

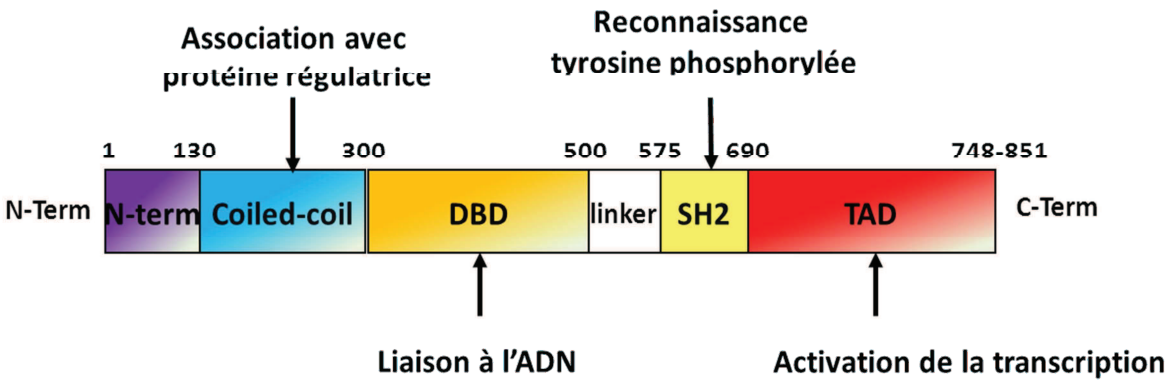

Figure 6 : Structure des protéines STAT

Les protéines STAT possèdent 6 domaines conservés : un domaine N-terminal (impliqué dans la dimérisation des STAT non phosphorylés et dans leur maintien à l'état inactif), un domaine Coiled-coil, un domaine de liaison à l'ADN (DBD pour « DNA Binding Domain »), un domaine de liaison « linker », un domaine SH2 et un domaine de transactivation (TAD) en C-terminal.

3.3.1.3. Régulation négative de la voie JAK/STAT

- La famille des tyrosines phosphatases SHP (SH2-containing phosphatases) comprend deux membres SHP1 (PTPN6) et SHP2 (PTPN11). La régulation négative des protéines SHP est également retrouvée dans les voies RAS/MAPK et PI3K/AKT. L'expression de SHP1 est quasi exclusivement restreinte au système hématopoïétique, alors que l'expression de SHP2 (PTPN11) est ubiquitaire. Les SHP sont constituées de deux domaines SH2 consécutifs et d'un domaine PTP (Protein Tyrosine Phosphatase) (Tonks and Neel 2001). Ces protéines régulent l'activité des kinases en déphosphorylant les résidus tyrosines des protéines JAK (**Figure 7**). Les protéines SHP interrompent ainsi la voie JAK/STAT en aval du récepteur en inhibant le recrutement des facteurs STAT.

- La famille des protéines SOCS (suppressors of cytokine signaling) comprend 8 membres (CIS et SOCS1-7). Les membres de la famille SOCS ne sont pas exprimés au niveau basal. Leur transcription est induite par de nombreuses cytokines, hormones et FC dont l'IFNγ, la GH et plusieurs interleukines qui activent les voies JAK/STAT (Linossi *et al.* 2013). Ils régulent par trois mécanismes la voie JAK/STAT : - ils entrent en compétition avec les JAK et les STAT pour la fixation au récepteur, - se fixent au niveau du domaine JH1 des protéines JAK, ou - ubiquitinent les JAK entrainant leur dégradation par le protéasome (**Figure 7**).

- La famille PIAS (protein inhibitor of activated STAT) comprend 5 membres PIAS1, PIAS3, PIASxα, PIASxβ et PIASy (Shuai and Liu 2005). PIAS1 semble être spécifique à STAT1 (suite à une stimulation à l'IFN ou à l'IL6) alors que PIAS3 se lie à STAT3. Les PIAS semblent être exprimées constitutivement. Par contre, leur interaction avec les protéines STAT nécessite une stimulation des cellules par des cytokines (Valentino and Pierre 2006) (**Figure 7**).

- La famille CBL (Casitas B-cell Lymphoma) comprend trois membres CBL, CBLB et CBLC impliqués dans la dégradation des RTK en augmentant leur ubiquitinylation et leur élimination par le protéasome (Mohapatra *et al.* 2012) (**Figure 7**).

- La famille SH2B comprend trois membres SH2B1 (SH2B), SH2B2 (APS), SH2B3 (LNK). Les membres de la famille interagissent directement avec les protéines JAK via leur domaine SH2 et leur tyrosine phosphorylée p.Y813. Cette association est requise pour la régulation de l'activité de JAK2 et la phosphorylation des adaptateurs par la kinase (Maures *et al.* 2006; Kurzer *et al.* 2006) (**Figure 7**).

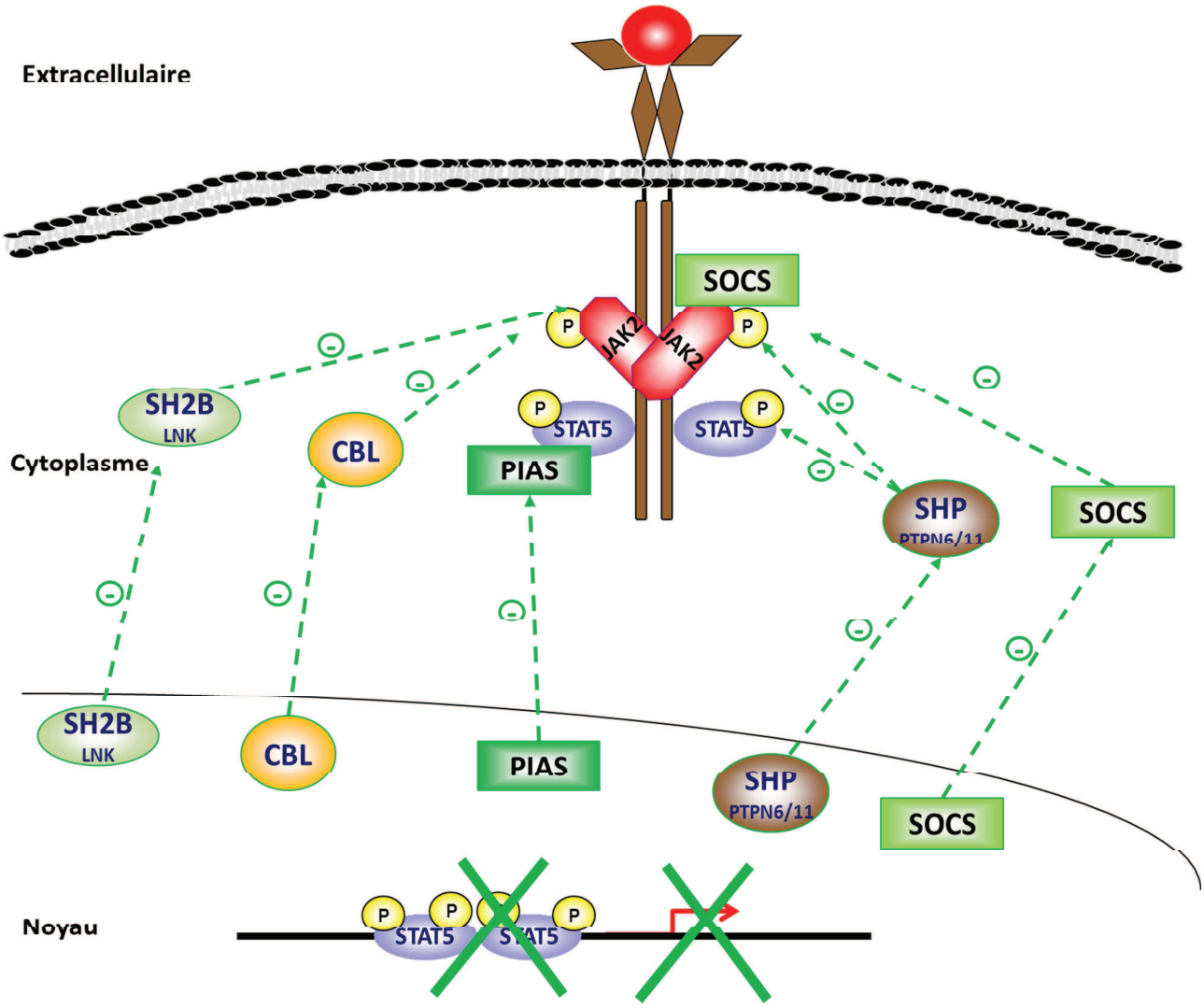

Figure 7 : Régulation négative de la voie JAK/STAT

La voie JAK/STAT est sous le contrôle de régulateurs négatifs tels que les protéines SH2B (LNK), CBL, PIAS, SHP (PTPN6, PTPN11) et SOCS.

3.3.2. Transduction du signal par la voie RAS/MAPK

La voie RAS (Rat Sarcoma)/MAPK (Mitogen Activated Protein Kinase) est une voie de signalisation intracellulaire qui joue un rôle important dans la régulation de la prolifération, de la survie, de la différenciation, de la migration cellulaire, et de l'angiogenèse. La voie est en général activée par le biais d'un récepteur membranaire, lui-même stimulé par un FC qui, sans entrer dans la cellule, va transmettre le signal extracellulaire via d'autres kinases (**Figure 8**). La voie RAS/MAPK constitue, avec la voie PI3K (Phosphatidyl Inositol 3-Kinase)/AKT (AK transforming), une des voies de transmission du signal les mieux connues. Celle-ci aboutit, après une cascade de phosphorylations successives, à la mise en jeu de FT capables d'activer la transcription de gènes impliqués dans la prolifération cellulaire, mais aussi dans l'invasion et la migration, la survie cellulaire et l'angiogenèse. Parmi les récepteurs de FC capables d'activer la voie RAS/MAPK, les principaux sont l'EGFR (Epidermal Growth Factor Receptor), les membres de la famille ERBB/EGFR (Epidermal Growth Factor Receptor), l'IGFR (Insulin-like Growth Factor Receptor), le FGFR et le PDGFR.

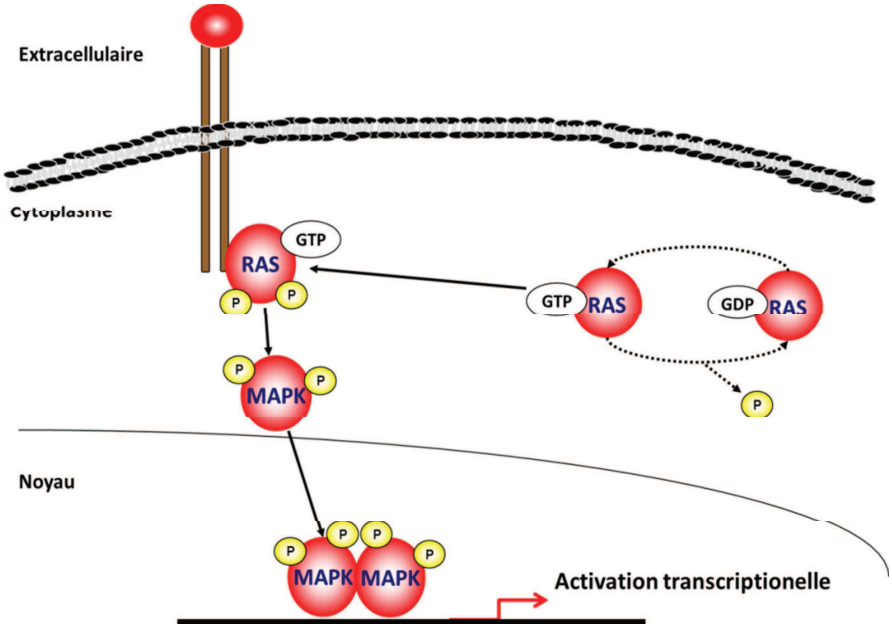

Figure 8 : Transduction du signal par la voie RAS/MAPK

La voie RAS/MAPK est activée par la liaison de la protéine RAS phosphorylée à un récepteur membranaire lui-même stimulé par un facteur de croissance. Cette voie va aboutir à une cascade de phosphorylations successives capable d'induire plusieurs mécanismes tels que la prolifération cellulaire, l'invasion, la migration, la survie et l'angiogenèse.

3.3.2.1. *Les protéines RAS*

Les protéines HRAS, KRAS et NRAS font partie de la famille des GTPases. Une fois activée par des RTK, elles se lient au GTP (Guanosine Tri-Phosphate) induisant une cascade de phosphorylation des protéines kinases (MAPK). L'interaction transitoire de RAS avec d'autres molécules intracellulaires effectrices engendre l'activation des différentes voies de signalisation et de gènes codant pour des FT.

3.3.2.2. *Régulation négative de la voie RAS*

Les tyrosines phosphatases PTPN6 et PTPN11 régulent l'activité des voies JAK/STAT et RAS/MAPK en déphosphorylant les résidus tyrosines des kinases (**Figure 9**). La neurofibromine (NF1) appartient à la famille des protéines GAP à activité GTPasique. Elle catalyse la conversion de la forme active de p21/RAS (RAS-GTP) en forme inactive RAS-GDP, et constitue un régulateur négatif de la voie RAS/MAPK. La neurofibromine intervient dans le contrôle de la différenciation et de la prolifération cellulaire (**Figure 9**). La forme active de RAS (RAS-GTP) active les protéines RAF et par conséquent la voie des MAPK. L'inhibition de cette voie est médiée par PTPN11 qui dérégule les résidus tyrosines et par NF1 qui catalyse l'hydrolyse de RAS-GTP en RAS-GDP.

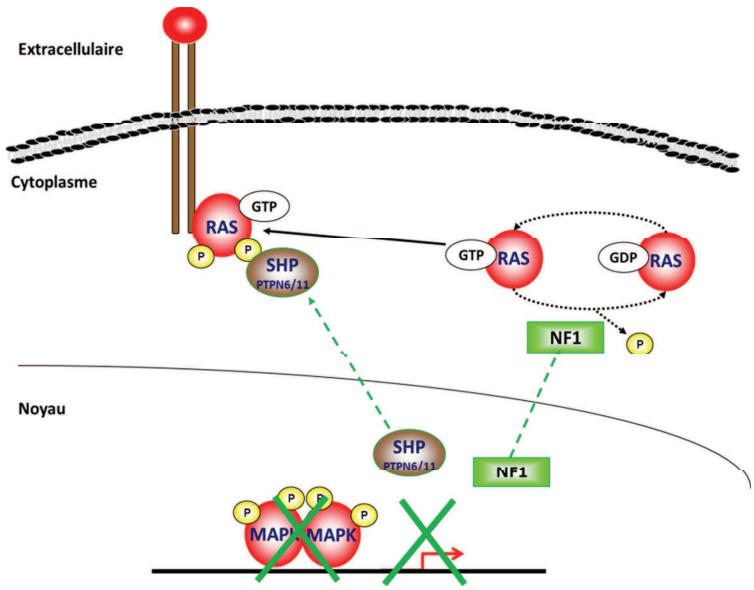

Figure 9 : Régulation négative de la voie RAS/MAPK

La voie RAS/MAPK est sous le contrôle de régulateurs négatifs tels que les protéines SHP (PTPN6, 11) et NF1.

3.3.3. Transduction du signal par la voie des SMAD

La voie de signalisation des SMAD permet la transduction du signal des ligands de la famille du TGFβ qui sont le TGFβ lui-même, les activines, les inhibines, les « bone morphogenetic protein » (BMP) et les « growth and differentiation factors » (GDF). Leurs activations requièrent des récepteurs serine/thréonine kinases de type I (ALK : activine receptor like kinase 1, 2, 3, 4, 5, 6, 7) et de type II pour transmettre un signal (**Figure 10**). C'est un système de signalisation très dynamique qui selon le type cellulaire et l'état de différenciation, contrôle la prolifération, la différenciation, la motilité, l'adhésion ou la mort.

3.3.3.1. *Le TGFβ*

Il existe trois protéines TGFβ1, TGFβ2 et TGFβ3, codées par trois gènes distincts. Elles sont synthétisées par de nombreux types cellulaires dont les plaquettes, les macrophages, les fibroblastes. Le TGFβ est sécrété sous forme d'un complexe latent qui devra être activé avant de pouvoir déclencher un effet biologique.

3.3.3.2. *Les protéines SMAD*

Cette famille est divisée en trois groupes fonctionnels différents (Kamato *et al.* 2013):

i) les SMAD activées par le récepteur : R-SMAD (SMAD1, 2, 3, 5, 8)
ii) les SMAD partenaires : co-SMAD (SMAD4, 4B)
iii) les SMAD inhibiteurs : I-SMAD (SMAD6, 7)

C'est une famille de FT, qui exerce son rôle régulateur par coopération entre membres SMAD, et/ou par des interactions avec l'ADN. Les SMAD possèdent deux domaines, MH1 qui permet la liaison à l'ADN et MH2 qui permet le recrutement des co-activateurs de la transcription (ex : les histones acétyltransférase) et des co-répresseurs (ex : les histones désacétylases).

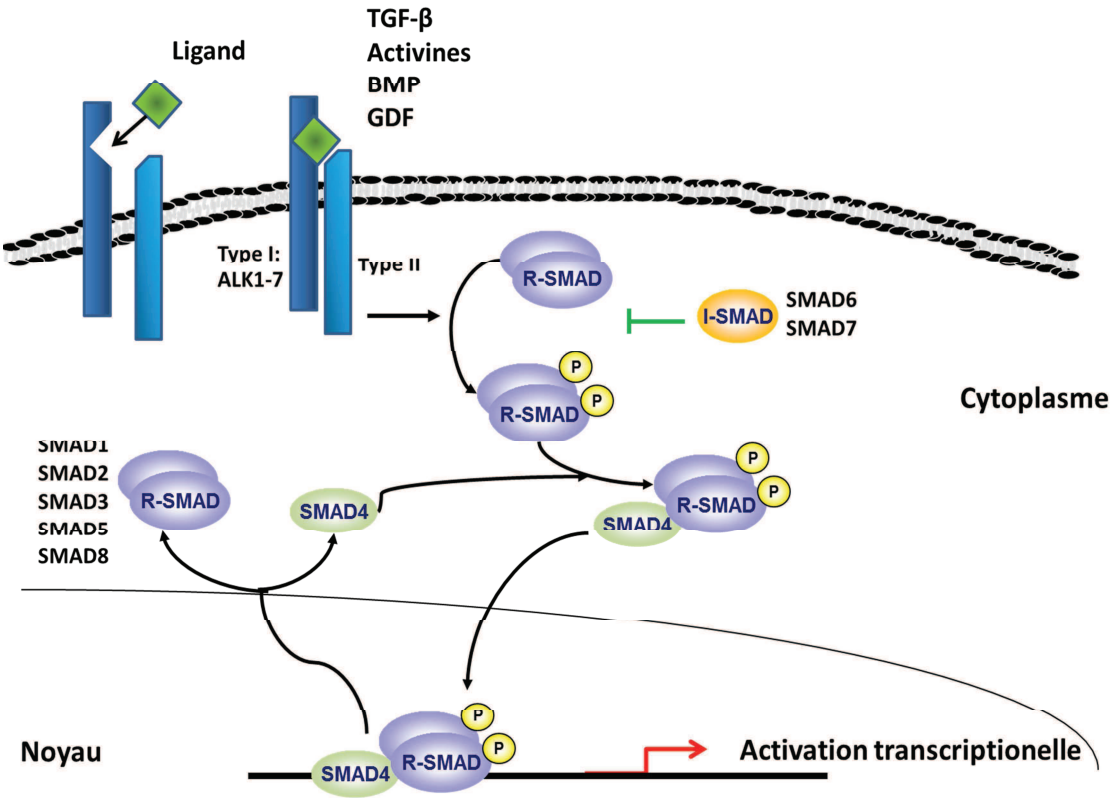

Figure 10 : Voie de signalisation des SMAD

D'après (Söderberg *et al.* 2009). Dans le cytoplasme, les R-SMAD phosphorylés s'associent aux co-SMAD, formant un complexe. Celui-ci dans le noyau par liaison avec des partenaires, est capable d'activer la transcription de gènes cibles. Les I-SMAD, agissent par un mécanisme de rétrocontrôle négatif, en inhibant l'activation des R-SMAD.

4. EPIGENETIQUE et REGULATION DE L'HEMATOPOÏESE

Les régulations épigénétiques participent activement aux processus de différenciation des cellules. Elles sont transmissibles lors de la mitose et/ou la méiose, mais ne découlent pas de modifications dans la séquence de l'ADN (Wolffe and Matzke 1999). Les mécanismes régulateurs de l'épigénétique sont la méthylation des gènes, la modification covalente des histones et la régulation épigénétique des ARN non-codants.

4.1. La méthylation des gènes

La méthylation des gènes joue un rôle important dans de nombreux processus cellulaires, comme l'inactivation des rétrotransposons, l'empreinte parentale, l'inactivation du X, ou encore la régulation de l'expression génique. Ce type de modification se concentre au niveau des îlots CpG, positionnés au niveau du promoteur et/ou du premier exon de plus de 60% des gènes humains (Bird 2002). Plusieurs familles de protéines participent à la méthylation de l'ADN notamment les DNMT, TET et IDH.

4.1.1. La famille DNMT

Les DNA méthyltransférases (DNMT) 1, 3A et 3B transfèrent des groupements méthyl sur les cytosines des îlots CpG (**Figure 11**). DNMT1 agit comme une enzyme de maintenance et se localise au niveau de la fourche de réplication durant la phase S ; DNMT3A et DNMT3B sont impliquées dans la méthylation de novo durant le développement (Okano *et al.* 1999; Tadokoro *et al.* 2007). De nombreux gènes impliqués dans l'auto-renouvellement des CSH sont réprimés sous l'action de ces enzymes, notamment *RUNX1* et *GATA3* (Trowbridge and Orkin 2012).

4.1.2. La famille TET

TET1, TET2 et TET3 catalysent la conversion de la 5-methylcytosine (5mc) de l'ADN en 5-hydroxymethylcytosine (5hmC) sous la dépendance de fer et d'α-cétoglutarate (Ko *et al.* 2010; Tahiliani *et al.* 2009) (**Figure 11**). Les 5hmC ont été identifiées comme une nouvelle marque épigénétique impliquée dans l'inactivation génétique.

4.1.3. La famille IDH

Les isocitrates déshydrogénases (IDH) IDH1 (cytoplasmique) et IDH2 (mitochondriale) catalysent la décarboxylation oxydative d'un isocitrate en α-cétoglutarate (cofacteur des enzymes de la famille TET) (**Figure 11**). (Pardanani, Lasho, Finke, Mai, *et al.* 2010; Green and Beer 2010). Les patients mutés pour ces enzymes ont des hyperméthylations aberrantes de

leur génome, dues à une production d'un métabolite anormal : 2-hydroxyglutarate à la place de α-cétoglutarate qui inhibe la déméthylation amorcée par TET2 (Figueroa *et al.* 2010).

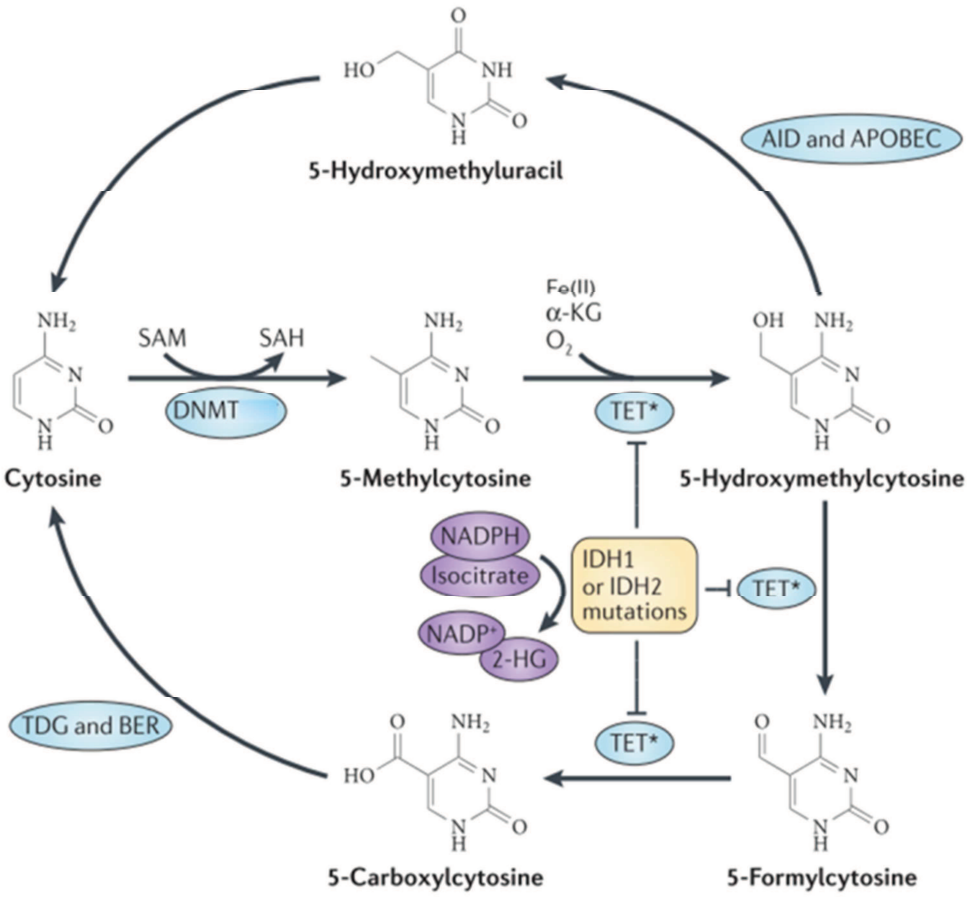

Figure 11 : Cycle de la méthylation de la cytosine

(Shih *et al.* 2012). La 5hmC initie la déméthylation de l'ADN de façon passive ou active. Les protéines TET sont capables de poursuivre l'oxydation des 5hmC en 5 formylcytosines (5fC) et 5 carboxycytosines (5caC) (He *et al.* 2011; Ito *et al.* 2011). Les 5fC et 5caC peuvent ensuite être excisées par des DNA glycosylases comme la thymine DNA glycosylase (TDG), conduisant à la formation d'un site abasique (Maiti and Drohat 2011). Ce site est par la suite reconnu par le complexe BER (Base Excision Repair) qui réintroduit une cytosine. Les 5hmC peuvent être déaminées par les enzymes de la famille AID/APOBEC pour produire des 5hmU.113. Le mismatch 5hmU/G est ensuite réparé par l'action successive des DNA glycosylases puis du BER (Cortellino *et al.* 2011).

4.2. Les modifications covalentes des histones

Les histones sont des protéines basiques assemblées sous forme de nucléosome qui jouent un rôle important dans la compaction de l'ADN. Au niveau des queues d'histones, des modifications chimiques post-traductionnelles sous l'effet de méthylases, d'acétylases sont responsables des modifications épigénétiques. Celles-ci sont chargées de modifier la chromatine et notamment l'accessibilité de l'ADN à la machinerie transcriptionelle, régulant ainsi positivement ou négativement la transcription. Strahl et Allis ont proposé que les modifications des queues des histones constituent un code compréhensible par la cellule : « le code histone » (Strahl and Allis 2000). De nombreuses modifications covalentes ont été mises en évidence la phosphorylation, l'ubiquitinylation, la SUMOylation, l'ADP-ribosylation, la biotinylation, l'hydroxylation et la crotonylation (Tan *et al.* 2011) (**Figure 12**). Toutefois, les plus étudiées aujourd'hui sont l'acétylation et la méthylation des résidus lysines (K).

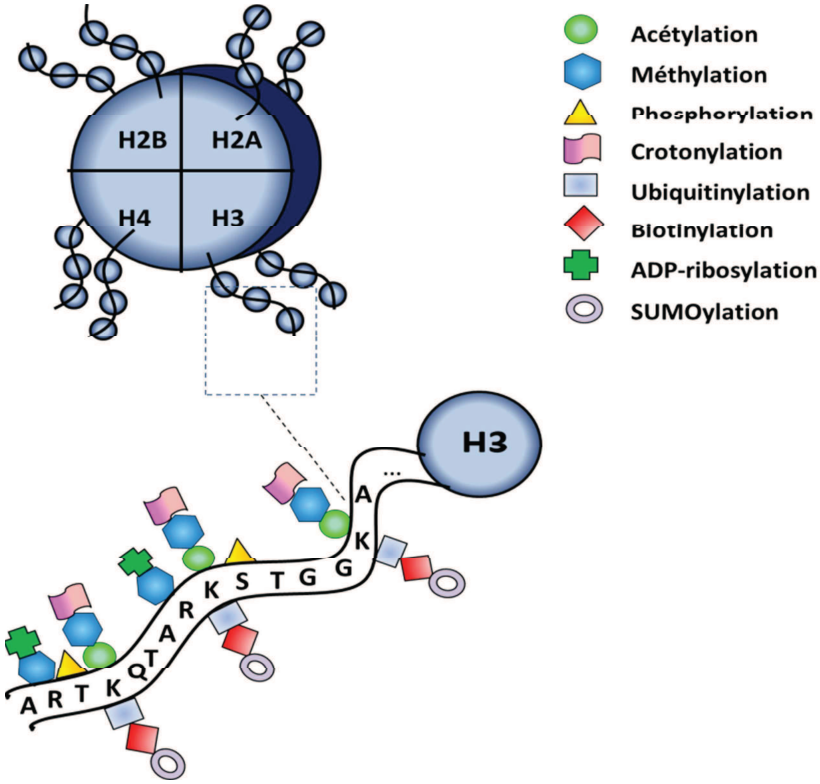

Figure 12 : Les modifications covalentes des histones

D'après (Kato *et al.* 2011; Tan *et al.* 2011). Les modifications covalentes des histones telles que l'acétylation, la méthylation, la phosphorylation, la crotonylation, l'ubiquitinylation, la biotinylation, l'ADP-ribosylation et la SUMOylation modulent la compaction de la chromatine.

4.2.1. L'acétylation des histones

L'acétylation des histones est effectuée par des acétyltransférases (HAT) qui ajoutent sur les lysines de 4 histones (H2A, H2B, H3, H4) un groupement acétyle. Ces ajouts vont neutraliser partiellement les charges positives des histones et ainsi diminuer leur affinité pour l'ADN chargé négativement. L'acétylation des histones est associée à l'activation transcriptionelle et la désacétylation est associée à la répression effectuée par des histones désacétylases (HDAC). En plus de son effet sur la transcription, le niveau d'acétylation intervient également au niveau de la réparation (Ikura *et al.* 2000; Bird *et al.* 2002) et de la réplication (Miotto and Struhl 2010) de l'ADN.

4.2.2. La méthylation des histones

Le rôle de la méthylation semble plus complexe. Les histones méthyltransférases peuvent ajouter un, deux ou trois groupements méthyles sur les fonctions amines des lysines (K) et des arginines (R), et les histones déméthylases les enlèvent. La position et le nombre de groupement méthyles va avoir des effets différents sur la chromatine (Izzo and Schneider 2010). Certaines méthylations sont associées à une chromatine active, par exemple : H3K4me(1/2/3), H3K36me(1/2/3), H4K20me1, H3R17me(1/2). D'autres méthylations sont associées à une chromatine inactive, par exemple : H3K9me(1/2/3), H3K27me(2/3), H4K20me3, H3R2me2.

4.2.3. Les complexes Polycomb (PcG)

Les complexes Polycomb (PcG) et Trithorax (TrxG) sont des régulateurs clés des modifications des histones. Ces protéines ont été initialement identifiées comme des répresseurs stables des gènes *HOX* au cours du développement de la drosophile (Lewis, 1978). Les protéines du groupe TrxG et les protéines du groupe PcG agissent de façon antagoniste. Le groupe TrxG active la transcription d'un certain nombre de gènes alors que le groupe PcG la réprime. Les protéines de ce dernier appartiennent à deux complexes majeurs PRC1 et PRC2 (Polycomb Repressive Complex1/2) (**Figure 13**). Ces complexes sont capables de maintenir l'inactivation de la chromatine en interagissant avec des séquences d'ADN régulatrices définies comme les "PcG response elements" (PRE).

4.2.3.1. *Le complexe Polycomb répresseur 1*

Le complexe PRC1 reconnait les marques de méthylation des H3K27 et H3K9 et peut induire la répression des promoteurs cibles. Les PRC1 sont conservés de la drosophile à l'homme (Woo *et al.* 2013). Chez les mammifères, on compte plusieurs homologues pour chaque composant de PRC1 : les CBX (Pc) ; les PHC (Ph), les RING1A et RING1B (Sce), les L3MBTL, BMI1, MEL18, RNF68, RNF3, et RNF159 (Psc) (**Figure 13**). Parmi les co-facteurs qui viennent s'ajouter, les protéines ASXL (Additionnal sex Combs like) (Fisher *et al.* 2006) possèdent un domaine PHD (Plant Homeo Domain) en C-terminal qui est un motif structural retrouvé dans les protéines nucléaires impliquées dans les modifications de la chromatine (Bienz 2006), comme les histones déméthylases (Cloos *et al.* 2008).

4.2.3.2. *Le complexe Polycomb répresseur 2*

Le complexe PRC2 est composé principalement par EED, SUZ12, EZH1 et EZH2 (**Figure 13**). Il est responsable de la di- et triméthylation de H3K27 (H3K27me2/3), marque associée à une répression de la transcription (Cao *et al.* 2002), et H3K9. PRC2 initie la répression des gènes cibles, par l'activité méthyltransférase d'EZH2.

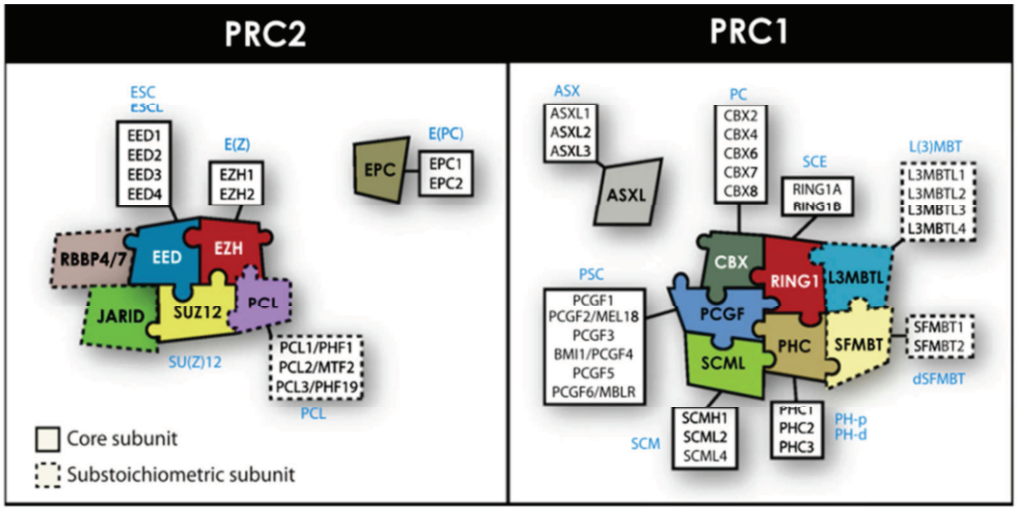

Figure 13 : Les complexes Polycomb PRC1 et PRC2

(Sauvageau and Sauvageau 2010). Les homologues de la drosophile de chaque sous-unité sont indiqués en bleu clair. De multiples combinaisons de sous-unités paralogues peuvent générer une diversité de complexes (PRC1 et PRC2), qui ont sans doute des fonctions spécifiques et partagées. Les contacts illustrés dans les schémas ne sont pas destinés à représenter les interactions réelles.

4.3. La régulation épigénétique des ARN non-codants

La régulation épigénétique des ARN non-codants se fait par les micro-ARN et les longs ARN non codants. Les micro-ARN sont de petites chaines d'environ 22 nucléotides, qui régulent l'expression génique au niveau post-transcriptionnel. Ils se fixent sur la région 3' non codante des ARNm cibles pour induire une répression de leur traduction ou leur dégradation (Vasilatou *et al.* 2013). Les longs ARN participent également à l'inactivation fonctionnelle des gènes et ont un rôle clef dans la différenciation et le développement cellulaire mais leur mode d'action est encore peu caractérisé. D'autres ARN participent à la régulation des ARNm tels que les SNORNA (small nucleolar RNA), qui jouent un rôle dans l'incorporation de modifications chimiques d'autres ARN (par méthylation du ribose en position 2' et par transformation de l'uridine en pseudouridine) (Falaleeva and Stamm 2013; Ferreira *et al.* 2012).

5. EPISSAGE et REGULATION DE L'HEMATOPOÏESE

L'épissage est une étape essentielle du processus de maturation des ARNm, assuré par le spliceosome qui est un ensemble de complexes ribonucléoprotéiques (ARN et protéines). Le spliceosome localisé dans le noyau assure plusieurs fonctions : la reconnaissance des sites d'épissage, l'excision des introns, des régions non-codantes de l'ARN pré-messagers et enfin la suture des exons. Il est composé de cinq particules ribonucléoprotéiques, appelées snRNP (U1, U2, U4, U5 et U6). Plusieurs protéines participent au contrôle l'épissage de l'ARN dont SF3A1, SF3B1, SAP130, SRSF2, SRSF6, ZRSF2, U2AF1, U2AF2, U2AF26, LUC7L2, PRPF8, SF1 (**Figure 14**). L'épissage de l'ARN est souvent couplé à la transcription, et de ce fait il peut aussi être affecté par les modifications d'histones et de structure de la chromatine.

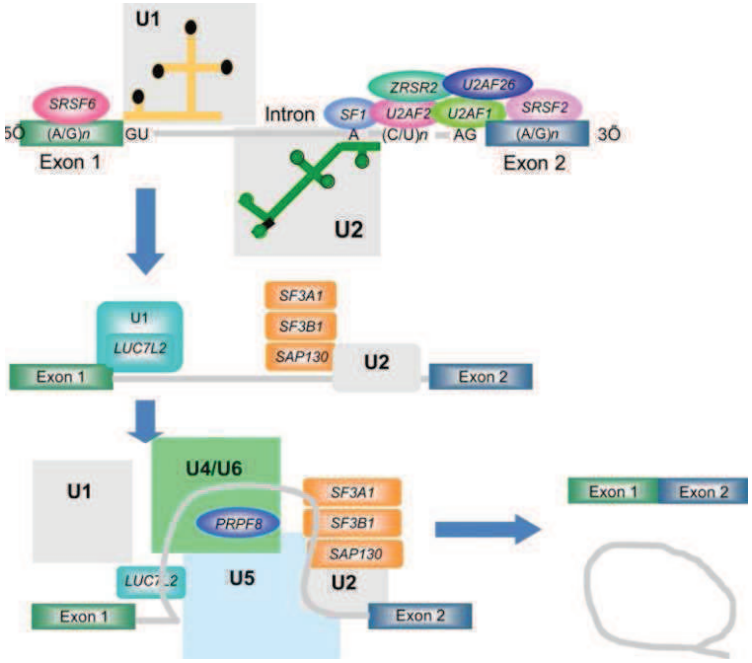

Figure 14 : L'épissage du pré-ARNm se fait en plusieurs étapes catalysées par le spliceosome

(Maciejewski and Padgett 2012). L'épissage est un mécanisme en deux étapes, catalysé par le spliceosome et par trois éléments spécifiques de la séquence ARN appelés : site d'épissage 5', le site d'épissage 3' et le site de branchement (comportant une adénosine qui joue un rôle central dans l'épissage). Dans la première étape, il y a une attaque nucléophile du 2'-OH du ribose de l'adénosine du site de branchement sur le phosphate de la jonction exon-intron en 5'. Après cette coupure, le 3'-OH libéré au niveau de l'exon en amont attaque le phosphate de la jonction intron-exon en aval. Les produits de cette réaction sont les deux exons ligaturés correctement et l'excision de l'intron sous forme de lasso.

6. LA NICHE HEMATOPOIETIQUE

6.1. Le concept de niche hématopoïétique

Schofield en 1978 a été le premier à proposer le concept de niche hématopoïétique. Il a émis l'hypothèse que, dans la moelle osseuse, les CSH devaient être en association avec d'autres cellules afin de maintenir leur état de quiescence et d'auto-renouvellement. Depuis il a été démontré que les niches sont constituées d'ostéoblastes, d'ostéoclastes, de cellules endothéliales, de fibroblastes, d'adipocytes et de plusieurs molécules comme les collagènes qui forment un réseau de soutien au sein de la matrice extracellulaire. La niche

hématopoïétique maintient et procure aux CSH les facteurs d'attraction (chimiokines), les facteurs d'adhésion, de survie, de prolifération et de différenciation.

6.2. Une ou plusieurs niches hématopoïétiques

Plusieurs modèles de niche hématopoïétique existent soutenant des hypothèses sans cesse controversées. A ce jour, la dernière hypothèse soutient l'existence de trois types de niches (**Figure 15**) :

i) La niche ostéoblastique ou endostéale, qui au contact de l'os serait impliquée dans le maintien en quiescence des CSH. Elle est constituée d'ostéoblastes, de fibroblastes et d'adipocytes ayant tous pour origine la cellule souche mésenchymateuse, ainsi que d'ostéoclastes, d'origine hématopoïétique, impliqués dans la résorption osseuse. Les ostéoblastes produisent des facteurs qui régulent les propriétés de quiescence, d'auto-renouvellement et de différenciation des CSH tels que l'angiopoitéine-1, l'ostéopontine ou le SDF1/CXCL12.

ii) La niche vasculaire, qui consiste en un réseau de vaisseaux fenêtrés formés par des cellules endothéliales dérivant de la cellule souche endothéliale. Les facteurs angiogéniques sont sécrétés par les cellules endothéliales, cadhérine+, situées près des capillaires sinusoïdes médullaires (Kiel *et al.* 2005) et permettent une balance entre l'expansion et la différentiation des CSH (Butler et al., 2010).

iii) La niche réticulaire, constituée de cellules réticulaires appelées cellules CAR (pour CXCL12-abundant reticular). Les cellules CAR (et surtout leur production de SCF et CXCL12/SDF1) sont importantes pour maintenir le nombre de CSH (Omatsu et al., 2010).

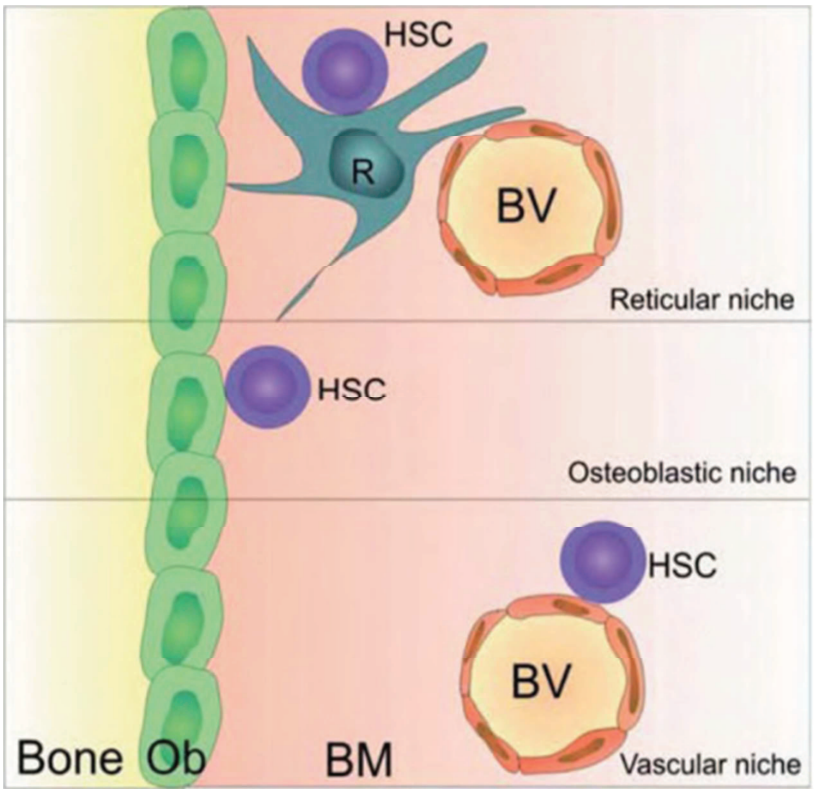

Figure 15 : Les trois niches hématopoïétiques

(Ellis and Nilsson 2012) Il existe 3 types de niches : la niche ostéoblastique (Ob), la niche vasculaire (BV) et la niche réticulaire (R).

6.3. Cas particulier du couple SDF-1(CXCL12)/CXCR4

En conditions physiologiques, les CSH résident dans la niche hématopoïétique et très peu sont mobilisées dans la circulation sanguine. Contrairement aux états de stress (inflammation, traumatismes, chimiothérapies) où les interactions entre CSH et niche hématopoïétique sont relâchées afin que les CSH soient mobilisées dans la circulation sanguine. La mobilisation résulte à la fois de la perte des contacts entre les cellules ou avec la matrice extracellulaire (via la sous-expression des molécules d'adhérence) et d'une désensibilisation de la signalisation des chimiokines. La désensibilisation est faite notamment par le couple SDF1(CXCL12)/CXCR4. A l'inverse, le « nichage », c'est-à dire le maintien des cellules souches dans leur niche, nécessite la surexpression des molécules d'adhésion et une activation de l'axe SDF1/CXCR4 (Lapidot, Dar, & Kollet, 2005).

B- LES HEMOPATHIES MYELOIDES

Les hémopathies malignes (HM) sont dues à une prolifération clonale des cellules hématopoïétiques de la moelle. Les HM sont réparties en deux groupes selon la voie de différenciation affectée : myéloïde et lymphoïde. Les HM myéloïdes affectent les lignées myéloïdes : érythrocytaire, mégacaryocytaire et granuleuses (neutrophile, monocytaire, éosinophile, basophile). La 4ème édition de la classification de l'Organisation Mondiale de la Santé (OMS) de 2008 répartit les HM myéloïdes appelées néoplasmes en 5 sous-groupes majeurs (Tefferi, Thiele, *et al.* 2009) (**Table 1**).

1. La Leucémie Aigüe Myéloïde (LAM)
2. Les Syndromes Myélodysplasiques (SMD)
3. Syndromes ou Néoplasmes myéloprolifératifs (SMP) 3.1. La Leucémie Myéloïde Chronique (LMC) 3.2. La Polyglobulie de Vaquez (PV) 3.3. La Thrombocytémie Essentielle (TE) 3.4. La Myélofibrose Primitive (MFP) 3.5. La Leucémie Chronique à polynucléaires Neutrophiles (LCN) 3.6. La Leucémie Chronique à Eosinophiles (LCE) 3.7. Les Mastocytoses 3.8. Les SMP inclassables
4. Les formes frontières SMD/SMP 4.1. La Leucémie Myélomonocytaire Chronique (LMMC) 4.2. La Leucémie Myélomonocytaire Juvénile (LMMJ) 4.3. La LMC atypique 4.4. Les formes frontières SMD/SMP inclassables
5. Les syndromes myéloïde et lymphoïde avec éosinophilie et anomalies de PDGFRA, PDGFRB et FGFR1

Table 1 : Classification des néoplasmes myéloïdes selon la classification OMS 2008

C- LES SYNDROMES ou NEOPLASMES MYELOPROLIFERATIFS

1. GENERALITES SUR LES SYNDROMES MYELOPROLIFERATIFS (SMP)

1.1. Définition des SMP

William Dameshek a été le premier en 1951 à introduire le terme de syndrome myéloprolifératif (SMP) pour décrire un groupe d'hémopathies ayant des caractéristiques cliniques et biologiques communes (Dameshek 1951). Initialement, il a réuni quatre syndromes sous le terme de SMP : la polyglobulie de Vaquez (PV), la thrombocytémie essentielle (TE), la myélofibrose primaire ou idiopathique (MFP) et la leucémie myéloïde chronique (LMC). Les SMP dérivent de la transformation d'une cellule souche hématopoïétique (CSH) et sont caractérisés par :

i) une hématopoïèse augmentée avec une production excessive de cellules myéloïdes sans anomalie de maturation.
ii) des anomalies de réponse aux cytokines.
iii) une chronicité.

Les cellules sont morphologiquement normales, à la différence des syndromes myélodysplasiques (SMD). Depuis 2008, la classification OMS inclut 8 entités dans le groupe des SMP, nommées sous le terme de néoplasmes myéloprolifératifs (**Table 1**) : la LMC, la PV, la TE, la MFP, la leucémie chronique à polynucléaires neutrophiles, la leucémie chronique à polynucléaires éosinophiles, les mastocytoses et les SMP inclassables.

A noter, que cette nouvelle classification a renommé les SMP en néoplasmes myéloprolifératifs (NMP) ce qui implique et souligne le fait que ces pathologies soient reconnues comme des HM. Dans ce manuscrit, j'utiliserai par préférence et habitude le terme de SMP, ne faisant pas de distinction entre les termes NMP et SMP.

1.2. Répartition des SMP

Les 8 entités de la classification OMS des SMP ont des fréquences de survenues différentes. Ces hémopathies sont relativement rares dans la population générale mais assez fréquentes pour les hématologistes. La TE est le SMP le plus fréquent (40%), suivi par la PV (35%), la LMC (16%) et la MF (5%). Les autres SMP ont des fréquences plus faibles (<4%) et regroupent la LCN, LCE, les mastocytoses et les SMP inclassables (**Figure 16**).

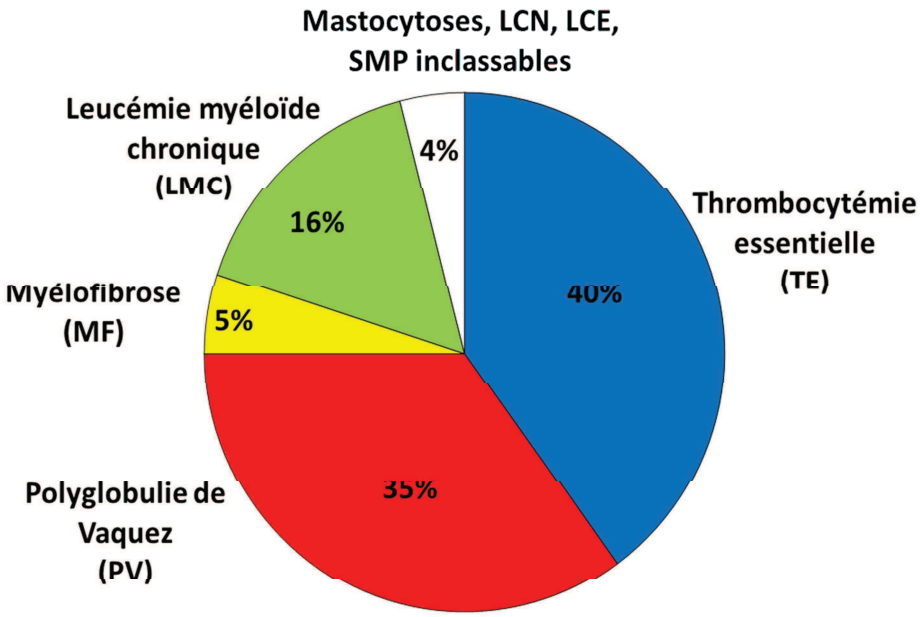

Figure 16 : Répartition des sous-types SMP

Les travaux présentés dans cette thèse se sont consacrés à l'étude des SMP classiques non LMC (PV, TE et MF). Ainsi dans ce manuscrit, les autres SMP : la LMC, la LCN, la LCE, les mastocytoses et les SMP inclassables ne seront pas abordés.

2. LES SMP CLASSIQUES NON LMC

Par définition, les SMP classiques non LMC sont caractérisés par l'absence du chromosome Philadelphie. On regroupe sous le terme de SMP classiques non LMC : la PV, la TE et la MF. Les caractéristiques générales de ces hémopathies et les critères diagnostiques selon l'OMS sont répertoriés respectivement dans les **Tables 2** et **3**.

2.1. Epidémiologie des SMP classiques non LMC

L'incidence annuelle globale des SMP classiques non LMC est de 4 à 5 cas pour 100 000 habitants (Girodon *et al.* 2009). Sans cause actuellement identifiée, les SMP sont près de deux fois plus fréquents chez les hommes que chez les femmes (sexe ratio = 1.8). La médiane d'âge au moment du diagnostic des SMP se situe autour de 65 ans (Girodon *et al.* 2009). Les incidences de la PV et la TE sont proches, avec environ pour chacune 2 cas pour 100 000 habitants et environ 1 cas pour 100 000 habitants pour la MF (**Table 2**).

Caractéristiques générales	PV	TE	MF
Incidence (nombre de cas pour 100 000 habitants/an)	0.9-1.97	1.55-2	0.3-0.7
Prévalence (nombre de cas pour 100 000 habitants/an)	30-35	39	10
Age (ans)	68-72	61-73	>65
Sexe	H>F	H/F=0.76	H>F
Symptômes	Prurit Erythrose faciale Céphalées Acouphènes Vertiges Splénomégalie (rare)	Erythromélagies Gonflements des extrémités (mains, pieds) Splénomégalie (rare)	Prurit Perte de poids Asthénie Sueurs nocturnes Douleurs osseuses Splénomégalie
Complications	Thromboses	Thromboses Hémorragies	Thromboses Hémorragies Infections Défaillances cardiaques
Evolution en PV	-	5-15% (à 15 ans)	-
Evolution en MF	10-15% (à 15 ans)	5-15% (à 15 ans)	-
Evolution en LAM	15% (à 15 ans)	15% (à 15 ans)	20%
Survie médiane	93% (à 5 ans) 46% (à 20 ans)	83% (à 10 ans) 47% (à 20 ans)	3.5-5 ans
Mutation JAK2V617F	98%	55%	65%
Traitements	En fonction du risque thrombotique : agents cytoréducteurs et/ou anti-aggrégants	En fonction du risque thrombotique : agents cytoréducteurs et/ou anti-aggrégants	En fonction du score pronostique : agents cytoréducteurs et/ou greffe allogénique

Table 2 : Caractéristiques générales des SMP classiques non LMC

D'après (Girodon *et al.* 2009) (Phekoo *et al.* 2006) (Bonicelli *et al.* 2013), et l'association FIM (France Intergroupe des syndromes Myéloprolifératifs)

2.2. Diagnostic des SMP classiques non LMC

Le diagnostic de ces SMP se fait la plupart du temps lors d'examens médicaux réalisés de façon fortuite ou suite à un évènement vasculaire :

i) **examen biologique** (numération formule sanguine) révélant une augmentation de l'hémoglobine, de l'hématocrite, une thrombocytose et/ou une hyperleucocytose. Dans le cas des myélofibroses, on observe une érythromyélémie avec présence de signes indirects de myélofibrose (dacryocytes).

ii) **examen clinique**, révélant une splénomégalie associée ou non à une hépatomégalie.

Des examens complémentaires sont ensuite réalisés afin d'affirmer le diagnostic de SMP (**Table 3**). Devant une numération sanguine suspecte, la recherche de la mutation JAK2V617F du gène *JAK2* est l'examen clé. Lorsque la mutation est présente, le diagnostic d'hémopathie maligne est certain et permet d'éliminer les polyglobulies et thrombocytoses réactionnelles. Néanmoins, si la recherche de mutation JAK2V617F est négative, il faut trouver des preuves de clonalité afin d'affirmer le caractère malin de ces hémopathies.

	PV	TE	MF
Diagnostic	2 critères majeurs et de 1 critère mineur ou le 1er critère majeur associé à 2 critères mineurs	**4** critères majeurs	**3** critères majeurs et de **2** critères mineurs
Critères majeurs	1. Hémoglobine > 18,5 g/dL chez l'homme et 16,5 g/dL chez la femme ou toute autre preuve de l'augmentation de la masse globulaire érythrocytaire	1. Augmentation persistante du nombre de plaquettes ≥ 450 x 10^9/L	1. La biopsie ostéo-médullaire montre une prolifération de la lignée mégacaryocytaire avec des atypies morphologiques accompagnées d'une fibrose réticulinique ou collagène, ou en l'absence de fibrose réticulinique significative, augmentation de la cellularité médullaire caractérisée par une prolifération granuleuse et souvent une érythropoïèse diminuée
	2. Présence de JAK2V617F ou d'autres mutations fonctionnellement similaires (par exemple mutation de *JAK2* exon 12)	2. La biopsie ostéo-médullaire montre une prolifération prédominante sur la lignée mégacaryocytaire faite d'une majorité d'éléments matures et dystrophiques de grande taille. Pas d'augmentation significative de la granulopoïèse neutrophile, ni de l'érythropoïèse et pas d'excès d'éléments immatures dans ces deux lignées	2. Absence des critères retenus par l'OMS en faveur du diagnostic de : PV, LMC, MDS ou d'une autre maladie maligne de la lignée myéloïde
		3. Absence des critères retenus par l'OMS en faveur du diagnostic de PV, MF primaire, LMC, syndrome myélodysplasique ou d'un néoplame myéloïde	3. Démonstration de la mutation JAK2V617F ou d'un autre marqueur de clonalité (e.g. MPLW515L/K). Ou, en l'absence de marqueur de clonalité, démonstration de l'absence d'argument en faveur d'une myélofibrose due à la présence d'une maladie inflammatoire sous-jacente ou d'une affection néoplasique
		4. Démonstration de la mutation JAK2V617F ou d'un autre marqueur de clonalité ou en l'absence de marqueur de clonalité : absence d'argument en faveur d'une thrombocytose réactionnelle	
Critères mineurs	1. La biopsie ostéo-médullaire montre en fonction de l'âge, une hyperplasie cellulaire portant sur les trois lignées : érythrocytaire granulocytaire,mégacaryocytaire (panmyélose)		1. Leuco-érythroblastose sanguine (érythromyélémie)
	2. Taux d'érythropoïétine sérique au-dessous des valeurs normales de référence		2 .Augmentation des taux sériques de lactate déshydrogénase (LDH)
	3. Pousse spontanée des progéniteurs érythrocytaires in vitro		3. Anémie
			4 .Splénomégalie palpable

Table 3 : Critères diagnostiques selon l'OMS des SMP classiques non LMC

3. LA POLYGLOBULIE DE VAQUEZ

En 1982, Louis Henri Vaquez est le premier à décrire la présence d'une polyglobulie chez un patient présentant une érythrocytose importante et une hépatomégalie (Vaquez 1892)*. Il faudra attendre 1903, pour que William Osler nomme cette nouvelle entité clinique : maladie de Vaquez* (Osler 1903)*.*

Aujourd'hui, le diagnostic de PV est évoqué devant une polyglobulie et un taux d'hémoglobine élevé. Une hyperleucocytose et/ou une thrombocytose modérées sont parfois observées. L'analyse médullaire montre une hyperplasie des lignées érythroblastique et mégacaryocytaire avec une dystrophie mégacaryocytaire. Il existe une hypersensibilité des progéniteurs érythroblastiques à l'érythropoïétine (EPO) qui se traduit par une pousse endogène des progéniteurs en l'absence d'EPO. Toutefois, le taux sérique d'EPO est bas chez ces patients. Le diagnostic repose sur un ensemble de critères majeurs et mineurs définis par la classification OMS (**Table 3**). Les causes de polyglobulies secondaires telles que l'hypoxie chronique, une tumeur rénale ou hépatique, un kyste rénal doivent être systématiquement exclues.

4. LA THROMBOCYTEMIE ESSENTIELLE

En 1934, Emil Epstein et Alfred Goedel identifient des présentations avec thrombocytose, sans érythrocytose, comme un syndrome clinique distinct : la thrombocytémie essentielle (Epstein and Goedel 1934)*.*

La TE est caractérisée par une thrombocytose supérieure à 450.10^9/L. L'analyse médullaire montre une hyperplasie mégacaryocytaire avec des mégacaryocytes nombreux et dystrophiques au myélogramme. Le diagnostic de TE demande d'exclure les thrombocytoses réactionnelles tels qu'une carence martiale, un syndrome inflammatoire, infectieux ou tumoral. La TE est le SMP le plus fréquent avec une incidence > 1.5 cas pour 100 000 habitants. La TE est observée dès l'adulte jeune (> 20 ans) et il existe une discrète prédominance féminine.

5. LA MYELOFIBROSE

5.1. Physiopathologie de la myélofibrose

En 1879, le médecin allemand Gustave Heuck décrit pour la première fois chez deux patients la présence de splénomégalie associée à des symptômes constitutionnels et une hématopoïèse extra-médullaire (Heuck 1879).

Plusieurs synonymes existent pour définir la MF : splénomégalie myéloïde, métaplasie myéloïde avec MF, ceci témoigne de la difficulté à définir cette hémopathie de façon simple. Il existe deux types de MF : la MF primaire (MFP) qui se développe spontanément et la MF secondaire qui survient après une phase plus ou moins longue à l'état de PV et TE (MF post-PV/TE) (Mesa *et al.* 2007). Il n'existe cependant pas de différences cliniques et biologiques entre les MFP et les MF post-PV/TE avec une prise en charge clinique identique. La MF est une pathologie inflammatoire caractérisée par deux versants biologiques qui sont la prolifération médullaire et l'insuffisance médullaire.

5.1.1. La prolifération médullaire

Dans la moelle, la prolifération médullaire se traduit par une hyperplasie des lignées granuleuse et mégacaryocytaire. Les mégacaryocytes sont dystrophiques et groupés « en amas ». Ces derniers sécrètent des cytokines et principalement le TGFβ qui vont stimuler la prolifération de fibroblastes polyclonaux (Le Bousse-Kerdilès 2012) (**Figure 17**). La fibrose médullaire n'est pas pathognomonique de la maladie car ce signe est également rencontré dans d'autres maladies systémiques. Plusieurs études ont rapporté que dans la MF, les mégacaryocytes et les plaquettes circulants des patients produisaient des quantités accrues de TGFβ sous forme latente (Martyré *et al.* 1997). Le TGFβ est une cytokine aux multiples fonctions, qui régule négativement les fonctions des CSH mais qui agit cependant comme un puissant facteur fibrogénique stimulant l'expression génique des collagènes de types I, III et IV, de fibronectine et de protéoglycanes.

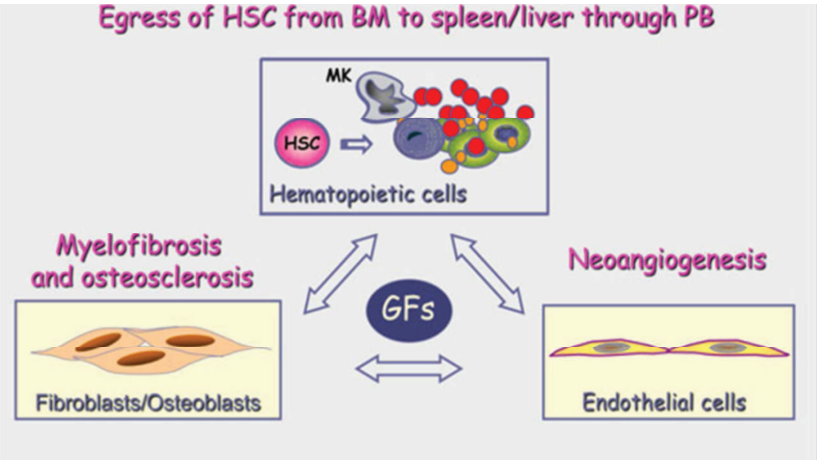

Figure 17 : Caractéristiques physiopathologiques de la myélofibrose primaire

(Le Bousse-Kerdilès 2012). La MFP est caractérisée par une amplification clonale de CSH et une prolifération importante de mégacaryocytes (MK) dystrophiques qui est associé à une migration des CSH de la moelle osseuse vers la rate et le foie à travers le sang périphérique. La myéloprolifération est associée à des altérations du stroma aboutissant à une myélofibrose, une ostéosclérose et une néangiogénèse. La réaction stromale serait secondaire à une stimulation des cellules stromales telles que les fibroblastes, les ostéoblastes et les cellules endothéliales par les facteurs de croissances (GFs) produits en excès à partir d'un clone hématopoïétique et spécifiquement par les MK.

5.1.2. L'insuffisance médullaire

Après une phase de prolifération plus ou moins longue, l'insuffisance médullaire s'installe. Celle-ci se traduit principalement par une anémie. La moelle devenue totalement fibrotique, ne peut plus être le siège de l'hématopoïèse. Cette altération médullaire conduirait à une augmentation des cellules souches circulantes (myéloprolifération) et à une hématopoïèse extra-médullaire dans la rate et le foie (spléno- et hépatomégalie) (**Figure 17**). *Bien que la mobilisation des CD34+ dans le sang ne fasse pas partie des critères de diagnostic OMS* (Vardiman *et al.* 2009)*, les cliniciens utilisent parfois ce phénomène pour affiner le diagnostic de MF.* Une des hypothèses est que dans la rate et dans le foie, les cellules souches mésenchymateuses/fibroblastes et les progéniteurs endothéliaux pourraient contribuer à un microenvironnement favorable à la prolifération et à la différenciation de CSH pathologiques. Ainsi, selon le modèle « Bad seed in bad soil» de l'équipe de MC. Le Bousse-Kerdilès, la MFP est une maladie de la CSH (bad seed) et des niches niches hématopoïétiques (bad soil) altérées (Le Bousse-Kerdilès 2012) (**Figure 18**).

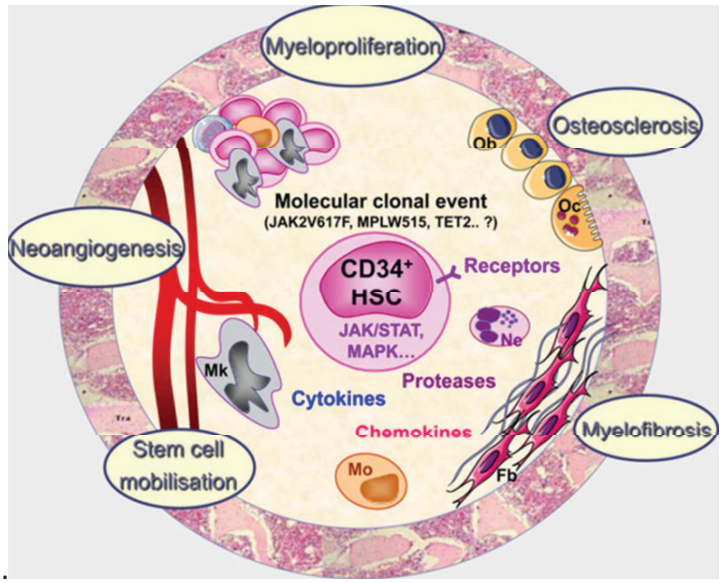

Figure 18: Modèle « Bad Seed and Bad Soil »

(Le Bousse-Kerdilès 2012). Le modèle de « Bad Seed and Bad Soil » décrit le dialogue altéré entre les cellules hématopoïétiques pathologiques (Bad seeds) et les cellules des niches hématopoïétiques (Bad soil).

5.1.3. Le contexte inflammatoire

Il est actuellement admis qu'un déséquilibre entre la synthèse accrue de collagène et une diminution de sa dégradation joue un rôle clé dans le développement de la fibrose. La MF est un processus réactionnel et inflammatoire due à un déversement trop important de cytokines par les mégacaryocytes, les monocytes activés et les CD34+ pathologiques (pour revue Le Bousse-Kerdilès *et al.* 2008; Hasselbalch 2013).

5.1.4. Traitement de la myélofibrose

Plusieurs traitements de ce SMP agressif existent et agissent essentiellement sur les symptômes de la maladie. L'anémie est traitée par les corticoïdes, androgènes, EPO, transfusions, immumodulateurs ; la myéloprolifération est diminuée par l'hydroxyurée, l'anagrelide et le pipobroman. Les patients dont la médiane de survie est inférieure à 5 ans et/ou qui sont réfractaires aux autres traitements, la greffe allogénique de CSH représente

l'ultime option thérapeutique. Les essais thérapeutiques de phase II/III utilisant un inhibiteur de JAK1/JAK2 ont montré une réduction importante de la splénomégalie entrainant une amélioration de la qualité de vie chez des patients JAK2 mutés et non mutés(Pardanani, Vannucchi, Passamonti, Cervantes, *et al.* 2010; Verstovsek *et al.* 2013; Mesa *et al.* 2013). Toutefois, la charge allélique de la mutation JAK2 n'est pas diminuée et aucune modification de la fibrose n'est observée (Quintás-Cardama 2013).

5.2. La MF primaire et les scores pronostiques

La MF est le SMP classique le plus agressif avec une survie médiane faible de 3.5-5 ans. Ainsi afin d'adapter au mieux la thérapeutique, plusieurs scores pronostiques ont été développés par les experts internationaux afin de classer les patients en groupes pronostiques. A noter que ces scores ne sont actuellement applicables que pour les MF primaires.

i) ***IPSS***, International Prognostic Scoring System
ii) ***DIPSS***, Dynamic International Prognostic Scoring System
iii) ***DIPSSplus***, Dynamic International Prognostic Scoring System plus.

Les **scores IPSS** et **DIPSS** sont calculés selon 5 facteurs de risques biologiques et cliniques : l'âge, le taux d'hémoglobine (Hb), le taux de globules blancs, la présence de blastes circulant dans le sang et la présence de symptômes constitutionnels (sueurs nocturnes, perte de poids (10%), fièvre non infectieuse). Chaque facteur de risque est pondéré d'un point, excepté l'Hb qui dans le score DIPPS est pondérée de deux points. Les scores se calculent en faisant la somme de tous les points (**Table 4**).

Le **score DIPSSplus** prend en compte les mêmes facteurs de risques que IPSS et DIPSS, mais également le besoin transfusionnel, le taux de plaquettes et la présence d'anomalies cytogénétiques spécifiques (caryotype complexe ou la présence d'une ou deux anomalies telles que +8, -7/7q, i(17q), -5/5q, -12p, inv(3) ou réarrangements 11q23). Ces trois derniers facteurs sont pondérés d'un point chacun. Pour calculer le score DIPPSplus, il faut additionner le nombre de points associés au score DIPSS (normal) et le nombre de points supplémentaires apporté par les facteurs de risques spécifiques du score DIPPSplus. A noter que le score IPSS est calculé au diagnostic de la MF primaire alors que les scores DIPSS et DIPSS plus qui sont des scores dits « dynamiques », peuvent être calculés à tout moment.

Facteurs de risque	IPSS	DIPSS
Age > 65 ans	1	1
GB > 25.10^9/L	1	1
Hb > 10g/dL	1	**2**
Blastes dans le sang circulant ≥ 1%	1	1
Symptômes constitutionnels*	1	1

* : sueurs nocturnes, perte de poids (10%), fièvre non infectieuse

Facteurs de risques	DIPSSplus
score DIPSS faible	0
score DIPSS intermédiaire-1	1
score DIPSS intermédiaire-2	2
score DIPSS élevé	3
Besoin transfusionnel	1
Plaquettes< 100.10^9/L	1
Anomalies du caryotype**	1

** : caryotype complexe ou la présence d'une ou deux anomalites tels +8, -7/7q, i(17q), -5/5q, -12p, inv(3) ou réarrangements 11q23

Catégories	IPSS score	DIPSS score	DIPSSplus score
Faible	0	0	0
Intermédiaire-1	1	1-2	1
Intermédiaire-2	2	3-4	2-3
Elevé	> ou = 3	5-6	4-6

Table 4 : Les scores pronostiques de la myélofibrose primaire : IPSS, DIPSS et DIPSSplus

Les scores IPSS (International Prognostic Scoring System), DIPSS (Dynamic IPSS) sont calculés selon cinq facteurs de risques biologiques et cliniques pondérés chacun de un point (excepté pour l'hémoglobine avec deux points dans le calcul du DIPSS). Le score DIPSSplus prend en compte les mêmes facteurs mais également le besoin transfusionnel, le taux de plaquettes < 100.109/L et la présence d'anomalies cytogénétiques spécifiques. Les trois scores IPSS, DIPSS et DIPSSplus se calculent en faisant la somme de tous les points. Le total des points permet de classer les patients en quatre groupes pronostiques (faible, intermédiaire 1, intermédiaire 2 et élevé).

5.3. La myélofibrose secondaire à une PV ou à une TE

La PV et la TE évoluent dans 10 à 15% des cas à 15 ans en MF (post-PV/ET MF). La baisse des trois lignées hématopoïétiques est un des signes de l'évolution en MF pour les patients déjà suivis pour une PV ou une TE. La MF primaire et la MF secondaire (MF post-PV/TE) n'ont pas de différences cliniques et histologiques (Mesa *et al.* 2007) ; et la prévalence des anomalies du caryotype n'est pas différente entre les trois sous-types (MFP, MF post-PV et MF post-TE) (Tefferi *et al.* 2001). Toutefois, les évènements génétiques déclenchant la MF primaire ou secondaire ne sont pas connus et peu d'étude existe sur les comparaisons génomiques (mutations et aberration du nombre de copies) entre les MF primaires et secondaires.

5.4. La myélofibrose préfibrotique

La myélofibrose préfibrotique est souvent révélée par une thrombocytose isolée. Seule l'analyse médullaire (et notamment la biopsie ostéo-médullaire) permet de faire le diagnostic différentiel entre une TE et une MF préfibrotique (Barbui *et al.* 2013). Elle est décrite comme faisant partie du continnum de la MF (Barosi *et al.* 2012). Une étude faite sur une importante cohorte de 891 TE et 180 MF préfibrotiques a montré que ces dernières avaient de façon significative un risque plus élevé de transformation en MF et en LAM (Barbui *et al.* 2011).

D- PHYSIOPATHOLOGIE DES SMP CLASSIQUES NON LMC

1. HISTORIQUE DU DIAGNOSTIC

Afin de différencier les SMP des polyglobulies et thrombocytoses réactionnelles, la preuve de clonalité est indispensable pour affirmer le diagnostic.

1.1. La pousse spontanée des progéniteurs hématopoïétiques

Dans les années 1970-80, le diagnostic des SMP, se faisait le plus fréquemment par culture cellulaire des progéniteurs érythroïdes endogènes (BFU-E). Afin de discriminer les PV vraies des réactionnelles, les cliniciens utilisaient une des propriétés des cellules de SMP : la pousse endogène ou spontanée en absence de facteurs de croissance (IL-3, GM-CSF , CSF, TPO, IGF-1) (Prchal and Axelrad 1974).

1.2. L'inactivation des gènes portés par le chromosome X

Dans les années 1970-80, le diagnostic de PV ou de TE chez la femme se faisait également par la recherche du marqueur « X-chromosome-linked glucose-6-phosphate dehydrogenase (G6PD)». Le principe s'appuyait sur le fait que les patientes atteintes de PV et TE, avaient toutes les cellules sanguines monoclonales et homozygotes pour le gène *G6PD* porté par le chromosome X. Ce qui n'était pas le cas chez les individus sains puisque les cellules sont polyclonales et donc hétérozygotes pour G6PD. Ces observations peuvent également s'appliquer pour les monocytes et les lymphocytes B, témoignant de l'implication d'un progéniteur multipotent (Raskind *et al.* 1985).

1.3. L'hyper-expression de PRV1

Dans les années 2000, Temerinac et al, après avoir cloné le récepteur de surface des cellules hématopoïétiques CD177/PRV1 (Polycythemia Rubra Vera 1), ont montré que l'ARNm *PRV1* était hyper-exprimé dans les granulocytes des patients atteints de PV contrairement aux patients atteints de LMC, de TE, de polyglobulies réactionnelles et aux personnes saines (Temerinac *et al.* 2000). Toutefois, la protéine elle-même n'est pas hyper-exprimée. L'étude de l'expression de l'ARNm n'a pas été retenue comme marqueur diagnostique des PV, car PRV1 est également hyper-exprimé dans certains cas de TE (Teofili 2002) souvent en association avec la formation des BFU-E (Liu *et al.* 2003), et chez des patients non SMP hospitalisés présentant une leucocytose post-traumatique/chirurgicale et chez des patients stimulés sous G-CSF (Passamonti *et al.* 2004).

1.4. Les anomalies cytogénétiques au caryotype

En 1960, Nowell et Hungerford découvrent l'existence du chromosome Philadelphie chez des patients atteints de LMC. L'anomalie moléculaire mise en cause est une translocation (9;22) ayant pour conséquence la création de la protéine de fusion BCR-ABL. Par analogie avec cette anomalie cytogénétique, de nombreuses équipes ont recherché des anomalies similaires dans les SMP classiques non LMC, mais n'ont pas eu malheureusement le même succès. En effet, aucune anomalie cytogénétique spécifique des SMP n'existe. Les caryotypes sont la plupart du temps normaux puisque la fréquence des anomalies cytogénétiques est de 5% pour les TE, 15% pour les PV, et 35% pour les MF **(Table 5)** (Hussein *et al.* 2009)**.**

Les principales anomalies cytogénétiques au caryotype sont :

La délétion 20q : C'est l'anomalie la plus fréquente des SMP. Toutefois, elle n'est pas spécifique (Tam *et al.* 2009), puisqu'elle est également retrouvée dans les SMD, LMMC (leucémie myélomonocytaire chronique) et LAM (Hahm *et al.* 2012; Braun *et al.* 2011). Les délétions sont la plupart du temps larges et incluent de nombreux gènes. Parmi les nombreux gènes candidats, aucun n'a été retrouvé muté, suggérant que leur

haploinsuffisance est suffisante au processus de la leucémogenèse (Aziz *et al.* 2012). Peu d'études ont analysé son impact pronostique sur les SMP (Bench *et al.* 2001; Hasserjian and Dal Cin 2011). Contrairement aux études sur les SMD et les formes dysplasiques de la LMMC qui ont montré que la délétion 20q isolée, confère un pronostic favorable (Braun *et al.* 2011). Sur le plan morphologique, sa présence est associée à une dysplasie plus marquée des lignées érythroblastique et mégacaryocytaire.

La délétion 13q : Au sein des trois sous-types, cette anomalie est retrouvée de façon plus spécifique dans les MF (Tam *et al.* 2009). Les délétions 13q impliquent essentiellement la région 13q12-13q1414, une région riche en gènes autour du gène *FLT3* et *RB1* (Retinoblastoma 1). Des pertes d'hétérozygotie ou « Loss of heterozygoty » (LOH) par disomie uniparentale acquise sont observées dans 43% des SMP. Mais aucune mutation du gène *RB1* n'a été décrite à ce jour, suggérant l'existence d'un autre gène potentiellement intéressant ou comme la délétion 20q l'existence d'une haploinsuffisance (Bench *et al.* 2001).

La trisomie 8 : Cette anomalie n'est pas non plus spécifique des SMP. Elle est retrouvée dans 5% des LAM, 10 à 15% des SMD, et 35% des LMC en phase aiguë. Dans le score pronostique des MF primaire DIPSSplus, la trisomie 8 est associée à un mauvais pronostic.

La trisomie 9 : Elle est retrouvée plus spécifiquement dans la PV (Najfeld *et al.* 2002; Gangat *et al.* 2008). De façon intéressante mais non accessible par le caryotype, la perte d'hétérozygotie (LOH) au niveau du bras court du chromosome 9 est la lésion cytogénétique la plus fréquente ; elle contient plusieurs gènes dont *JAK2*. Sa fréquence est 34% dans la PV (34%), 22% dans la MF et 3% dans la TE (Kralovics *et al.* 2005). L'étude de cette zone a permis de « pointer » JAK2.

Les translocations équilibrées : Ce sont des anomalies très rares dans les SMP classiques non LMC (Bench *et al.* 2001). Certaines ont été décrites dont la t(1;7)(p31;q22), t(1;12)(p31;q21), t(1 ;20)(q32;q13.3).

Anomalies chromosomiques	PV n=534	TE n=456	MFP n=397
del (20q)	8.4 %	0.2 %	7.7 %
del (13q)	3 %	0.7 %	6.3 %
trisomie 8	6.9 %	0.9 %	5 %
trisomie 9	6.6 %	0.2 %	1 %
trisomie 1q	3.6 %	0	3.5 %
del (7q) ou -7	0.9 %	0	3.8 %
del (5q) ou -5	3.2 %	0	1.5 %
% patients avec une ou plusieurs anomalies	33.7 %	5 %	39.5 %

Table 5 : Fréquences des anomalies cytogénétiques dans les SMP classiques non LMC au diagnostic et en cours d'évolution. D'après (Bench *et al.* 2001)*.*

2. LE DEBUT DU DIAGNOSTIC MOLECULAIRE

2.1. Le gène *JAK2* est muté dans les SMP classiques non LMC

2.1.1. Découverte de la mutation JAK2V617F

C'est en 2005 que la découverte de la mutation JAK2V617F a révolutionné le diagnostic des SMP puisque celle-ci est décrite dans près de 97% des PV et environ la moitié des cas de TE et MF (Levine *et al.* 2007). Cette découverte a été faite par quatre équipes (James, Ugo, and Vainchenker 2005; Kralovics *et al.* 2005; Baxter *et al.* 2005; Levine *et al.* 2005) selon plusieurs approches :

i) **L'approche cellulaire** s'est faite sur un modèle de culture en milieu liquide de la différenciation érythroïde spontanée des érythroblastes de PV ; mis au point par le Dr Valérie Ugo. Ce modèle a été utilisé afin d'inhiber JAK2 par la technique de siRNA ; les résultats ont montré une inhibition de la différenciation érythroïde terminale indépendante de l'EPO. (James, Ugo, and Vainchenker 2005). Cette équipe a également montré par western blot, une phosphorylation anormale de la protéine JAK2 après sevrage en EPO, suggérant une activation constitutive de la voie.

ii) **La première approche génomique** a utilisé la technique de SNP-array (Single Nucleotide Polymorphism) qui a révélé une région perdue avec perte d'hétérozygotie sur le bras court du chromosome 9. L'étude de la zone minimale critique de 6.2 Mb d'environ 40 gènes contenait le gène *JAK2* (Kralovics *et al.* 2005).

iii) **La deuxième approche génomique** s'est intéressée au séquençage du gène *JAK2*. Cette approche a permis d'identifier la mutation « hotspot » JAK2V617F (Baxter *et al.* 2005; Levine *et al.* 2005; James, Ugo, and Vainchenker 2005; Kralovics *et al.* 2005).

Au niveau de la protéine, la mutation JAK2V617F se traduit par la substitution d'une Valine par une Phénylalanine en position 617 dans le domaine JH1. Dans les cellules mutées, les voies de signalisation régulées par JAK2 sont activées constitutivement en l'absence de cytokine, ce qui aboutit à une prolifération cellulaire excessive. La mutation JAK2V617F est acquise (non présente dans les lymphocytes T) et clonale (retrouvée dans les érythroblastes, les polynucléaires neutrophiles, les monocytes et les plaquettes). Toutefois Delhommeau et al ont montré que cette mutation était également présente dans les lymphocytes B et NK (natural killer) suggérant que la mutation est acquise au stade de progéniteur commun lympho-myéloïde (Delhommeau *et al.* 2007).

En 2007, de nouvelles mutations de *JAK2* ont été identifiées sur l'exon 12, exclusivement retrouvées dans les PV non mutées JAK2V617F (Scott *et al.* 2007). A l'heure actuelle, 17 mutations sur l'exon 12 de *JAK2* ont été décrites (Scott 2011; Passamonti *et al.* 2011).

2.1.2. Le pourcentage de la charge allélique ou « allele burden »

Dans les modèles murins, la modulation de la charge allélique JAK2V617F influe sur le phénotype de la maladie (Tiedt *et al.* 2008), la PV est obtenue avec une expression de JAK2V617F importante (Lacout *et al.* 2006), et la TE avec une expression plus faible (Li *et al.* 2010). Ainsi, la charge allélique ou « allele burden » de JAK2V617F mesuré sur les cellules sanguines peut aider au diagnostic car variable selon les pathologies : faible et très rarement supérieur à 50% dans la TE, supérieur à 50 % dans au moins un tiers des cas dans la PV et la MFP, et très élevé dans les MF post-PV. Ces variations du pourcentage JAK2V617F sanguin sont liées en partie à des différences de nombre d'allèles mutés dans les cellules : un allèle muté (hétérozygotie), deux allèles mutés (homozygotie), ou même trois (JAK2 est situé sur le 9p et la trisomie 9 est une des anomalies cytogénétiques les plus

fréquentes dans la PV). Dans la grande majorité des cas de PV et de MFP, des cellules mutées à l'état homozygote sont présentes, alors que les TE comportent plutôt des clones hétérozygotes (Scott *et al.* 2006; Dupont *et al.* 2007). Un pourcentage JAK2V617F supérieur à 50 % signe l'existence d'au moins un clone homozygote chez le patient, mais un pourcentage < 50 % ne l'exclut pas. En conséquence, les termes « homozygotes » et « hétérozygotes » ne sont pas adaptés pour définir des catégories pronostiques en clinique.

2.1.3. Impact pronostique de la mutation JAK2V617F

L'impact pronostique de la mutation de *JAK2* est débattu. En effet, selon certaines études, la mutation aurait un impact sur la survie et le risque de transformation en LAM (Barosi *et al.* 2007) alors que d'autres ne retrouvent aucun intérêt pronostique (Tefferi *et al.* 2008). Ces différences peuvent s'expliquer selon la charge allélique de la mutation JAK2V617F (Passamonti 2011). En effet les patients avec une faible charge allélique auraient une meilleure survie (**Figure 19**), l'acutisation serait freinée par une compétition entre les clones. Alors que pour les patients non-mutés JAK2 ou avec une forte charge allélique, l'instabilité génomique serait plus importante et favoriserait l'acutisation.

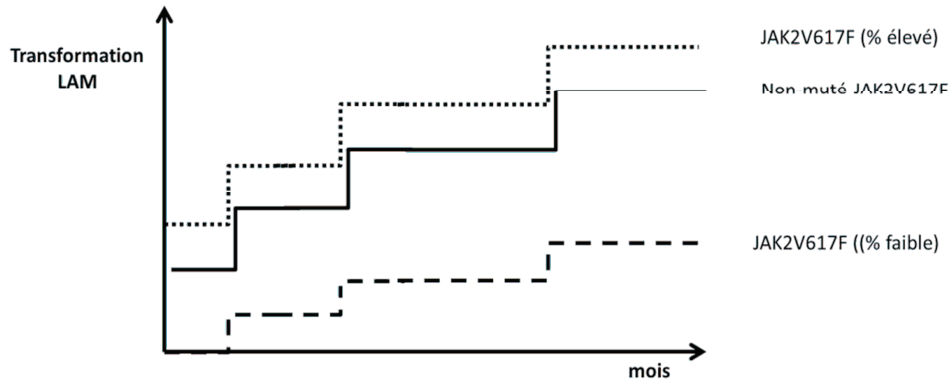

Figure 19 : Impact pronostic de la mutation JAK2V617F homozygote et hétérozygote

D'après la présentation du Dr Giovanni Barosi lors du congrès ESH Vienne 2013. Ce schéma illustre la transformation en LAM plus importante des patients mutés JAK2 (avec une forte charge allélique de la mutation JAK2V617F) et des patients non muté JAK2.

2.2. Le gène MPL est muté dans les SMP classiques non LMC

En 2006, Pikman et al ont étudié les SMP sporadiques JAK2 non mutés en séquençant plusieurs RCK : EPO-R, MPL, GCSF-R. La mutation « hotspot » W515L/K a été identifiée au niveau de l'exon 10 de du gène *MPL,* dans 5 à 10 % des SMP JAK2 non mutés et 1 à 5 % des TE JAK2 non mutées (Pikman *et al.* 2006; Pardanani *et al.* 2006). Le deuxième « hotspot » de MPL est retrouvé dans les formes familiales et sporadiques : W505A (**Figure 20**). Dans, les modèles murins-W515L, la mutation confère un phénotype « MFP-like » avec une thrombocytose, une splénomégalie et une fibrose (Heckl *et al.* 2011). Dans de rare cas, les mutations *MPL* et *JAK2* peuvent coexister comme deux clones indépendants ou deux sous-clones (Pardanani *et al.* 2006).

A ce jour, les études sur l'impact pronostique de la mutation *MPL* ont montré que la survie et la transformation leucémique ne différaient pas entre les mutés et non mutés (Pardanani *et al.* 2011; Beer *et al.* 2010).

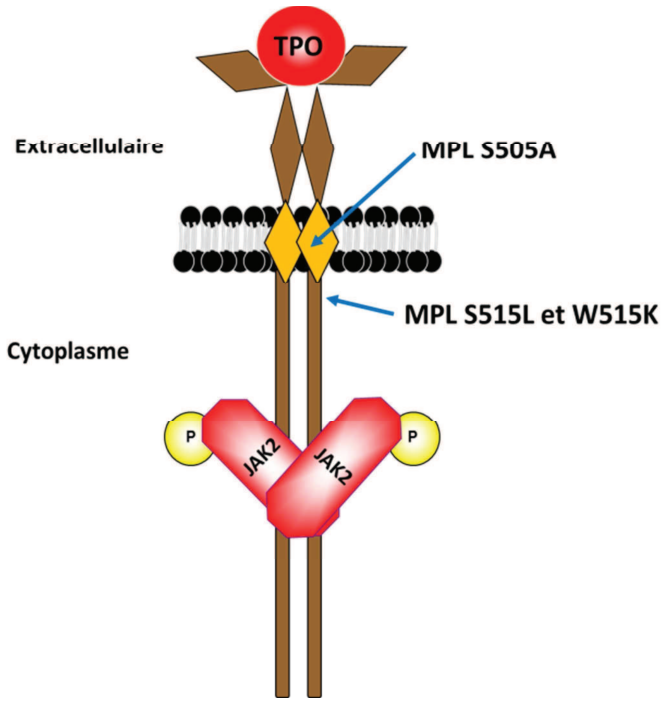

Figure 20 : Les mutations « hotspot » de MPL, récepteur de la thrombopoïétine

D'après (Bennett and Stroncek 2006).

Présentation des travaux

A. ETAT DES LIEUX EN 2009

L'année 2009 fût l'année pendant laquelle j'ai commencé mes travaux sur la caractérisation moléculaire des SMP classiques lors de mon stage de master 1. En 2009, les anomalies moléculaires connues étaient les mutations des gènes *JAK2* et *MPL*.

Selon les recommandations de l'OMS de 2008, la recherche de la mutation JAK2V617F (exon 14) fait partie du diagnostic de SMP. Pour les cas où la mutation JAK2V617F n'est pas retrouvée il est recommandé de rechercher les mutations de *JAK2* sur l'exon 12 pour la suspicion d'une PV ; et de rechercher des mutations du gène *MPL* en cas de suspicion de TE ou MF. Toutefois, il existe de nombreux SMP où aucune anomalie moléculaire n'a encore été détectée.

1. LES ANOMALIES DU GENE *TET2*

En 2009, Delhommeau et al ont rapporté une petite délétion du chromosome 4 en 4q24 dans une PV grâce à la technique d'hybridation génomique comparative (ou CGH-array) (Delhommeau *et al.* 2009). La région minimale délété contenait un seul gène : *TET2*. Le gène *TET2* a été séquencé et des mutations localisées sur tout le gène ont été identifiées. Les mutations de *TET2* ont été décrites dans les fractions CD34+/CD38-, et dans les fractions de cellules myéloïdes et lymphoïdes précédant l'acquisition de la mutation JAK2V617F. Ces anomalies ne sont pas spécifiques des SMP ; des mutations et des délétions ont été également observées dans d'autres hémopathies myéloïdes (SMD, LMMC, LAM). Delhommeau et al ont alors suggéré que les anomalies de *TET2* intervenaient de façon précoce au cours de la leucémogenèse et que l'inactivation de *TET2* engendrerait un état pré-leucémique. La survenue d'un 2ème évènement oncogénique comme JAK2V617F déclencherait le SMP du point de vue phénotypique. Toutefois, l'étude de l'architecture clonale de patients mutés JAK2 et TET2 a montré que les mutations de ce dernier pourraient survenir avant ou après la mutation de *JAK2* dans le même clone, mais

également un clone différent (Delhommeau *et al.* 2009). L'étude des transformations de SMP en LAM a ensuite mis en évidence des situations où des mutations de *TET2* pouvaient être détectées au moment de l'acutisation (Abdel-Wahab *et al.* 2010).

2. LES ANOMALIES DU GENE *ASXL1*

Parallèlement au sein du laboratoire, l'utilisation de la technique de CGH-array nous a permis d'identifier deux délétions impliquant les gènes *ASXL1* (20q11) et *ASXL2* (2p24.1) au sein d'un patient atteint de SMD. Ces deux gènes ont été séquencés. Aucune mutation n'a été identifiée sur *ASXL2* contrairement au gène *ASXL1* retrouvé muté dans 10 % des SMD, 40% des LMMC (Gelsi-Boyer *et al.* 2009) et dans 8% des SMP (Carbuccia *et al.* 2009). Ces études ont été les premières à décrire des mutations de ce gène dans des maladies humaines. Pour les SMD, LMMC et SMP, les mutations étaient présentes dans les cellules matures sanguines, ainsi que dans les fractions CD34+. Les mutations étaient de type décalage du cadre de lecture (« frameshift ») qui avaient pour conséquence la traduction d'une protéine tronquée, suggérant un domaine PHD en C-terminal non fonctionnel ou inexistant (Fisher *et al.* 2006) (**Figure 21**).

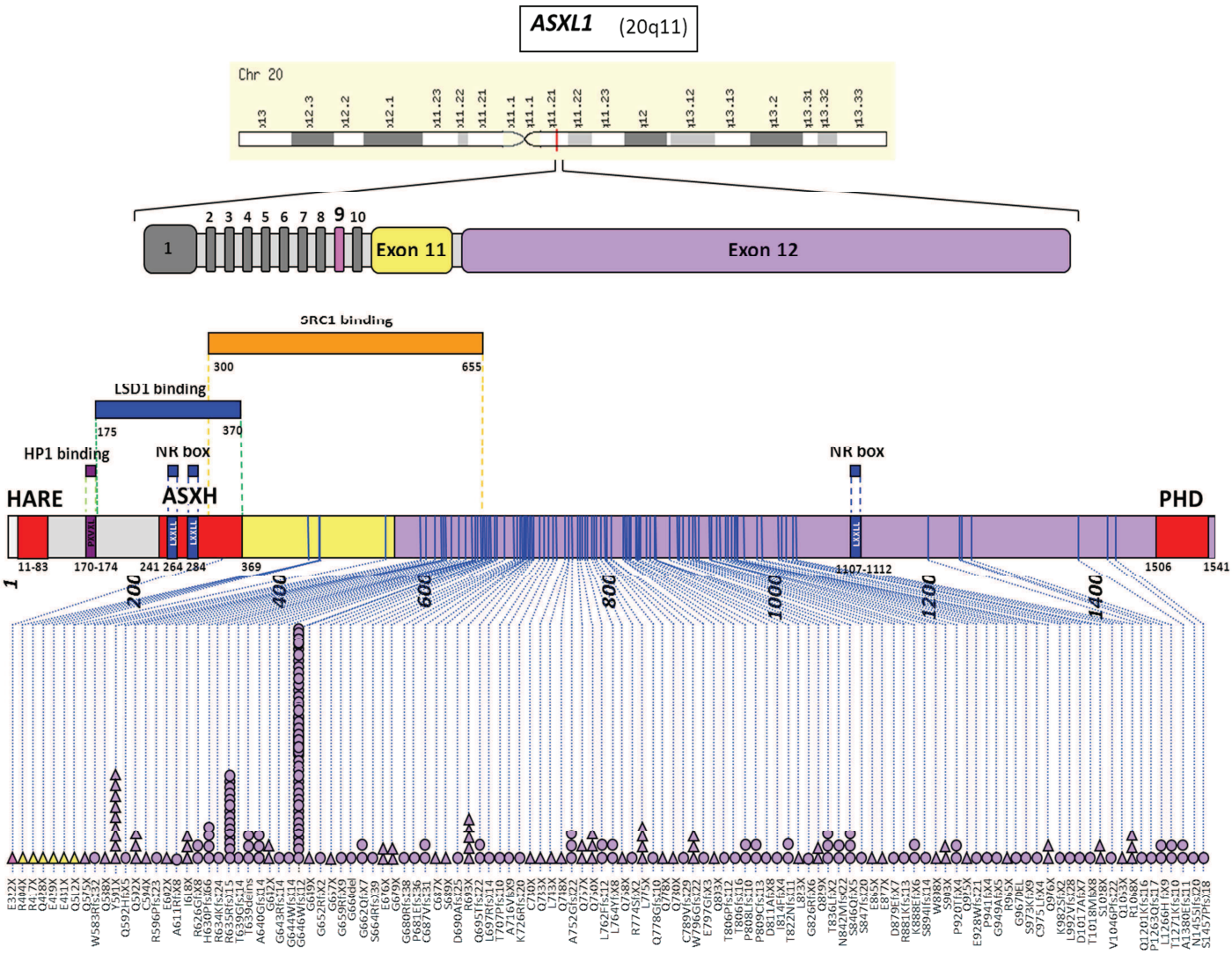

Figure 21 : Distribution des mutations le long de la protéine ASXL1, revue de la littérature

(Gelsi-Boyer, Brecqueville *et al.* 2012). Du haut vers le bas, sont représentées la localisation du gène *ASXL1* sur la région chromosomique 20q11, la structure exonique d'*ASXL1* et la protéine avec ses motifs et domaines conservés: la région HARE helix-turnhelix en N-terminal, la région où se fixe la protéine HP1/CBX5 binding region, le domaine ASXH, qui contient le motif LXXLL, et la région en C-terminale qui contient le domaine PHD (plant homeodomain) (PHD). En bas, sont reportées les mutations de la protéine décrites dans la littérature, les cercles indiquent des mutations « frameshift » et les triangles des mutations « non-sens ». Le code couleur est basé sur la localisation des exons (exons 9, 11 et 12).

B. QUESTIONS SOULEVEES EN 2009

Au sein de la voie JAK/STAT, la protéine JAK2 altérée participe activement à la physiopathologie des SMP. La mutation JAK2V617F est commune aux trois SMP classiques non LMC. Toutefois celle-ci n'est pas indispensable à la physiopathologie des SMP puisque dans de très rares cas de PV et près de 50% des cas de TE et MF, le gène *JAK2* n'est pas muté. En 2009, lors de mon arrivée au laboratoire, le « boom moléculaire » des hémopathies myéloïdes était à ses débuts (notamment avec la découverte des altérations des gènes *TET2* et *ASXL1*), conduisant mes travaux sur les SMP selon les questions ci-dessous (**Figure 22**) :

- Existent-ils d'autres acteurs impliqués dans la signalisation des facteurs de croissance hématopoïétiques ?
- Quels sont les acteurs aux autres fonctions cellulaires altérées ?
- Quelle est la proportion des gènes mutés dans les PV, TE et MF ?
- Existent-t-ils des anomalies spécifiques d'un sous-type de SMP?
- Quelles sont les particularités moléculaires dans les myélofibroses ?
- Comment expliquer l'évolution des PV/TE en MF ?
- Existent-t-ils des anomalies spécifiques d'une évolution en LAM ?
- Quelles sont les combinaisons de gènes mutés dans les LAM post-SMP ?
- Quel modèle proposé pour expliquer la physiopathologie des SMP ?
- Existent-t-il des mutations spécifiques d'un pronostic ?

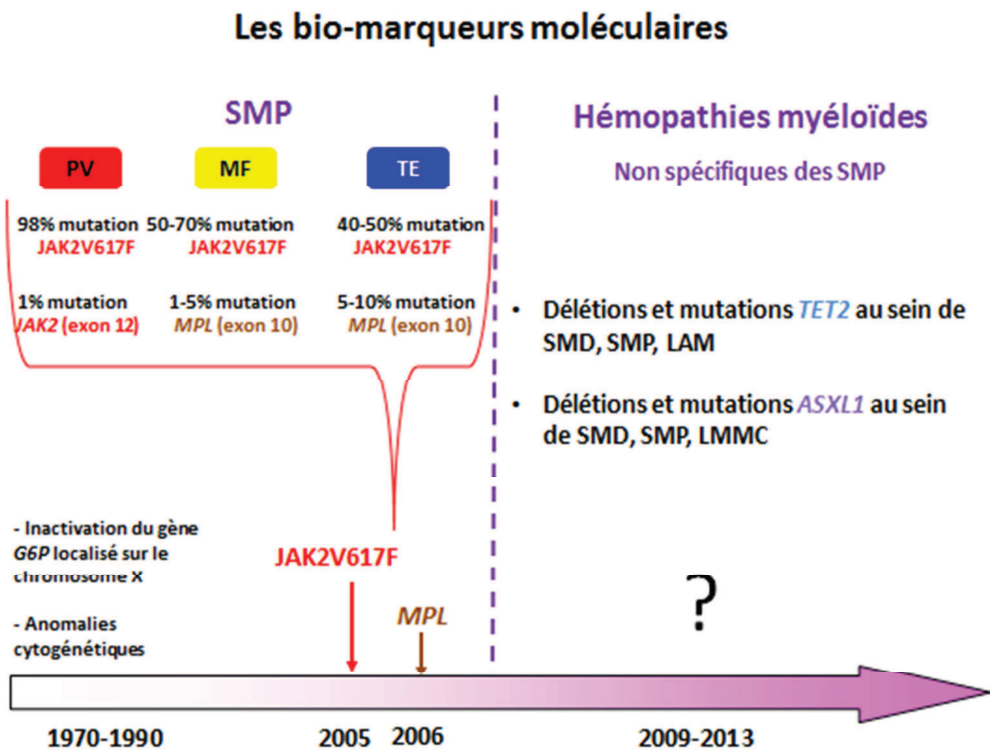

Figure 22 : Etat des lieux de la caractérisation moléculaire des SMP en 2009 lors de mon arrivée au laboratoire

Dans les années 1970-1990, le diagnostic moléculaire des SMP se faisait par l'étude d'inactivation du gène *G6P* localisé sur le chromosome X, ainsi que par des analyses cytogénétiques. En 2005, la découverte de la mutation JAK2V617F a véritablement révolutionné le diagnostic des SMP, suivi en 2006 par la découverte des mutations du gène *MPL*. En 2009, deux gènes *TET2* et *ASXL1* ont été identifiés délétés et mutés dans plusieurs hémopathies. Mes travaux de caractérisation moléculaire se sont poursuivis dans cette dynamique insufflée par la découverte de ces deux gènes.

C. LA DEMARCHE

Mes travaux se sont principalement focalisés sur les cellules différenciées du sang périphérique, afin de rechercher les évènements moléculaires impliqués dans la physiopathologie des SMP. Nos objectifs principaux étaient d'améliorer notre compréhension sur les mécanismes d'altérations et d'identifier de nouveaux marqueurs biologiques pour le diagnostic, le suivi et le pronostic. Pour cela, nous avons choisi plusieurs approches moléculaires : le séquençage, l'hybridation génomique comparative (CGH-array) et l'étude des profils d'expression géniques (transcriptome). Ces approches ont été réalisées de façon concomitante selon une analyse génomique intégrée.

1. LE MATERIEL

Pour réaliser ces approches, il a été capital de constituer une banque d'échantillons. La première partie de mes travaux a été de poursuivre la collecte des échantillons de patients (projet préalablement initié par ma directrice de thèse le Dr Anne Murati). Le matériel d'étude était majoritairement du sang périphérique (SP) disponible grâce aux examens diagnostiques et au suivi des patients atteints de SMP au sein de l'Institut Paoli-Calmettes (Marseille).

Nous avons choisi d'étudier les **cellules totales du SP** des patients atteints de SMP comportant une majorité de polynucléaires neutrophiles, quelques lymphocytes et monocytes. Nous sommes partis du postulat que si une mutation était présente dans le compartiment myéloïde, elle serait représentée dans le SP. Néanmoins, nous avons triés certains SP afin de définir quelles étaient les populations affectées (tri CD34+ et CD3+).

Les **cellules CD34+** du sang périphérique ont été isolées des autres cellules (CD34-) afin d'identifier si les anomalies retrouvées dans les cellules différenciées étaient également présentes dans le contingent des progéniteurs. L'antigène de surface CD34 est porté par les CSH et les progéniteurs capables de se différencier en cellules myéloïdes (Verfaillie *et al.* 1990), en lymphocytes B (Baum *et al.* 1992), en « natural killer » (Miller *et al.* 1992), en lymphocytes T (Srour *et al.* 1993).

Les **cellules triées CD3+** ont été isolées afin d'avoir des cellules contrôles. En effet les cellules marquée CD3+ sont des lymphocytes T, qui ne sont probablement pas affectées par les anomalies retrouvées dans les cellules myéloïdes des SMP (Delhommeau *et al.* 2007). Toutefois, nous ne pouvons pas exclure formellement une anomalie acquise par le progéniteur commun des lignées myéloïde et lymphoïde. Ainsi l'obtention d'ADN à partir de cellules non-hématopoïétiques telles que les cellules de **l'épithélium buccal** a été nécessaire afin d'identifier si la mutation est germinale ou somatique.

2. LES METHODES

2.1. Le séquençage

Les mutations sont des anomalies d'une ou plusieurs bases de la séquence génomique d'un gène. Selon la localisation des mutations, les conséquences peuvent varier. Nous avons choisi cette approche afin d'étudier la prévalence dans les SMP de mutations déjà décrites dans les hémopathies myéloïdes et afin de rechercher de nouvelles mutations sur des gènes candidats révélés par la CGH-array ou par le transcriptome. Les mutations peuvent appartenir à plusieurs catégories i) **faux-sens** (remplacement d'un nucléotide par un autre entrainant une modification de l'acide aminé) ; ii) **non-sens** (remplacement d'un nucléotide par un autre entrainant un codon-stop avec pour conséquence, une traduction d'une protéine tronquée) ; iii) **silencieuse** (remplacement d'un nucléotide par un autre mais n'entrainant aucune modification de l'acide aminé du fait de la redondance du code génétique) ; iv) « **frameshift** » (délétion ou insertion d'un ou plusieurs nucléotides entrainant un décalage du cadre de lecture et pouvant entrainer de façon prématurée l'apparition d'un codon stop).

Dans notre étude, nous avons pris en compte les mutations ponctuelles de type faux-sens, non-sens et les mutations « frameshift ». Les mutations silencieuses ont été exclues. Les mutations ont été recherchées dans les exons codants et à proximité des exons où sont localisés les sites donneurs et accepteurs d'épissage. Toutes les mutations ont été vérifiées par une PCR indépendante à partir de l'échantillon « mère ». Les mutations ont été

également vérifiées dans l'ADN « contrôle » des patients afin d'affirmer le caractère somatique ou germinale. L'ADN contrôle a été extrait soit à partir d'écouvillonnage de l'épithélium buccal, soit à partir de cellule triée CD3+ (contingent lymphoïde T). Toutes les mutations de type polymorphisme ou SNP, ont été exclues de nos analyses après avoir été vérifiées au sein de bases de données.

La recherche des mutations a été effectuée par la technique de séquençage Sanger qui est une technique classique pour les laboratoires de biologie. En effet en 2009, les outils de séquençage de génome à haute résolution, ou personnalisé (next generation sequencing ou NGS) étaient peu répandus. La technique de séquençage Sanger a plusieurs avantages : peu de matériel est nécessaire (moyenne de 50 à 100 ng selon le gène), rapide, reproductible. Néanmoins, la technique a des inconvénients, des faux positifs peuvent être révélés, l'analyse de la charge allélique (« allele burden ») n'est pas possible, trouver un SNP hétérozygote est délicat car les bases ne sont pas représentées au ratio 1 :1, le coût est assez élevé, la sensibilité n'est pas optimale, elle est de 10 à 20 % (une mutation homozygote n'est détectable que si elle est présente dans plus 10% des cellules et une mutation hétérozygote dans plus de 20% des cellules).

2.2. L'hybridation génomique comparative ou la CGH-array

La technique d'hybridation génomique comparative de type haute résolution (CGH-array) détecte les aberrations du nombre de copies (CNA) d'un gène (gain ou délétion). Son principe est une hybridation compétitive entre de l'ADN tumoral et de l'ADN contrôle sur une puce pangénomique à oligonucléotides (Pollack *et al.* 1999, 2002). La CGH-array a été utilisée en complément des informations fournies par le caryotype. La CGH-array permet de révéler au sein de nombreuses pathologies malignes des CNA non détectées par le caryotype. Toutefois, celui-ci reste indispensable dans la détection des clones minoritaires (Tiu *et al.* 2011; Das and Tan 2013; Tefferi, Sirhan, *et al.* 2009). Les différences entre ces deux techniques sont répertoriées dans la **Table 6**.

Comparaisons	Caryotype	CGH-array
Prélèvement	moelle totale (++) sang périphérique (+)	moelle totale (++) sang périphérique (++)
Echec de la méthode	Possible	Très rare
Etape du cycle cellulaire	en division (bloquées en métaphase)	en division et en non-division
Résolution	5-2 Mb	36 kb (théorie), précision point cassure (56kb)
Nombre de cellule	20 cellules	1 ug d'ADN soit environ 10^5 cellules
Population cellulaire	Sélectionnée (pousse des progéniteurs)	Totale (cellules différenciées présentes)
Détection des mosaïques	oui	non
Détection réarrangements équilibrées et non équilibrées (translocations, insertions, inversions)	oui	non

Table 6 : Comparaison des différences des techniques de caryotypes et de CGH-array

D'après (Coe *et al.* 2007; Tefferi, Sirhan, *et al.* 2009)*.*

2.3. Les profils d'expression génique ou le transcriptome

Afin d'étudier les profils d'expression des gènes des patients atteints de SMP, nous avons choisi d'utiliser la technique des puces à ADN. Celle-ci permet de caractériser et de quantifier les ARNm. Son principe repose sur la quantification de l'hybridation complémentaire entre les fragments d'ARNc marqués (appelés cibles, qui correspondent aux ARNm de l'échantillon) et les oligonucléotides (appelés sondes) fixés sur la puce (correspondants aux ARNm du transcriptome humain).

Nous avons choisi de travailler sur l'ensemble de la population cellulaire totale étant donné que la présence de la mutation du gène *JAK2* dans les polynucléaires neutrophiles confirme le caractère malin de cette population.

Résultats

Publications

- **Article 1 - Mutations of *ASXL1* gene in myeloproliferative neoplasms.** Carbuccia N., Murati A, Trouplin V, Brecqueville M, Adélaïde J, Rey J, Vainchenker W, Bernard O, Chaffanet M, Vey N, Birnbaum D & Mozziconacci MJ.(2009). *Leukemia*, 23(11), 2183–2186.

- **Article 2 - Rare mutations in *DNMT3A* in myeloproliferative neoplasms and myelodysplastic syndromes.** Brecqueville M, Cervera N, Gelsi-Boyer V, Murati A, Adélaïde J, Chaffanet M, Rey J, Vey N, Mozziconacci MJ & Birnbaum D.(2011). *Blood Cancer Journal*, *1*(5), e18.

- **Article 3 - Mutations and deletions of the *SUZ12* polycomb gene in myeloproliferative neoplasms.** Brecqueville M, Cervera N, Adélaïde J, Rey J, Carbuccia N, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D, Gelsi-Boyer V & Murati A.(2011). *Blood Cancer Journal*, 1(8), e33.

- **Article 4 - Alterations of polycomb gene *BMI1* in human myeloproliferative neoplasms.** Brecqueville M, Adélaïde J, Bertucci F, Finetti P, Chaffanet M, & Murati A.(2012). *Cell Cycle*, *11*(16), 3141–3142.

- **Article 5 - Mutation Analysis of *ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12* and *TET2* in Myeloproliferative Neoplasms.** Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, Cervera N, Gelsi-Boyer V, Arnoulet C, Gisserot O, Verrot D, Slama B, Vey N, Mozziconacci MJ, Birnbaum D & Murati A.(2012).*Genes, Chromosomes & Cancer*, 51(8), 743–755.

- **Article 6 - Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase.** Brecqueville M, Rey J, Devillier R, Guille A, Gillet R, Adélaide J, Gelsi-Boyer V, Arnoulet C, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D & Murati A. (2013) *Haematologica*, in press.

ARTICLE n°1

Mutations of *ASXL1* gene in myeloproliferative neoplasms.

Carbuccia N., Murati A, Trouplin V, Brecqueville M, Adélaïde J, Rey J, Vainchenker W, Bernard O, Chaffanet M, Vey N, Birnbaum D & Mozziconacci MJ.

Leukemia, 23(11), 2183–2186. (2009)

Article 1 - Mutations of *ASXL1* gene in myeloproliferative neoplasms.

En 2009, les gènes *TET2* et *ASXL1* avaient été rapportés altérés dans plusieurs hémopathies myéloïdes (Delhommeau *et al.* 2009) (Gelsi-Boyer *et al.* 2009). Cependant peu d'études décrivaient les fréquences des mutations de *TET2* au sein des sous-types PV, TE et MF ; et *ASXL1* n'avait jamais été étudié au sein des SMP. Nous avons donc recherché les mutations de ces deux gènes au sein d'une série de 64 patients atteints de SMP (10 PV, 35 TE, 10 MFP, 1 SMP préfibrotique, et 3 SMP inclassables), ainsi que dans une série contrôle de formes réactionnelles (7 thrombocytoses secondaires et 5 polyglobulies secondaires). La recherche des mutations du gène *TET2* était inscrite dans un projet INCA multicentrique français initié par le Dr François Delhommeau où environ 1000 cas de SMP ont été colligés.

Ces travaux ont mis en évidence les éléments suivants :

- Nous avons identifié des mutations des gènes *TET2* dans 6 % (4/64) et *ASXL1* dans 8 % (5/64) des cas, soulignant la dérégulation des voies épigénétiques dans la physiopathologie des SMP. A noter que parmi les patients mutés pour ces deux gènes, un patient atteint de MFP portait ces deux mutations, mais pas la mutation JAK2V617F.
- Les mutations des gènes *TET2* et *ASXL1* étaient présentes au sein des cellules matures et immatures (fractions CD34+), suggérant que l'atteinte de ces deux gènes était précoce au cours de l'hématopoièse.
- Ces résultats soulignent l'intérêt potentiel de la recherche des mutations des gènes *TET2* et *ASXL1* afin d'améliorer le diagnostic des SMP. Ces mutations sont-elles prédominantes ou spécifiques d'un sous-type de SMP (PV, TE ou MF) ? Sont-elles mutuellement exclusives ? Sont-elles de mauvais pronostic? Existe-il d'autres gènes associés à l'épigénétique mutés dans les SMP ?

induce expression of the pump. As we are unable to directly measure epoxomicin uptake we utilized another P-gP substrate to determine if drug efflux is altered in KMS11R cells. Doxorubicin is an established substrate for P-gP and it can be easily measured in the cell because it fluoresces red. We compared the fluorescence of KMS11 and KMS11R cells treated with various concentrations of doxorubicin for 24 h. No differences in background fluorescence were observed and both cell lines could take up doxorubicin in a dose-dependent fashion (Figure 2b). However, the uptake observed was 3–5 fold lower in the resistant line. In addition, KMS11R cells are significantly resistant to doxorubicin-induced cell death (not shown).

Finally, we determined the role of P-gP in the acquired resistance to epoxomicin by observing the effects of inhibiting P-gP function with verapamil. Verapamil had no effect on the viability of either cell line nor did it alter the sensitivity of KMS11 cells to epoxomicin (Figure 3a) or doxorubicin (not shown). In contrast verapamil resensitized KMS11R to both drugs (Figure 3a and not shown). FACS analysis of cells treated with doxorubicin and verapamil revealed that the addition of verapamil to the KMS11R cells resulted in an increase in red fluorescence in doxorubicin-treated cells (Figure 3b).

Given the promise of bortezomib it is not surprising that other proteasome inhibitors that are either irreversible and/or inhibit the other activities of the proteasome are currently being tested in the clinic. As proteasome inhibitors become more commonly used in newly diagnosed diseases it will be important to understand mechanisms of acquired resistance to this class of agents. In addition, we will need to know how acquired resistance to proteasome inhibitors alter the response to other therapeutics used to treat myeloma patients.

Acquired expression of P-gP has been observed in myeloma patients and cell lines. P-gP is rarely seen in newly diagnosed patients, however, increased expression was observed in cells from approximately 75% of patients treated with vincristine, doxorubicin and dexamethasone.[6] We observed that P-gP is expressed and the P-gP inhibitor verapimil sensitizes KMS11R to epoxomicin. These data suggest that the acquired resistance observed was the result of P-gP expression. This is the first report to demonstrate the acquisition of P-gP with a proteasome inhibitor. Although it is possible that the induction of P-gP could be the result of inhibition of protein turnover, this does not appear to be a likely mechanism. First epoxomicin does not acutely induce P-gP (Figure 2). More importantly we also observe increased expression of the MDR mRNA in these cells (not shown). Thus the mechanism is likely to be due to increased gene expression possibly because of gene amplification. This remains to be determined.

Finally, our data suggest that carfilzomib could be ineffective in the treatment of P-gP-positive myeloma. Consistent with this possibility, a recent study demonstrated that the P-gP-positive cell line, RPMI8226-Dox40, is resistant to carfilzomib; however, they can be sensitized by co-treatment with verapamil.[7] Unfortunately such resensitization of cells to P-gP substrates has not proven to be effective clinically.[6,8] Several agents that inhibit drug efflux have been tested and either proven to be too toxic or had little effect on efficacy. In addition to confirming that epoxomicin-based compounds may be subject to this type of resistance mechanism we demonstrated that they can initiate resistance in this fashion. Therefore careful consideration of the use of these compounds both as single agents and in combination therapies will be needed to assure both efficacy of this therapy as well as subsequent treatment regimens.

Conflict of interest

The authors declare no conflict of interest.

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Mutations of *ASXL1* gene in myeloproliferative neoplasms

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Leukemogenesis is a complex process resulting from the alterations of many genes that perturb the processes leading from a hematopoietic stem cell to a differentiated blood cell. The *ASXL1* (additional sex combs like 1) gene is located in the chromosomal region 20q11 and may be affected in hematologic malignancies.[1] *ASXL1* belongs to a family of three identified members that encode poorly characterized proteins regulating chromatin remodeling.

The ASXL proteins contain a C-terminal PHD (plant homeodomain) finger and belong to polycomb and mixed lineage leukemia/trithorax chromatin modifier complexes. We have recently shown that *ASXL1* is mutated in roughly 10% of myelodysplastic syndromes (MDS) and 40% of chronic myelomonocytic leukemias;[2] mutations were mostly heterozygous, frameshifts, and located in exon 12. The predicted truncated protein would lack its PHD domain, thus compromising the function of the associated chromatin modifiers. In contrast, we did not find mutations of *ASXL2* in MDSs and chronic myelomonocytic leukemias (N Carbuccia and MJ Mozziconacci, unpublished). By using high resolution array-comparative genomic hybridization, we also detected a heterozygous loss of *ASXL1* in one MDS case.[2] These observations suggest that *ASXL1* haploinsufficiency plays a role in leukemogenesis.

To determine whether *ASXL1* could be involved in other types of myeloid diseases, we studied the *ASXL1* gene in 64 myeloproliferative neoplasms (MPNs). Our series comprised 10 cases of polycythemia vera, 35 cases of essential thrombocythemia (ET), 10 cases of primary myelofibrosis (PMF), 1 case of prefibrotic PMF, 5 MPNs at blast phase and 3 unclassifiable MPNs. We also searched for mutations in 12 non-MPN cases comprising 7 secondary thrombocytosis and 5 secondary erythrocytosis. All patients signed an informed consent and the study was approved by our ethical committee. We searched for *ASXL1* deletions by array-comparative genomic hybridization on 28 samples by using high-density oligonucleotide microarrays (Hu-244A, Agilent Technologies, Massy, France), as previously described.[3] *ASXL1* mutations were searched by DNA sequencing, as described.[2] We also searched for mutations of JAK2 (V617F) and *TET2* (all exons). *TET2* mutations have been found in 5–20% of MPN[4,5] and in other myeloid diseases,[6–8] and may be predicted to affect gene regulation.[9] *TET2* missense mutations were considered when located in conserved regions, spanning amino acids 1134–1444 and 1842–1921.[4]

We did not find any deletion of *ASXL1* or *TET2* by array-comparative genomic hybridization (not shown). One deletion at 1p31, comprising *NFIA,* has been reported previously.[10] We found heterozygous *TET2* frameshift mutations in 4 out of the 64 MPN cases (6.2%), 2 ET and 2 PMF (Table 1). We found heterozygous frameshift mutations of *ASXL1* in 5 cases (7.8%) including 1 ET out of 35, 3 PMF out of 10 (1 was in accelerated phase) and 1 acute myeloid leukemia (AML) post-ET (Figures 1 and 2). We also found three cases of substitutions (shown with footnote 'a' in Table 1) that we did not take into account because they could represent polymorphisms. None of the five *ASXL1*-mutated cases carried a JAK2 V617F mutation. Only one (a PMF) of these five cases was also mutated for *TET2*. The four other cases did not have a *TET2* mutation but two of them showed an abnormal karyotype; case HD-0535 showed a t(3;12) with upregulation of *HMGA2*.[11] Table 1 shows summarized results on the cases with a sequence variation in either TET2 or ASXL1 (the remaining cases did not show variations of TET2 or ASXL1 deduced amino acid sequences).

We analyzed the same sequences in DNA extracted from CD34-purified cells of three patients with *ASXL1* and/or *TET2* mutation in their blood cell DNA (HD-0496, HD-0536 and HD-0540). The same *ASXL1* and *TET2* mutations were detected in the corresponding CD34 DNA. This is in agreement with what is known of the physiopathology of MPNs and suggests that *ASXL1* mutations occur early during disease evolution.

Our study shows that several potential tumor suppressors may be inactivated in MPNs, especially in PMF. However, larger studies are required to better define the prevalence and prognostic impact of *ASXL1* mutations, and their association with *TET2* and *JAK2* mutations. It further suggests that cumulated haploinsufficiency may play a role in leukemogenesis; however, truncated proteins may also have a dominant negative effect, and inactivation may also occur though epigenetic regulation. A first hypothesis is that *TET2* and *ASXL1* alterations (single or combined) lead to an increase in the program of self-renewal in

Table 1 *ASXL1, JAK2* and *TET2* mutations in patients with MPN

Case	*Sex/age*	*Diagnosis*	*Karyotype at diagnosis*	*JAK2Val617Phe*	*TET2*	*ASXL1 (exon 12)*
HD-0433	F/68	ET	ND	20–30%	c.4010-4025del p.Tyr1337LeufsX21	
HD-0496	M/63	PMF	ND	Negative	c.1864C>T p.Gln622X	c.1934dupG p.Gly646TrpfsX12
HD-0528	M/57	PMF	46, XY [20]	~75%	c.4062delA p.Ala1355HisfsX8	
HD-0535	M/56	AML (post-ET MF in blast phase)	46, XY, t(3;12) (q26–27;q13–14) [20]	Negative		c.2285dupT p.Leu762PhefsX12
HD-0536	M/55	PMF accelerated phase	46, XY, add (15)(q25–26) [5]/46, XY [19]	Negative		c.1934dupG p.Gly646TrpfsX12
HD-0537	F/61	ET	ND	Negative		c.2385delC p.Trp796GlyfsX22
HD-0540	M/55	PMF	46, XY [20]	Negative		c.1934dupG p.Gly646TrpfsX12
HD-0545	F/66	ET	46, XX [20]	~15%	c.3965_3966insA p.Glu1323GlyfsX16	c.4189G>A[a] p.Gly1397Ser
HD-0552	F/50	ET	46, XX [20]	Negative		c.3173G>T[a] p.Gly1058Val
HD-0595	M/59	SE	ND	Negative		c.3935C>T[a] p.Ala1312Val

Abbreviations: ET, essential thrombocythemia; F, female; M, male; ND, not done; PMF, primary myelofibrosis; SE, secondary erythrocytosis.
[a]Possible polymorphism.
References for nucleotide and protein sequences of TET2 and ASXL1 are, respectively, NM_001127208.1, NP_001120680.1 and NM_015338.4, NP_056153.2.

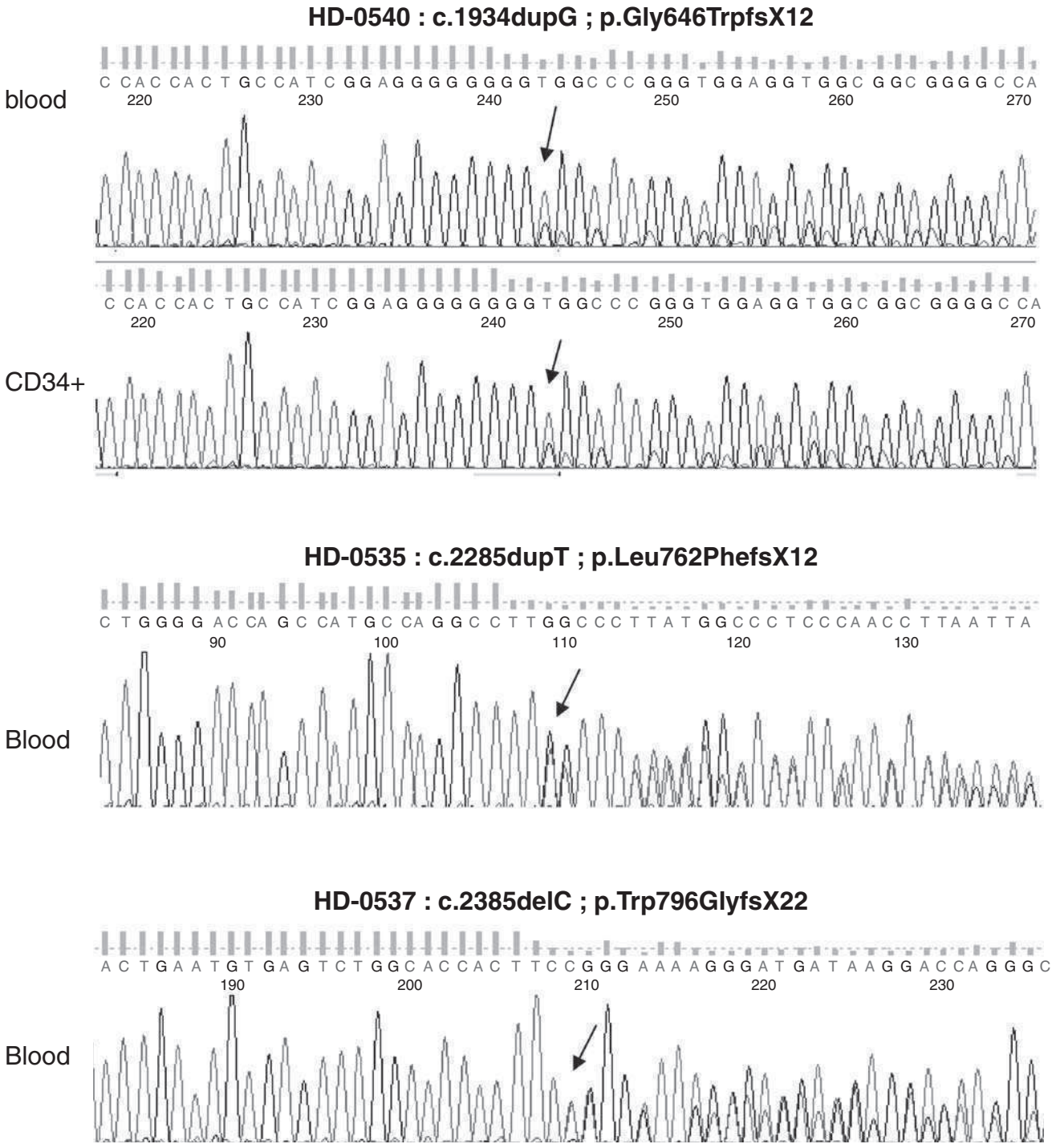

Figure 1 Examples of nucleotide sequences for three myeloproliferative neoplasm (MPN) cases. Sequence of the mutated *ASXL1* allele shows change at the position indicated by an arrow. The corresponding sequence is shown above the tracks.

MPN progenitors through modifications of DNA and histone regulatory marks. Mutations of *TET2* and *ASXL1* are also found in MDSs,[2,3,6,7] suggesting that additional alterations are responsible for the differences between the two types of chronic myeloid disease. JAK2 V617F is one of these, but in the JAK2-negative cases they are unknown. Upregulation of *HMGA2*, which also controls chromatin structure, may participate.[11,12] In our study, *ASXL1* and JAK2 V617F mutations were mutually exclusive. Thus, a second hypothesis is that ASXL1 plays the role of JAK2 in MPNs non-mutated for JAK2. However, our series is too small to allow any conclusion.

Together with the recent report on *UTX* mutations,[13] our study further suggests that some modifications of epigenetic status in tumor cells are consequences of mutations in specific genes. The questions are how many such genes do exist and how many are needed to trigger a disease; in other words, what is the

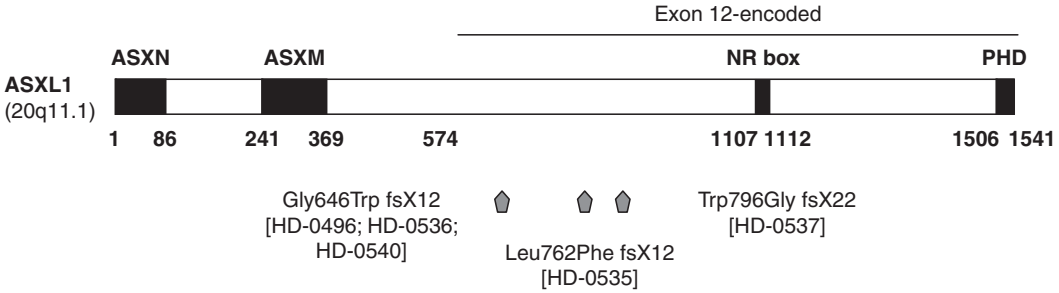

Figure 2 Representation of the ASXL1 protein with known motifs and domains. Localization of frameshift mutations in studied myeloproliferative neoplasm (MPN) samples is indicated below by arrowheads (for example, Gly646TrpfsX12 denotes a frameshifting change with Glycine as first affected amino acid, change in a Tryptophan and a new reading frame ending in a stop at position 12).

repertoire and what are the combinations? It is probable that in the future we will have to determine the number of mutated alleles for many genes in any given case.

Conflict of interest

The authors declare no conflict of interest.

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ARTICLE n°2

Rare mutations in *DNMT3A* in myeloproliferative neoplasms and myelodysplastic syndromes.

Brecqueville M, Cervera N, Gelsi-Boyer V, Murati A, Adélaïde J, Chaffanet M, Rey J, Vey N, Mozziconacci MJ & Birnbaum D.

Blood Cancer Journal, 1(5), e18. (2011)

<u>Article 2</u> - Rare mutations in *DNMT3A* in myeloproliferative neoplasms and myelodysplastic syndromes.

L'utilisation du séquençage de nouvelle génération a permis d'identifier une nouvelle classe de protéines mutées dans la leucémogenèse, les enzymes métaboliques.

En effet en 2009, des mutations des gènes codant pour *IDH1* cytoplasmique et son homologue mitochondrial *IDH2* ont été rapportées dans les LAM de novo (9%) (Mardis *et al.* 2009; Ward *et al.* 2010), dans les LAM post-SMP (20%) et dans les MFP (4%) (Pardanani, Lasho, Finke, Mai, *et al.* 2010; Green and Beer 2010). IDH est une enzyme qui catalyse la décarboxylation oxydative de l'isocitrate en α-cétoglutarate. Les patients mutés pour cette enzyme ont des hyperméthylations aberrantes de leur génome, dues à une production d'un métabolite anormal : 2-hydroxyglutarate à la place de α-cétoglutarate qui inhibe la déméthylation amorcée par TET2 (Figueroa *et al.* 2010).

Des mutations d'une autre enzyme métabolique, DNMT3A, avaient été rapportées dans une série de LAM *de novo* (4-20%) (Ley, Ding, Walter, McLellan, *et al.* 2010), ainsi que dans des SMD (8%) (Walter *et al.* 2011) et des MFP (7%) (Abdel-Wahab *et al.* 2011). Les protéines DNMT sont des méthyltransférases qui catalysent l'addition d'un groupement méthyle sur l'ADN au niveau des cytosines des îlots CpG. Les mutations de ce gène sont principalement localisées au niveau de l'exon 23 avec un « hotspot » en position R882. Les études faites par Ley et Walter avaient montré que les mutations de *DNMT3A* étaient associées à une survie plus courte dans les LAM *de novo* et les SMD.

Afin d'identifier si les gènes *IDH1, IDH2* et *DNMT3A* étaient mutés dans les SMP, nous les avons séquencé dans une série de 135 échantillons de SMP composée de 33 PV, 56 TE et 46 MF. Parallèlement, nous avons également séquencé ces deux gènes au sein de 66 SMD.

Ces travaux ont mis en évidence les éléments suivants :

- <u>Au sein des 135 SMP</u>**,** nous n'avons identifié aucune mutation des gènes *IDH1* et *IDH2*. Toutefois, nous avons décrit deux mutations du gène *DNMT3A* (1.5%) dans une MFP non mutée JAK2V617F et pour la première fois au sein d'une PV. Ceci a permis de souligner la possible concomitance des mutations des gènes *DNMT3A* et *JAK2.* Nous

avons identifié 13 mutations du gène *TET2* (9 %) dont 2 mutations chez un même patient (SMP/SMD).

- Au sein des 66 SMD, nous avons identifié 5 mutations d'*IDH1/2* (7.6 %), 4 mutations de *DNMT3A* (6 %) et 12 mutations de *TET2* (18.2 %),
- Parmi les 6 mutations de *DNMT3A* identifiées, la mutation R882 a été retrouvée seulement une fois dans une MFP et pas dans les SMD de faible pronostic. Bien que le gène *DNMT3A* soit muté dans plusieurs hémopathies, la mutation R882 semble être spécifique des phénotypes agressifs (Walter *et al.* 2011; Ewalt *et al.* 2011), suggérant l'association à des phénotypes et à des fonctions différentes des autres mutations.
- Dans les hémopathies chroniques et aiguës, les spectres mutationnels des gènes *IDH1, IDH2* et *DNMT3A* étaient différents. En effet, les mutations d'*IDH1/2* et *DNMT3A* étaient rares dans les formes chroniques contrairement aux LAM (Ley, Ding, Walter, Mclellan, *et al.* 2010). De plus au sein des LAM, les mutations prédominaient dans les LAM *de novo* comparé aux LAM secondaires (Pardanani, Lasho, Finke, Mai, *et al.* 2010; Abdel-Wahab *et al.* 2011). Ainsi, les mutations des gènes *TET2, IDH1/2, DNMT3A* qui sont impliqués dans la méthylation de l'ADN, ne semblent pas jouer les mêmes rôles au cours de la leucémogenèse. En effet, l'inactivation de *TET2* serait précoce puisque ce gène peut être inactivé à la fois dans les formes chroniques et aiguës ; à la différence des mutations de *DNMT3A* et d'*IDH1/2* qui surviennent plutôt de façon tardive (LAM).

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LETTER TO THE EDITOR

Rare mutations in DNMT3A in myeloproliferative neoplasms and myelodysplastic syndromes

Blood Cancer Journal (2011), e18; doi:10.1038/bcj.2011.15; published online 13 May 2011;

Alterations of epigenetic marks are thought to play an important role in myeloid malignancies. In particular, aberrant DNA methylation is a hallmark of these diseases. DNMT3A and DNMT3B methyltransferases have predominant role in *de novo* methylation of DNA. Mutations in DNMT3A have been found in roughly 20% of acute myeloid leukemia (AML).[1–3] The precise mechanism by which DNMT3A may affect DNA methylation is not known. The *TET2* gene encodes an enzyme that favors the transformation of 5-methylcytosines in 5-hydroxymethylcytosines. TET2 function requires alpha-ketoglutarate (αKG). TET2 is frequently mutated in myeloid diseases. Mutation in IDH1 and IDH2 changes their enzymatic activity and induces an hypermethylation of AML DNA.[3] Mutated IDH1/2 enzymes catalyze αKG into 2-hydroxyglutarate (2HG). Production of 2HG impairs TET2 function. This explains why mutations in TET2 and in IDH1/2 are mutually exclusive.[4] In contrast, mutations in IDH1/2 are more frequent in AML cases with DNMT3A mutations.[2]

We searched for mutations and deletions of *DNMT3A*, *TET2* and *IDH1/2* in a series of 201 chronic myeloid diseases including 135 myeloproliferative neoplasms (MPNs) and 66 myelodysplastic syndromes (MDSs). The MPN cases comprised 33 polycythemia vera (PV) and 5 post-PV myelofibrosis (MF), 56 essential thrombocythemia (ET) and 10 post-ET MF, 25 primary myelofibrosis (PMF), 3 MPN- unclassifiable and 3 MDS/MPN cases. The MDSs comprised 5 refractory anemia (RA), 13 RA with ring sideroblasts (RARS), 7 refractory cytopenia with multilineage dysplasia, 16 RA with excess blasts (RAEB) type 1, 20 RAEB type 2 and 5 MDS-unclassifiable cases.

We determined the sequence of all exons of *TET2*, exons 4 of *IDH1* and *IDH2*, and exons 15 to 23 of *DNMT3A* (which encode the C-terminal half of the protein, including the catalytic domain, where most mutations have been found so far), as described.[2,5] High density array-comparative genomic hybridization[5] provided information on the status of the respective loci.

In MPNs, we found 13 mutations in *TET2* in 12 patients (2 PV, 1 post-PV MF, 3 ET, 2 post-ET MF, 2 PMF, and 2 MDS/MPN one of which had two mutations), 0 mutations in *IDH1/2*, and 2 mutations in *DNMT3A* (1 in a JAK2 V617F-positive PV, 1 in a JAK2 V617F-negative PMF) (see Table 1). The two mutations in *DNMT3A* were missense (c.2245C>T, p.Arg749Cys in the PV; c.2644G>A, p.Arg882Ser in the PMF). All mutations were heterozygous.

Table 1 Mutations in three DNA methylation-associated genes in patients with chronic myeloid diseases

	TET2[a]	*IDH1/2*[a]	*DNMT3A*[a]	*Total*[a]
MPNs ($N=135$)	12 (8.9)	0	2 (1.5)	14 (10.4)
MDSs ($N=66$)	12 (18.2)	5 (7.6)	4 (6)	21 (31.8)
Total ($N=201$)	24 (11.9)	5 (2.5)	6 (3)	35 (17.4)

Abbreviations: IDH1, isocitrate dehydrogenase 1; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm.
[a]Percentages are in parentheses.

In MDSs, we found 12 mutations and 1 deletion of *TET2* (all heterozygous), 5 mutations of *IDH1/2*, and 4 mutations (6%) and 1 deletion of *DNMT3A* (all heterozygous) (see Table 1). Mutations in *DNMT3A* were 1 nonsense (c.1681G>T, p.Glu561Stop), 1 frameshift (c.1872del, p.Pro625LeufsX26) and 2 missense (c.1723G>C, p.Ala575Pro; c.2141C>G, p.Ser714Cys). Mutations in *TET2*, *IDH1/2* and *DNMT3A* were all mutually exclusive. Thus, 23 MDS cases out of 66 (roughly one-third) showed one alteration (mutation or deletion) in either DNA methylation-associated gene. Strikingly, the 4 *DNMT3A*-mutated cases were 1 RA and 3 RARS. One RARS case had a trisomy 8.

DNMT3A mutations were very recently reported in two series of MDSs, including 62 RAEB cases[6] and 150 cases of various subclasses.[7] In the RAEB series,[6] 3 cases (4.8%) were mutated. In the second series,[7] 12 patients had DNMT3A mutations (8%). These results show that, in chronic myeloid diseases, TET2 mutations are prominent, whereas IDH1/2 and DNMT3A are less frequent. In MPNs, we did not find any IDH mutation; previous works had found that only 4% of PMF cases and few PV and ET were mutated in IDH1/2.[8,9] IDH1/2 mutations are also rare in MDSs, except in some subclasses such as MDSs with del(5q) or trisomy 8.[5,10,11] Only six cases were mutated in DNMT3A in our whole series of chronic cases. Overall, IDH1/2 and DNMT3A mutations are therefore more a feature of AMLs, especially primary AMLs with normal karyotype and intermediate prognosis.[2,3] This suggests that mutations in TET2, IDH1/2 and DNMT3A, although potentially all functionally linked to DNA methylation, may not be equivalent events in the initiation of leukemogenesis; TET2 mutation could be more efficient in triggering the process. In our series, mutations of the three genes were mutually exclusive, whereas DNMT3A mutations have been found to be associated with TET2 or IDH1/2 mutations in AMLs.[2] This may just be because of a low number of mutated samples in chronic cases. However, this may also suggest that IDH1/2 and DNMT3A mutations may participate, although less frequently than TET2, to the initial phases of the disease. This may be in collaboration with specific cooperating alterations such as trisomy 8 or del(5q).

All our DNMT3A-mutated MDSs were low-risk RA/RARS cases. The DNMT3A Arg882 amino-acid residue, which is a mutation hotspot in AMLs,[1–3] was only mutated once in our series of MPNs (in a PMF) and it was not mutated in our series of MDSs. In the reported RAEB series,[6] the three mutations affected the Arg882 residue. In the other published series,[7] three out of the four Arg882-mutated MDSs were RAEB/RAEB-T cases. The DNMT3A mutations can occur in the various subclasses of MDS. However, the Arg882 mutation may be more specific of RAEB and/or aggressive cases, whereas mutations at the other residues may have a different function and may be associated with a different (milder?) phenotype.

Conflict of interest

The authors declare no conflict of interest.

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ARTICLE n°3

Mutations and deletions of the *SUZ12* polycomb gene in myeloproliferative neoplasms.

Brecqueville M, Cervera N, Adélaïde J, Rey J, Carbuccia N, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D, Gelsi-Boyer V & Murati A.

Blood Cancer Journal, 1(8), e33. (2011)

ARTICLE n°4

Alterations of polycomb gene *BMI1* in human myeloproliferative neoplasms.

Brecqueville M, Adélaïde J, Bertucci F, Finetti P, Chaffanet M, & Murati A.

Cell Cycle, 11(16), 3141–3142. (2012)

<u>Article 3</u> - Mutations and deletions of the SUZ12 polycomb gene in myeloproliferative neoplasms.

<u>Article 4</u> - Alterations of polycomb gene *BMI1* in human myeloproliferative neoplasms.

Comme nous venons de le voir, des mutations des gènes impliqués dans la régulation épigénétique comme A*SXL1, DNMT3A, EZH2, IDH1/2 et TET2* ont été mis en évidence (pour revue Tefferi 2010; Vainchenker *et al.* 2011). Les enzymes DNMT3A, IDH1/2 et TET2 catalysent la méthylation de l'ADN ; alors que les protéines ASXL1 et EZH2 (PRC1/2) participent aux modifications covalentes des histones. Le complexe PRC1 est composé des protéines RING, RNF2, CBX, BMI1 et ASXL1, et le complexe PRC2, des protéines EZH1/2, EED et SUZ12. Etant donné le nombre élevé d'altérations des gènes *ASXL1* et *EZH2* rapportée dans la littérature, nous avons suspecté que d'autres membres des complexes PRC pouvaient être également impliqués.

Nos résultats de CGH-array réalisés sur 35 MF, nous avaient permis d'identifier deux micro-délétions d'une dizaine de gènes : la del(17q11) et la del(10p12.31). De façon intéressante la microdélétion (17q11) incluait *SUZ12* (complexe PRC2), et la microdélétion (10p12.31) incluait *BMI* (complexe PRC1). L'inactivation de *BMI1* avait été rapportée dans un modèle de souris impliquée dans la MF (Oguro et al. 2012). Nous avons donc séquencé ces deux gènes afin d'identifier si des mutations étaient présentes au sein des SMP en phase chronique (n=125 SMP) et en phase aiguë (n=14 LAM post-SMP).

Ces travaux ont mis en évidence les éléments suivants :

- Nous avons identifié deux mutations du gène *SUZ12,* dans une PV et dans une transformation en LAM de MF post-TE.
- Chez le patient atteint de PV muté *SUZ12*, après deux ans de traitement sous hydroxyurée la mutation c.1685 A>G a été détectée plus faiblement suggérant que le clone porteur de cette mutation était toujours présent mais en régression.

- Nous n'avons identifié aucune mutation du gène *BMI1*, suggérant que la mutation de ce gène n'est pas le mécanisme d'inactivation ; la délétion conduisant à une haploinsuffisance.
- Parmi les gènes inclus dans la délétion cryptique (17q11), *NF1* était également délété. *NF1* est un gène régulateur négatif de la voie RAS, ce qui suggère qu'en parallèle de la voie JAK/STAT, une autre voie de signalisation pouvait être altérée et participer à la physiopathologie des SMP. Le séquençage de ce gène dans les SMP en phase chronique n'ayant jamais été réalisé, la recherche de mutations nous est apparue nécessaire.

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LETTER TO THE EDITOR

Mutations and deletions of the *SUZ12* polycomb gene in myeloproliferative neoplasms

Blood Cancer Journal (2011) **1,** e33; doi:10.1038/bcj.2011.31; published online 26 August 2011

Alterations of epigenetic marks have an important role in myeloid malignancies.[1] Mutations have been found in several epigenetic regulators including ASXL1, DNMT3A, EZH2, IDH1/2 and TET2.[2,3] DNMT3A, IDH1/2 and TET2 are involved in the regulation of DNA methylation. EZH2 belongs to a complex of proteins called 'polycomb repressor complex 2' (PRC2).[4] ASXL1 is a regulator of PRC1, the other polycomb complex. Several other proteins belong to the two polycomb complexes; although alterations of ASXL1[5] and EZH2[6,7] are probably prominent in myeloid diseases, we suspected that these other PRC components might also be involved in some cases.

We searched for mutations in *SUZ12*, a gene encoding a PCR2 protein, in 186 whole blood samples from 125 myeloproliferative neoplasms (MPNs), 14 MPNs at the blast-phase stage and 47 chronic myelomonocytic leukemias (CMML). The MPNs comprised 33 polycythemia vera (PV) and 4 post-PV myelofibrosis (MF), 51 essential thrombocythemia (ET) and 9 post-ET MF, 22 primary myelofibrosis, 3 unclassifiable and 3 myelodysplastic syndrome/MPN cases. *SUZ12* is located on chromosome arm 17q (Figure 1a) and comprises 16 exons (Figure 1b). We determined the sequence of *SUZ12* exons 10 to 16 (the existence of a highly conserved *SUZ12* pseudogene prevented easy design of oligonucleotide primers for *SUZ12* exons 1 to 9). In the MPN samples, we also searched for mutations of *JAK2* (exon 14), *ASXL1* (exons 12), *DNMT3A* (exons 15–23), *IDH1* and *IDH2* (exon 4), *MPL* (exon 10) and *TET2* (all exons). Mutations were searched by Sanger DNA sequencing analysis except for the *JAK2* mutation, which was detected by semi-quantitative PCR. We searched for *SUZ12* deletions in 80 of the MPN samples and in the 47 CMML samples by using array-comparative genomic hybridization (aCGH) on high-density oligonucleotide microarrays (Hu-244A, Agilent Technologies, Massy, France), as described previously.[8] All patients signed an informed consent and the study was approved by our ethics committee.

We found mutations of *SUZ12* in two MPN patients: a PV, HD-0716 and a blast phase of post-ET MF, HD-1038 (Table 1). The two mutations were missense and apparently heterozygous (Figures 1c and d). In the PV case, sequencing of DNA from buccal swab or CD3-purified T-cells showed the absence of variation compared with reference, thus showing that the mutation was acquired (Figure 1e). The HD-0716 PV case was JAK2 V617F-positive (the mutated allele burden was 15–30%) and *TET2*-mutated. After diagnosis, the patient, a 66-year-old man, was treated with hydroxyurea. After 2 years of treatment a blood sample processed and studied as the initial one showed that the *SUZ12* mutation was barely detectable (Figure 1d). The HD-1038 blast phase of a post-ET MF was diagnosed in an 80-year-old woman. The chronic stage was not available for study. At the acute stage, the sample was JAK2 V617F-negative and *TET2*-mutated (c.3640C>T p.Arg1214Trp), the karyotype was complex and the aCGH profile showed, among other alterations, a heterozygous loss of *TET2*, 7q21-qter spanning *EZH2,* 12p12-p13 spanning *AEBP2* and 17q11-q21 spanning *NF1* and the other *SUZ12* allele (Figure 2). No germline DNA was available. We did not detect any mutation of *ASXL1* in the two cases, whereas 17 other MPN cases were mutated (Table 1). We found 2 mutations in *DNMT3A*,[9] 2 in *IDH2*, 1 in *MPL* and 15 in *TET2*. No *SUZ12* mutation was found in the 47 CMML cases.

Deletions of the *NF1* gene at 17q11 are frequent in MPNs.[10] By using aCGH we found that the *SUZ12* gene, which lies close to *NF1*, is often included in these deletions. We found *SUZ12* deletions in three MPNs and in two CMML cases (Figure 2). This was the case for HD-1038. No aCGH profile was available for HD-0716. Two other MPN cases (HD-0535 and HD-0728) showed a deletion of a *SUZ12* allele but were not mutated (Table 1); however, as mentioned above, the first nine exons of the gene were not studied. In another MPN case (HD-0689), the deletion encompassed *NF1* but not *SUZ12* (Figure 2), suggesting that *NF1* was the actual targeted gene of the deletion.

Our study shows that a mutation can affect a PRC2 component other than EZH2 in few cases of MPN. This mutation of SUZ12 may substitute for or cooperate with a mutation of EZH2 to compromise PRC2. This reinforces the current view that position epigenetic regulators as major players of leukemogenesis, together with signaling molecules and transcription factors. *SUZ12* mutations are rare but are actually in the same range as *DNMT3A*,[9] *IDH2* and *MPL*. Because we tested only the last exons of the gene our study may have underestimated the frequency of *SUZ12* mutations. Interestingly, in addition to mutations, *SUZ12* function could be affected by gene loss. The 17q11 region encompassing *NF1* and *SUZ12* is deleted in MPNs[10] and CMMLs. Our study suggests that both genes may participate to leukemogenesis, explaining why the *NF1-SUZ12* 17q11 region is often lost *en bloc*. It is also possible that other genes are involved; we sequenced the exons of *RAB11FIP4*, located between the *NF1* and *SUZ12*, in our series of MPN cases but found no mutation.

PRC2 is the major methyltransferase for H3K27 methylation, a modification of histone H3 that represses gene expression programs throughout development.[4] Mice with loss of function mutations in PRC2 components display enhanced activity of their hematopoietic stem cell/progenitor population and loss of SUZ12 function in particular enhances hematopoietic stem cell activity.[11] It remains to be determined whether the mutations we have identified lead to a loss of function of the protein. In the HD-0716 case, the c.1685A>G p.Asn562Ser mutation may affect the VEFS-box known to be critical for EZH2 interaction,[12] consequently disrupting PRC2 function. In the HD-1038 case, the deletion of an allele combined with the mutation of the other allele suggests a two-hit process associated with a tumor suppressor. Whether *SUZ12* mutations are present in other hematopoietic diseases should be determined. Whether other PRC2 components, such as AEBP2, EED and JARID2, are mutated and how they combined with other mutations in hematopoietic diseases should also be investigated.

Many genes contribute to leukemogenesis through mutations of their sequence; our study also shows that, beside the

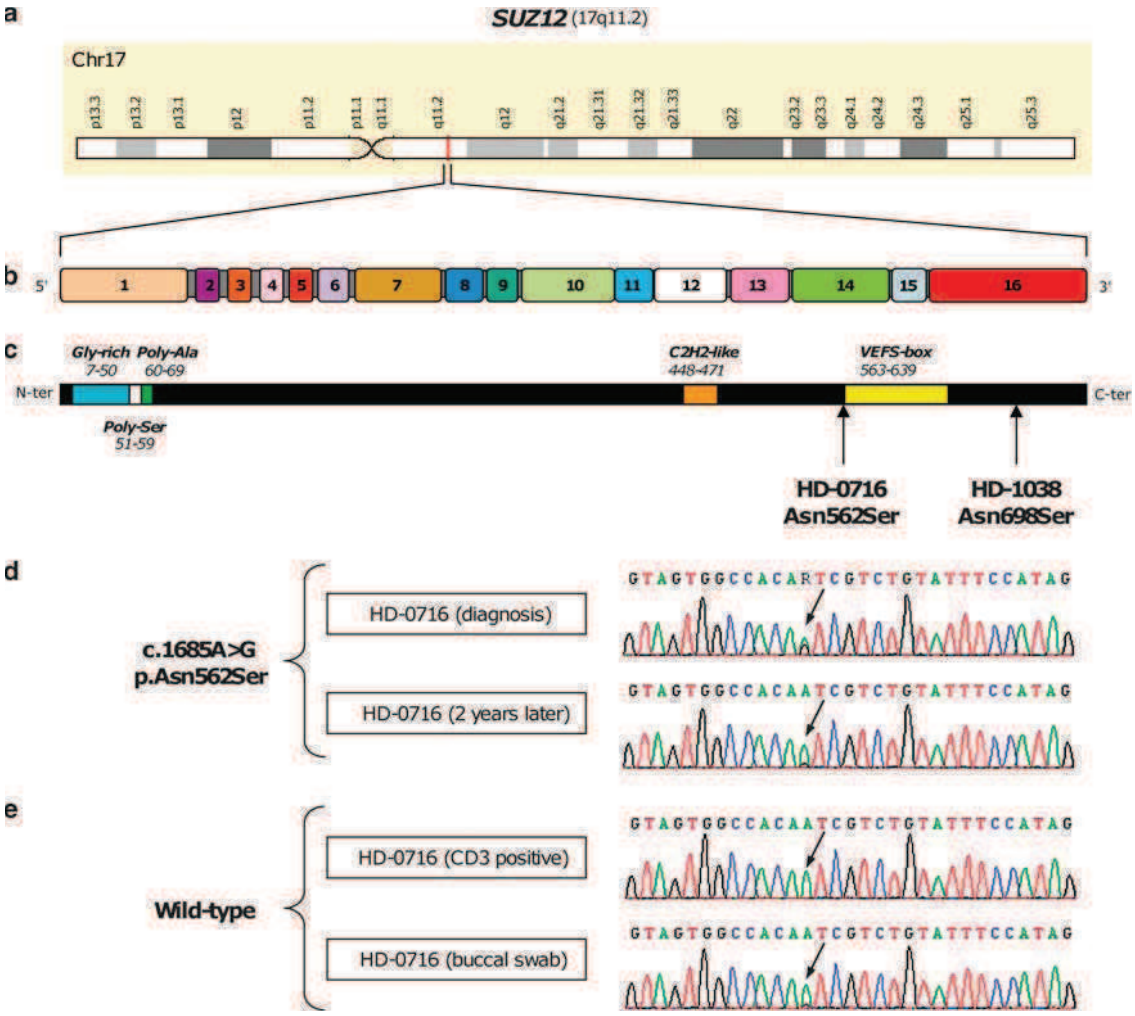

Figure 1 Mutation of the *SUZ12* gene in MPNs. (**a**) Localization of the *SUZ12* gene in chromosomal region 17q11. (**b**) Representation of the 16 *SUZ12* exons. (**c**) Representation of the 739-amino-acid-long SUZ12 protein with known motifs and domains. Localization of the mutations is indicated below by arrowheads. (**d**) Nucleotide sequences for HD-0716 PV case. Sequence of the mutated *SUZ12* allele demonstrates change at the position indicated by an arrow in the sample taken at diagnosis. (**e**) The mutation was hardly detectable after 2 years of treatment and is absent from CD3 T-cells and buccal swab samples. The corresponding sequence is shown above the tracks.

Table 1 Mutations and deletions of *SUZ12* in patients with myeloproliferative neoplasms

	SUZ12 mutation	*SUZ12 loss*	*ASXL1 mutation*	*ASXL1 loss*	*DNMT3A mutation*	*IDH1/2 mutation*	*JAK2 mutation*	*MPL mutation*	*TET2 mutation*	*TET2 loss*
Polycythemia vera	1/33	0/7	3/33	0/7	1/33	0/33	32/33	0/33	4/33	0/7
Essential thrombocythemia	0/51	0/19	2/51	0/19	0/51	0/51	32/51	0/51	3/51	0/19
Myelofibrosis	0/35	1/35	8/35	1/35	1/35	0/35	24/35	0/35	4/35	0/35
Other MPN	0/6	0/6	2/6	0/6	0/6	0/6	1/6	0/6	2/6	0/6
Blast phase MPN	1/14[a]	2/13[a]	2/14	0/13	0/14	2/14	4/14	1/14	2/14[a]	2/13[a]
Total MPNs	2/139[a]	3/80[a]	17/139	1/80	2/139	2/139	93/139	1/139	15/139[a]	2/80[a]

Abbreviation: MPN, myeloproliferative neoplasm.
[a]HD-1038 sample in common.

major contributors, most of which may be known already, there may be a number of rarely mutated contributors. This could render the molecular diagnosis of myeloid diseases more difficult to establish. Many of these rarely mutated genes (for example, *SUZ12*) may affect the same functions and pathways (for example, polycomb repression) as the frequently mutated ones (for example, *EZH2*).

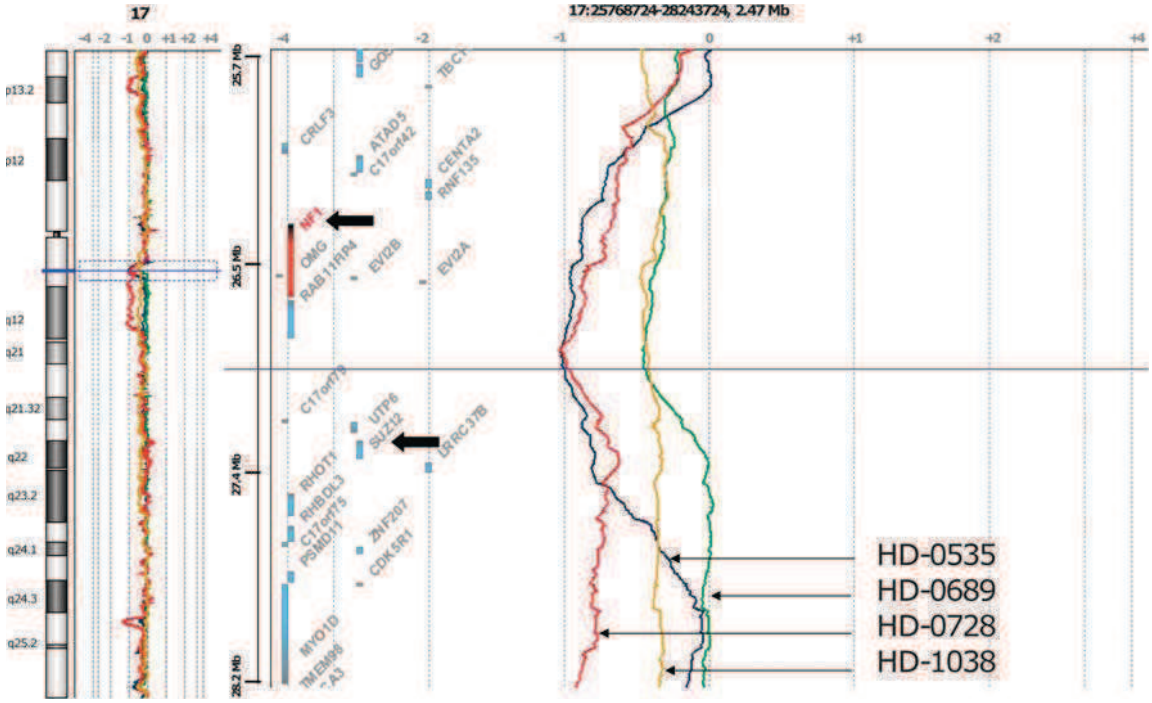

Figure 2 aCGH profile of chromosome 17 in four MPN cases showing loss of the *NF1-SUZ12* region. HD-0535 (blue profile), a blast phase primary myelofibrosis (PMF), HD-0728 (red profile), a PMF and HD-1038 (yellow profile), a blast phase post-ET MF show loss, among other genes, of *NF1* and *SUZ12*, whereas in HD-0689 (green profile), a PMF, no loss of *SUZ12* is observed. Deletion of *NF1* but not *SUZ12* in HD-0689, and mutation of *SUZ12* in HD-1038 suggest both genes participate in leukemogenesis.

Conflict of interest

The authors declare no conflict of interest.

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Alterations of polycomb gene *BMI1* in human myeloproliferative neoplasms

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Recent articles in the *Journal of Experimental Medicine* and in *Cell Cycle* by the laboratory of A. Iwama established a role for polycomb gene *BMI1* in leukemogenesis using a mouse model.[1,2] Polycomb proteins regulate gene expression through the control of chromatin structure and repressive histone marks. They play major roles in development and stem cell biology. They distribute into two complexes called polycomb repressive complexes 1 and 2 (PRC1 and PRC2). PRC1 contains several proteins, including BMI1. PRC1 monoubiquitylates lysine 119 of histone H2A via the ubiquitin ligases RING1 and RNF2.[3] PRC2 contains EED, EZH2 and SUZ12 proteins. PRC2 is a histone methyltransferase that trimethylates lysine 27 of histone H3, resulting in the H3K27me3 mark, which specifies transcriptional repression. Recent studies have shown that components of PRC2, especially EZH2, are targeted by inactivating mutations in human myeloid malignancies.[4-7] PRC2 genes are mutated in acute stages, such as acute myeloid leukemia (AML), and chronic stages, such as myelodysplastic syndromes (MDS) or myeloproliferative neoplasms (MPN). The latter comprise polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). EZH2 is mutated in 13% of PMF cases and in 12% of cases with MDS/MPN features.[5] Actually, through these direct inactivating mutations in its components or through alterations in PRC2-interacting proteins such as ASXL1,[8] PRC2 appears as a key tumor suppressor complex in myeloid diseases. No such results have been reported yet for PRC1 genes in human samples. In contrast, because the loss of *Bmi1* gene induces a defect in the self-renewal capacity of hematopoietic stem cells[9] it was thought that PRC1 and PRC2 played opposite roles in leukemogenesis. Oguro and colleagues[1] have just demonstrated that in an *Ink4a/Arf-l-* mouse, the loss of the *Bmi1* gene generated a disease similar to PMF. Mice repopulated with *Bmi1-/- Ink4a-Arf-/-* hematopoietic cells developed a lethal disease with clinical features observed in human PMF. This exciting result suggests that alterations of PRC1 complex components could, according to the genetic context (an abrogation of p16 and p19 tumor-suppressor control in the mouse model of Oguro and colleagues), play a role in the development of myeloid diseases similar to that of PRC2. PRC1 and PRC2 silencing functions are coordinated, and the two complexes may exert their leukemogenic effect through the deregulation of the same loci. Indeed, Oguro and colleagues identified oncogenic loci repressed by PRC1, such as *Hmga2,* and further demonstrated that *Hmga2* promoter is also under the control of EZH2.[1,2] A consequence of these results in human medicine is that alterations or deregulation of PRC1 components and controlled loci may substantially increase the proportion of hematopoietic diseases with defects in polycomb network genes. To determine whether *BMI1* may be altered in human MPNs, we studied the genome of 35 PMF cases by using array-comparative genomic hybridization (aCGH) on high-density oligonucleotide microarrays (Hu-244A, Agilent Technologies), as previously described.[10] We also searched for mutations in *BMI1* in 77 MPN cases comprising 4 PV, 20 ET and 36 myelofibrosis (MF) (25 PMF, 8 post-ET MF, 3 post-PV MF), 3 MPN/MDS, 3 MPN-unclassifiable, 5 blast phase post-ET, 3 blast phase post-ET MF, 3 blast phase PMF using Sanger DNA sequencing of *BMI1* coding exons 2 to 10. We did not find any mutations in *BMI1*. We found a heterozygous deletion of chromosome region 10p12.31 encompassing the *BMI1* locus and seven other genes (**Fig. 1**) in case HD-1095, a 64-year-old man diagnosed with PMF. The HD-1095 sample had a JAK2V617F mutation (50–60% allele burden), was not mutated in *ASXL1, DNMT3A, IDH1, IDH2* and *TET2*, and displayed other small chromosomal deletions that had led to the loss of *SOCS2, FBXL18* and *MYB*, but not of *CDKN2A/INK4A* (not shown). To our knowledge, this is the first example of *BMI1* locus alteration in human cancer. *BMI1* deletion could lead to haploinsufficiency; unfortunately, RNA was not available for this sample. We studied *BMI1* mRNA expression in 25 PMF profiled by Affymetrix microarrays. *BMI1* expression in PMF was similar to that in normal blood (not shown), suggesting that the *BMI1* locus is not inactivated by hypermethylation as a general rule. In this series studied by aCGH we did not find alterations of the *HMGA2* locus, but we had previously reported one case of *HMGA2* 3'UTR breakage and mRNA overexpression in an MPN case.[11] Finally, to determine whether *BMI1* alteration could be found in other myeloid diseases, we searched for a deletion of the locus in 253 non-PMF samples (73 MPNs, 53 chronic myelomonocytic leukemias, 63 MDSs, and 64 AMLs) by using aCGH but found no other deleted case. Our results show that, although structural alterations of *BMI1* and downstream effectors are rare, they do occur in human MPN samples. This supports both the findings of Oguro and colleagues in the mouse and the potential role of PRC1 in leukemogenesis. Whether other mechanisms

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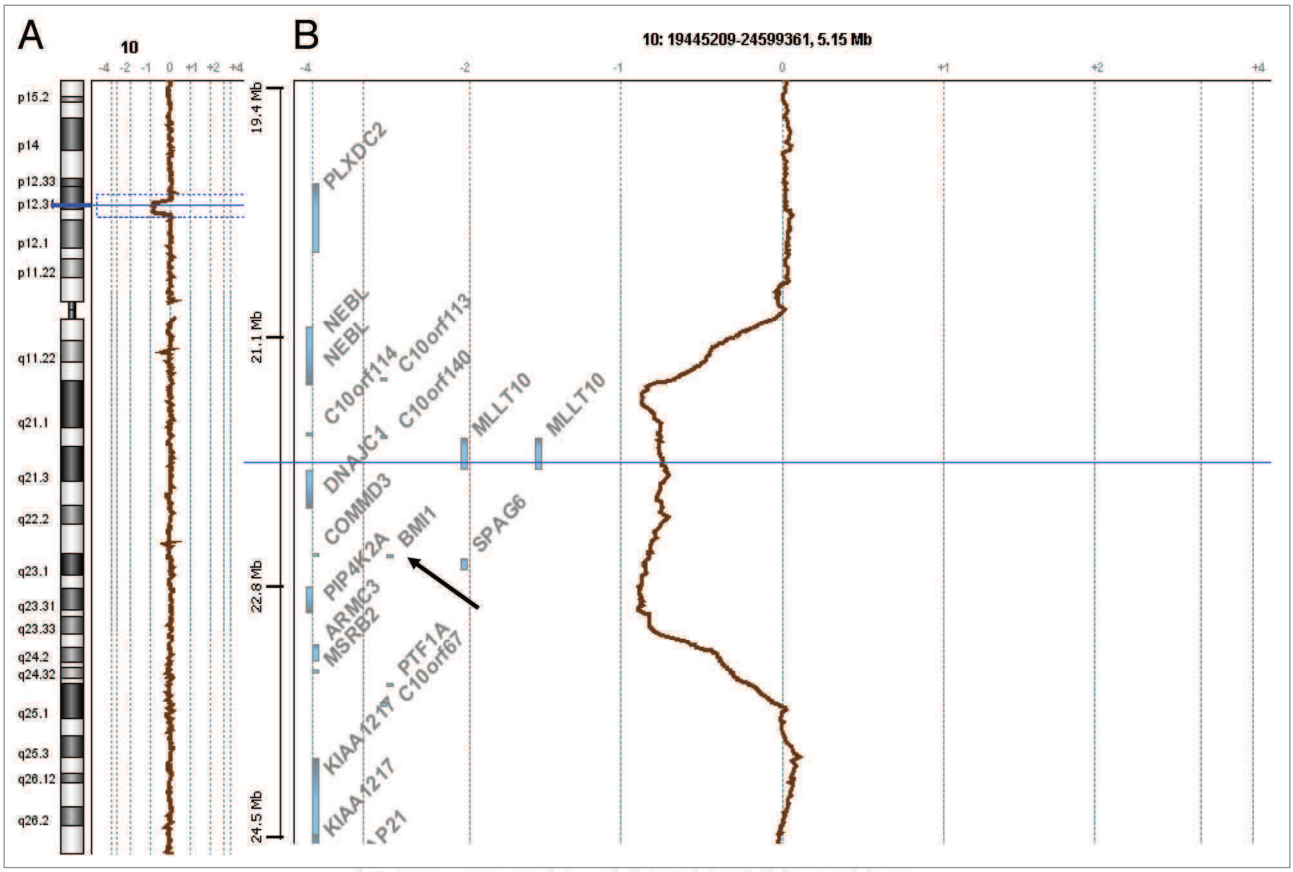

Figure 1. aCGH profile of chromosome 10 in one PMF case showing loss of the *BMI1* region. (A) Chromosome 10 ideogram and aCGH profile of HD-1095 case. (B) zoom on the 10p12.31 band showing the deletion that spans chr10:21,274,386–23,428,386, according to hg18 UCSC (http:/genome.ucsc.edu), and includes the *BMI1* gene (arrow).

of downregulation of *BMI1* or alterations in other PRC1 components can be found in hematopoietic diseases remains to be demonstrated.

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ARTICLE n°5

Mutation Analysis of *ASXL1*, *CBL*, *DNMT3A*, *IDH1*, *IDH2*, *JAK2*, *MPL*, *NF1*, *SF3B1*, *SUZ12*, and *TET2* in Myeloproliferative Neoplasms.

Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, Cervera N, Gelsi-Boyer V, Arnoulet C, Gisserot O, Verrot D, Slama B, Vey N, Mozziconacci, MJ, Birnbaum D & Murati A.

Genes, Chromosomes & Cancer, 51(8), 743–755. (2012)

<u>Article 5</u> - Mutation analysis of *ASXL1*, *CBL*, *DNMT3A*, *IDH1*, *IDH2*, *JAK2*, *MPL*, *NF1*, *SF3B1*, *SUZ12*, and *TET2* in myeloproliferative neoplasms.

Afin d'établir un état des lieux des mutations dans les sous-types de SMP classiques non LMC (PV, TE et MF), nous avons séquencé 11 gènes (*ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12* et *TET2*) au sein de 149 échantillons de patients (127 SMP classiques non LMC dont 30 PV, 53 TE et 44 MF), 14 LAM post-SMP et 8 SMP inclassables-SMP/SMD). Nos objectifs étaient d'établir la fréquence et la répartition des mutations, d'identifier la concomitance ou l'exclusivité des gènes mutés, et d'étudier leurs impacts pronostiques. Le gène *CBL* a été séquencé car il avait été décrit muté dans plusieurs hémopathies myéloïdes (SMP, SMD et LMMC) (Grand *et al.* 2009; Makishima *et al.* 2009; Adélaïde *et al.* 2010). *CBL* code pour une protéine qui régule négativement de nombreuses tyrosine kinases (comme FLT3 par exemple) et la voie JAK/STAT (Mohapatra *et al.* 2012). Nous avons également séquencé le gène *NF1* composé de 58 exons, trouvé délété lors de l'étude précédente (Brecqueville *et al.* 2011). *NF1* code pour un régulateur négatif de la voie RAS/MAPK. Le gène *SF3B1* a été également séquencé car décrit muté dans les SMD et notamment dans les SMP/SMD de type RARS-T (anémie réfractaire avec sidéroblastes en couronne et thrombocytose marquée) (Papaemmanuil *et al.* 2011; Malcovati *et al.* 2011; Yoshida *et al.* 2011; Visconte *et al.* 2012). Le gène *SF3B1* code pour une protéine appartenant au complexe de la machinerie d'épissage.

Ces travaux ont mis en évidence les éléments suivants :

- Parmi les 11 gènes séquencés au sein des SMP non LMC, trois étaient mutés de façon récurrente dans les SMP au stade chronique: *JAK2* (67%), *TET2* (12%) et *ASXL1 (11%).* Aucune mutation du gène *IDH2* n'a été retrouvée au stade chronique (seulement dans les transformations en LAM).
- Nous n'avons identifié aucune mutation dans 30% des TE et 21 % des MF.
- Nous avons confirmé le caractère somatique de la mutation ASXL1 : c.1934dupG, p.Gly646TrpfsX12.
- Pour la première fois, des mutations du gène *NF1* ont été identifiées au sein des SMP classiques non LMC (deux échantillons de MF étaient mutés).

- Les mutations du gène *TET2* ont été retrouvées de façon homogène au sein des trois sous-types.
- Par contre les mutations d'*ASXL1* étaient prédominantes au sein des MF (20%) par rapport aux PV (7%) et TE (4%), suggérant que l'altération d'ASXL1 joue un rôle dans la physiopathologie de la myélofibrose.
- Parmi les nombreuses mutations présentes dans les MF, celles des gènes *ASXL1*, *JAK2* et *TET2* pouvaient être concomitantes, ce qui témoigne de la complexité moléculaire importante de ce sous-type.
- Les MF présentaient une forte proportion d'altérations de gènes impliqués dans les modifications d'histones et d'ADN contrairement aux PV et TE, ces altérations pourraient être associées à la transition des stades PV/TE vers la MF.
- Chez un patient atteint de MF, l'acquisition de la mutation d'*ASXL1* a précédé l'acquisition de la mutation *MPL*, suggérant que l'altération d'*ASXL1* survient précocement dans la physiopathologie des SMP ou que le clone muté *MPL* a davantage proliféré devenant détectable par séquençage Sanger.
- Une deuxième mutation du gène *TET2* a été acquise au cours de l'évolution de la maladie (rechute) chez un patient atteint de MF malgré une allogreffe, suggérant que les mutations de *TET2* peuvent être acquises au stade précoce ou tardif de la maladie.
- Bien que notre série de MF était modeste (n=44), nous avons montré pour la première fois, que les mutations d'*ASXL1* étaient associées à une survie globale plus faible (p=0.02). Ce qui n'était pas le cas pour les mutations de *TET2*.

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Mutation Analysis of *ASXL1*, *CBL*, *DNMT3A*, *IDH1*, *IDH2*, *JAK2*, *MPL*, *NF1*, *SF3B1*, *SUZ12*, and *TET2* in Myeloproliferative Neoplasms

Mandy Brecqueville,[1,2] Jérôme Rey,[2,3] François Bertucci,[1,2] Emilie Coppin,[1,2] Pascal Finetti,[1] Nadine Carbuccia,[1] Nathalie Cervera,[1] Véronique Gelsi-Boyer,[1,2,4] Christine Arnoulet,[4] Olivier Gisserot,[5] Denis Verrot,[6] Borhane Slama,[7] Norbert Vey,[2,3] Marie-Joelle Mozziconacci,[1,4] Daniel Birnbaum,[1] and Anne Murati[1,4]*

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Since the discovery of the JAK2V617F tyrosine kinase-activating mutation several genes have been found mutated in non-chronic myeloid leukemia (CML) myeloproliferative neoplasms (MPNs), which mainly comprise three subtypes of "classic" MPNs; polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). We searched for mutations in *ASXL1*, *CBL*, *DNMT3A, IDH1, IDH2*, *JAK2*, *MPL, NF1*, *SF3B1*, *SUZ12*, and *TET2* genes in 149 non-CML MPNs, including 127 "classic" MPNs cases. *JAK2* was mutated in 100% PV, 66% ET and 68% MF. We found a high incidence of *ASXL1* mutation in MF patients (20%) and a low incidence in PV (7%) and ET (4%) patients. Mutations in the other genes were rare (*CBL, DNMT3A, IDH2, MPL, SF3B1, SUZ12, NF1*) or absent (*IDH1*).

INTRODUCTION

Myeloproliferative neoplasms (MPNs) originate from genetically transformed hematopoietic stem cells with a capacity for multilineage differentiation and efficient myelopoiesis. Non-chronic myeloid leukemia (CML) MPNs mainly comprise polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). MF can either occur *de novo* as primary MF (PMF) or result from the progression of PV or ET (hereafter called post-PV MF and post-ET MF). An MPN transforms into acute leukemia in ~4–6% of the patients (Noor et al., 2010).

The JAK2V617F somatic mutation is found in >95% of PV, 50% of ET and 50% of PMF (Panani, 2009; Delhommeau et al., 2010). However, the mechanism by which this single mutation contributes to the pathogenesis of three clinically distinct disorders remains unclear. Several novel mutations have been described recently in chronic or blast-phase MPNs (Tefferi, 2010). They affect *ASXL1* (Additional sex combs-like 1; Exon 12) (Carbuccia et al., 2009), *CBL* (Casitas B-lineage lymphoma; Exons 8 and 9) (Makishima et al., 2009), *DNMT3A* (DNA methyl transferase 3 alpha; several exons) (Ley et al., 2010; Abdel-Wahab et al., 2011a; Brecqueville et al., 2011a), *EZH2* (Enhancer of zeste homolog 2; several exons) (Ernst et al., 2010; Nikoloski et al., 2010), *IDH1* and *IDH2* (Isocitrate dehydrogenase 1 and 2; Exon 4) (Pardanani et al., 2010a), *JAK2* (Janus kinase 2; Exon 12) (Scott et al., 2007), *LNK* (Lymphocyte-specific adaptator protein; Exon 2), (Oh et al., 2010; Pardanani et al., 2010b), *MPL* (Myeloproliferative leukemia virus; Exon 10) (Pikman et al., 2006), splicing factor genes (Papaemmanuil et al., 2011; Yoshida et al., 2011), *SUZ12* (Suppressor of zeste 2 homolog; Exons 14

Additional Supporting Information may be found in the online version of this article.

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and 16) (Brecqueville et al., 2011b; Score et al., 2012), and *TET2* (Ten–Eleven Translocation, oncogene family member 2; several exons) (Delhommeau et al., 2009; Tefferi et al., 2009a; Tefferi et al., 2009b) genes.

We have studied 11 genes, including *SF3B1* and *NF1*, whose mutations have been recently identified (Yoshida et al., 2011; Haferlach et al., 2012), and *ASXL1*, whose mutations are frequent in myeloid malignant diseases and often associated with a poor prognosis. This combination of genes has never been studied so far in MPNs. *ASXL1* maps to chromosome band 20q11 (Fisher et al., 2003). The ASXL1 protein is a component of various DNA and histone regulatory complexes (Cho et al., 2006; Fisher et al., 2006) and participates in the deubiquitination of these complexes (Scheuermann et al., 2010). Mutations in *ASXL1* have been reported in both chronic and blast-phase MPNs by us and others (Carbuccia et al., 2009; Abdel-Wahab et al., 2011b; Stein et al., 2011); however, our reported series of cases was small, the study of Abdel-Wahab et al. did not take into account the frequent c.1934dupG; p.Gly646TrpfsX12 mutation (Abdel-Wahab et al., 2011b) and two recent studies reported mutations of *ASXL1* in MPNs but did not examine other candidate genes (Ricci et al., 2011; Stein et al., 2011).

MATERIALS AND METHODS

Patients and Samples

MPN samples were selected according to the WHO criteria (Vardiman et al., 2009). A total of 149 cases of non-CML MPN were included: 30 PV, 53 ET, 44 MF (30 PMF, 4 post-PV MF, 10 post-ET MF), 3 unclassifiable MPNs, 5 MPN/Myelodysplastic syndromes (MDS), and 14 blast-phase PV, ET, and MF. PV, ET, and MF are hereafter designed as "classic" MPNs (Supporting Information Table S1A). Paired samples were obtained at different times of chronic-phase disease for 11 patients (2 PV, 1 ET, 7 PMF, and 1 post-ET MF), (Supporting Information Table S1B). Samples were obtained at diagnosis or after diagnosis free of disease progression. Prognosis for PMF was evaluated with the Dynamic International Prognostic Scoring System (DIPSS)-plus (Gangat et al., 2011). Information on patients (individual thrombotic risk for ET and PV, DIPSS-plus risk for PMF, time from diagnosis to sampling, biological factors, therapies used prior to sampling, transfusion dependence are shown in Supporting Information Table S1A, S1B). We obtained paired normal DNA from 55 buccal swabs and 8 nonclonal CD3+ cells of these MPN patients.

We also searched for mutations in 24 non-MPN cases including seven reactive thrombocytosis (RT) due to inflammatory syndrome ($n = 1$), splenectomy ($n = 3$), or iron deficiency ($n = 3$), and 17 JAK2V617F-negative reactive erythrocytosis (RE) with normal or elevated serum erythropoietin due to pulmonary dysfunction ($n = 2$), heavy smoking ($n = 9$), severe sleep apnea ($n = 4$), or high oxygen affinity ($n = 2$). The majority of samples were collected at the Institut Paoli-Calmettes. Of 149 MPN samples, 17 were collected from three other Centers in France (Hôpital Saint-Joseph, Marseille; Hôpital d'instruction des armées Sainte-Anne, Toulon; Centre Hospitalier Général d'Avignon). For molecular analysis, all samples were processed similarly at the Institut Paoli-Calmettes. All patients signed an informed consent for research and the study was approved by our institutional review board ("Comité d'Orientation Scientifique" of the Institut Paoli-Calmettes).

Nucleic Acids Extraction

Peripheral blood samples, CD3+ cell population and buccal swabs were collected from patients and processed immediately or cryoconserved at −80°C. CD3+ cell population was separated by the CD3 microbead kit (Miltenyi Biotech, Germany). High-quality DNA from blood samples was extracted by the Allprep DNA/RNA isolation kit (Qiagen, Germany). DNAs from buccal swab and CD3+ cells were extracted by the QIAmp DNA micro kit (Qiagen, Germany).

DNA Sequencing

Genes were selected for their known involvement in leukemogenesis. DNA Sanger-sequencing of exon-coding sequences of *ASXL1* (Exon 12), *CBL* (Exons 8, 9), *DNMT3A* (Exons 15–23), *IDH1* (Exon 4), *IDH2* (Exon 4), *JAK2* (Exon 14), *MPL* (Exon 10), *NF1* (55 exons), *SF3B1* (Exons 15, 16), *SUZ12* (Exons 10–16), and *TET2* (Exons 3–11) was done as follows. The primers used for PCR sequencing are described in Supporting Information Table S2A. PCR amplifications of DNA were done in a total volume of 25 µl PCR mix containing at least 5 ng template DNA, Taq

buffer, 200 μmol of each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 U of Hot Star Taq (Qiagen). PCR amplification conditions were 95°C 10 min; 95°C 30 sec, variable temperature 30 sec, 72°C 30 sec to 1 min depending on PCR product length for 35 cycles (for *TET2*, the PCR program is described in Supporting Information Table S2B), 72°C 10 min. Because of the controversy about the *ASXL1* c.1934dupG mutation that occurs within a series of guanine, we tested four different polymerases (Hot star Taq from Qiagen, AmpliTaq Gold® from Applied Biosystems, Phire® from Finnzymes, Kapa2G Robust® from Kapabiosystems) to eliminate a possible polymerase failure leading to false positive detections. PCR products were purified using Millipore plate MSNU030. Two microliters of the purified PCR products were used for sequencing using the Big Dye terminator v1.1 kit (Applied Biosystems) including the forward or reverse primer. After G50 purification, sequences were loaded on ABI 3130*xl* and 3730 automats (Applied Biosystems). The sequence data files were analyzed using SeqScape software. The genetic alterations identified were cross-referenced to information from the Ensembl Genome Browser (http://www.ensembl.org) and all mutations were confirmed on an independent PCR product. *TET2* intronic mutations were validated by cDNA sequencing (SuperScript™ II Reverse Transcriptase, Invitrogen) and primers used are described in Supporting Information Table S2A). References for nucleotide and protein sequences are respectively for ASXL1: NM_015338.5, NP_056153.2; for CBL: NM_005188.2, NP_005179.2; for DNMT3A: NM_022552.3, NP_072046.2; for IDH1: NM_005896.2, NP_005887.2; for IDH2: NM_002168.2, NP_002159.2; for MPL: NM_005373.2, NP_005364.1; for NF1: NM_001042492.2, NP_001035957.1; for SF3B1: NM_012433.2, NP_036565.2; for SUZ12: NM_015355.2, NP_056170.2; and for TET2: NM_001127208.1, NP_001120680.1.

Statistical Analysis

Collected clinical and biological data were those observed at the time of sampling for molecular analyses. Correlations between sample groups and clinico-biological data were calculated with the Fisher's exact test for qualitative variables with discrete categories, and the Mann–Whitney *U*-test for continuous variables. Overall survival (OS) was calculated for MF as the time (in months) between the date of diagnosis and the date of death (for patients who deceased) or last follow-up (for censored patients). Causes of death for MF included leukemic transformation, progressive disease with marrow failure, and complications from infections. Survival curves were defined with the Kaplan–Meier method and compared with the log-rank test. Two-tailed *P* values <5% were considered as significant for all statistical tests.

RESULTS

Gene Mutations in non-CML MPNs

We searched for mutations in 11 selected genes in 149 MPN cases comprising 127 classic MPNs (PV, ET, and MF), 14 PV/ET/MF blast-phases and 8 unclassifiable MPN and MPN/MDS forms. A series of 24 non-MPN reactive cases was similarly studied.

We found *ASXL1* mutations in 17 MPN cases (11%). They were all heterozygous, and comprised 14 frameshift (including 11 c.1934dupG; p.Gly646TrpfsX12) and 3 nonsense mutations presumed to truncate the protein from its C-terminus that includes the plant homeodomain (PHD) finger (Table 1, Fig. 1). Subclass-specific mutational frequencies were 7% (2/30) in PV, 4% (2/53) in ET, 20% (6/30) in PMF, 50% (2/4) in post-PV MF, 10% (1/10) in post-ET MF, 14% (2/14) in PV/ET/MF blast-phases and 40% (2/5) in MPN/MDS (Fig. 2). *ASXL1* mutations were more frequent in MF than PV and ET ($P = 0.023$), (Fig. 2B). *TET2* mutations (Fig. 1) were present in 18 of 149 MPN patients (12%) and somewhat more evenly distributed across subtypes than *ASXL1* mutations (Table 1, Fig. 2). In one PMF (HD-0528) with a *TET2* mutation (c.4062delA; p.Ala1355HisfsX8) at diagnosis, we observed a second *TET2* mutation (c.1648C>T; p.Arg550X) 48 months later (HD-1300; 7 months after allogeneic transplant) (Table 1, Supporting Information Fig. S1). Intron variant for *TET2* c.3803_3803+5delAgtaagt (HD-0432) was confirmed by a cDNA sequencing. This intronic mutation causes Exon 6 deletion and creates a premature stop codon leading to a truncated TET2 protein (p.Val1199ArgfsX31) (Table 1). We found a *CBL* mutation in one ET, *MPL* mutations in a PMF (HD-1138) (Supporting Information Fig. S1) and a post-ET blast-phase (HD-0989) and mutations in *IDH2* in two post-ET blast-phases (HD-0619 and HD-0923). Mutations of *DNMT3A* were detected in three cases: a

TABLE 1. Molecular Data of 46 MPNs Cases with ASXL1, SUZ12, TET2, DNMT3A, IDH2, JAK2, MPL, CBL, NF1, and SF3B1 Mutations

				Epigenetic					Signaling				Splicing
				Histone methylation		DNA methylation							
Samples	Sex/age (y)	Diagnosis	Karyotype	*ASXL1* (exon 12)	*SUZ12* (exons 10-16)	*TET2* (exons 3-11)	*DNMT3A* (exons 15-23)	*IDH2* (exon 4)	JAK2V617F allele burden (%)	*MPL* (exon 10)	*CBL* (exons 7-8)	*NF1* (55 exons)	*SF3B1* (exons 15-16)
HD-0432	F/31	PV	46,XX[20]	wt	wt	c.3803_3803+5delAgtaagt; p.Val1199ArgfsX31	wt	wt	30	wt	wt	ND	wt
HD-0507	M/74	PV	ND	wt	wt	c.2641dupC; p.Arg881LysfsX20	wt	wt	15–30	wt	wt	ND	wt
HD-0659	M/72	PV	ND	c.1934dupG; p.Gly646TrpfsX12	wt	wt	wt	wt	2	wt	wt	ND	wt
HD-0716	M/66	PV	ND	wt	c.1685A>G; p.Asn562Ser^{NC}	wt	wt	wt	15–30	wt	wt	ND	wt
HD-0746	M/56	PV	ND	wt	wt	c.34131A>C; p.Glu1144Ala^{NC}	wt	wt	50–75	wt	wt	ND	wt
HD-0842	M/65	PV	ND	c.2324T>A; p.Leu775X	wt	wt	wt	wt	50–60	wt	wt	ND	wt
HD-0880	M/49	PV	ND	wt	wt	wt	c.2245C>T; p.Arg749Cys^{NC}	wt	35	wt	wt	ND	wt
HD-0433	F/68	ET	ND	wt	wt	c.4010-4025del; p.Tyr1337LeufsX21	wt	wt	20–30	wt	wt	ND	wt
HD-0537	F/59	ET	ND	c.2385delC; p.Trp796GlyfsX22	wt	wt	wt	wt	wt	wt	wt	ND	wt
HD-0541	M/61	ET	ND	wt	wt	wt	wt	wt	wt	wt	wt	ND	c.2098A>G; p.Lys700Glu
HD-0545	F/65	ET	46,XX[20]	wt	wt	c.3965_3966insA; p.Glu1323GlyfsX16^{NC}	wt	wt	15	wt	wt	ND	wt
HD-0547	F/83	ET	ND	wt	wt	wt	wt	wt	2–5	wt	c.1258C>T; p.Arg420X	ND	wt
HD-0745	M/64	ET	ND	wt	wt	c.1648C>T; p.Arg550X^{NC}	wt	wt	50	wt	wt	ND	wt
HD-0757	M/83	ET	ND	c.1934dupG; p.Gly646TrpfsX12	wt	wt	wt	wt	30–50	wt	wt	ND	wt
HD-0860	M/73	ET	ND	wt	wt	c.4082G>T; p.Gly1361Val	wt	wt	12–30	wt	wt	ND	wt
HD-0497	F/82	PMF	46,XX,del(20)(q11q13)[6]/46,XX[3]	wt	wt	c.1996_1997delGA; p.Asp666ProfsX14^{NC}	wt	wt	20–30	wt	wt	wt	wt
HD-0528 =HD-1300	M/56	PMF	46, XY[20]	wt	wt	c.4062delA; p.Ala1355HisfsX8^{NC}	wt	wt	75	wt	wt	wt	wt
HD-1300 =HD-0528	M/60	PMF	ND	wt	wt	c.4062delA; p.Ala1355HisfsX8^{NC} and c.1648C>T; p.Arg550X^{NC}	wt	wt	80–90	wt	wt	ND	wt
HD-0540 =HD-1138	M/58	PMF	46, XY[20]	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	wt	wt	wt	wt	wt	wt	wt	wt
HD-1138 =HD-0540	M/62	PMF	ND	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	wt	wt	wt	wt	c.1543T>A; p.Trp515Arg^{NC}	wt	ND	wt
HD-0717	M/65	PMF	ND	wt	wt	c.1972delC; p.His658ThrfsX42	wt	wt	wt	wt	wt	wt	wt
HD-0719	M/85	PMF	46,XY[20]	wt	wt	wt	c.2644G>A; p.Arg882Ser	wt	wt	wt	wt	wt	wt
HD-0726	F/68	PMF	48,XX,+8,+9[7]/46,XX[13]	wt	wt	wt	wt	wt	50–70	wt	wt	c.8499T>G; p.Asp2833Lys^{NC}	wt
HD-0777	M/86	PMF	ND	wt	wt	c.5602C>T; p.His1868Tyr	wt	wt	wt	wt	wt	wt	wt
HD-0862	M/83	PMF	46, XY[20]	c.1934dupG; p.Gly646TrpfsX12	wt	wt	wt	wt	12–30	wt	wt	wt	wt
HD-0927	M/72	PMF	ND	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	wt	wt	wt	40–50	wt	wt	wt	wt
HD-1100	M/73	PMF	46,XY,del(13)(q13q22-31)[6]/46,sl,?del(17)(p12-13)[5]/44,sl,der(1)inv(1)(p32∼34q23)?dup(1)(q31q32),-7,-16,?der(17)t(16;17)(p11-12;p12-13)[6]/44,sdl1,?del(17)(p12-13)[5],+der(17)t(16;17)(p11-12;p12)[5]	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	wt	wt	wt	31–50	wt	wt	wt	wt

(Continued)

TABLE 1. Molecular Data of 46 MPNs Cases with ASXL1, SUZ12, TET2, DNMT3A, IDH2, JAK2, MPL, CBL, NF1, and SF3B1 Mutations (Continued)

				Epigenetic					Signaling				Splicing
				Histone methylation		DNA methylation							
Samples	Sex/age (y)	Diagnosis	Karyotype	*ASXL1* (exon 12)	*SUZ12* (exons 10-16)	*TET2* (exons 3-11)	*DNMT3A* (exons 15-23)	*IDH2* (exon 4)	JAK2V617F allele burden (%)	*MPL* (exon 10)	*CBL* (exons 7-8)	*NF1* (55 exons)	*SF3B1* (exons 15-16)
HD-1123	F/80	PMF	46,XX[20]	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	wt	wt	wt	50–70	wt	wt	c.3790G>A; p.Glu1264Lys^{NC}	wt
HD-1207	M/64	PMF	46,XY,ider(20)(q10)del(20)(q11q13)[20]	wt	wt	wt	wt	wt	wt	wt	wt	wt	c.2098A>G; p.Lys700Glu
HD-1289	M/75	PMF	ND	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	c.481_482insT; p.Lys161IlefsX10^{NC}	wt	wt	mutated	wt	wt	ND	wt
HD-0554	F/78	post-ET MF	46, XX[20]	wt	wt	wt	wt	wt	30–50	wt	wt	wt	c.2098A>G; p.Lys700Glu
HD-0601	M/76	post-ET MF	46,XY[20]	c.1888-1909del; p.His630ProfsX66	wt	c.5693C>T; p.Ser1898Phe	wt	wt	60–70	wt	wt	wt	wt
HD-0725	M/74	post-ET MF	46,XY[20]	wt	wt	c.3820C>T; p.Gln1274X^{NC}	wt	wt	40	wt	wt	wt	wt
HD-0758	F/80	post-PV MF	ND	c.1774C>T; p.Gln592X	wt	wt	wt	wt	50	wt	wt	wt	wt
HD-0789	M/66	post-PV MF	ND	c.1934dupG; p.Gly646TrpfsX12^{NC}	wt	wt	wt	wt	80–90	wt	wt	wt	wt
HD-0619	F/58	Blast-phase ET	ND	wt	wt	wt	wt	c.419G>A; p.Arg140Gln	wt	wt	wt	ND	wt
HD-0923	M/83	Blast-phase ET	46,XY[20]	wt	wt	wt	wt	c.419G>A; p.Arg140Gln	50	wt	wt	ND	wt
HD-0989	M/76	Blast-phase ET	46,XY[22]	wt	wt	c.4112T>A; p.Val1371Asp	wt	wt	wt	c.1544G>T; p.Trp515Leu	wt	ND	wt
HD-0646	F/61	Blast-phase post-ET	MF 46,XX,inv(3) (q21q26)[10]/46,XX[10]	wt	wt	wt	wt	wt	5–10	wt	wt	ND	c.2098A>G; p.Lys700Glu
HD-1038	M/80	Blast-phase post-ET	MF 44,X,-X[12],-7,del(9)(q22q34),-12,add(12)(q24)[3],-17,+mar1[9],+mar2[8],+mar3[4],+mar4[7],+mar5[4],+mar6[2] [cp17]/46,XX[3]	wt	c.2093A>G; p.Asn698Ser	c.3640C>T; p.Arg1214Trp	wt	wt	wt	wt	wt	wt	wt
HD-0535	M/69	Blast-phase PMF	46,XY,t(3;12)(q26-27;q13-14)[20]	c.2285dupT; p.Leu762PhefsX12	wt	wt	wt	wt	wt	wt	wt	wt	wt
HD-0536	M/54	Blast-phase PMF	46,XY,add(15)(q25-26)[5]/46XY[19]	c.1934dupG; p.Gly646TrpfsX12	wt	wt	wt	wt	wt	wt	wt	ND	wt
HD-1137	M/83	Blast-phase PMF	ND	wt	wt	wt	c.2644G>A; p.Arg882Ser	wt	ND	wt	wt	wt	wt
HD-0496	M/62	MPN/MDS	47,XY,+8 [18]/46, XY[2]	c.1934dupG; p.Gly646TrpfsX12	wt	c.1864C>T; p.Gln622X	wt	wt	wt	wt	wt	wt	c.2098A>G; p.Lys700Glu
HD-0531	M/70	MPN/MDS	46,XY,del(9)(q22q32-33)[20]	wt	wt	c.787delG; p.Glu263ArgfsX30 and c.3323delC; p.Pro1108LeufsX5	wt	wt	30–40	wt	wt	wt	wt
HD-0752	M/64	MPN/MDS	46,XY[20]	c.2278C>T; p.Gln760X^{NC}	wt	wt	wt	wt	wt	wt	wt	ND	wt

References for nucleotide and protein sequences of ASXL1, SUZ12, TET2, DNMT3A, IDH2, MPL, CBL, NF1, and SF3B1 are respectively NM_015338.5, NP_056153.2, NM_015355.2, NP_056170.2; NM_001127208.1, NP_001120680.1; NM_022552.3, NP_072046.2; NM_002168.2, NP_002159.2; NM_005373.2, NP_005364.1; NM_005188.2, NP_005179.2; NM_001042492.2, NP_001035957.1; NM_012433.2, NP_036565.2.

ET, essential thrombocythemia; MDS, myelodysplastic syndrome; MF, myelofibrosis; MPN, myeloproliferative neoplasms; NC, not constitutional mutation; ND, not determined; PMF, primary myelofibrosis; PV, polycythemia vera; wt, wild-type; y, years.

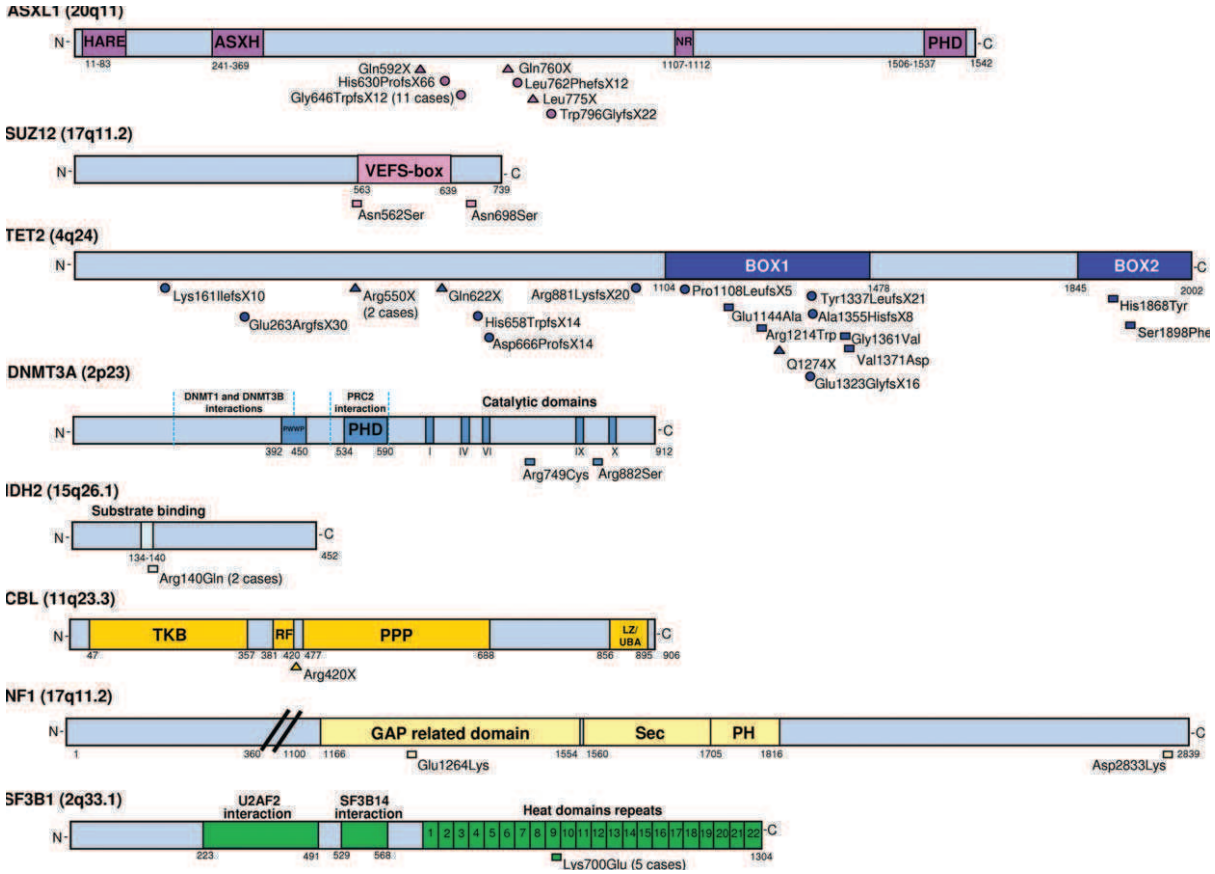

Figure 1. Deduced localization of mutations in myeloproliferative neoplasms. ASXL1 (ASXH: Additional sex combs homology domain; PHD: Plant Homeodomain; NR box: Nuclear Receptor), SUZ12 (VEFS-box: (VRN2-EMF2-FIS2-Su(z)12) box), TET2 (BOX1 and BOX2: conserved regions 1134–1444, 1842–1921), DNMT3A (PHD: Plant Homeodomain; PRC2: Polycomb Repressive Complex 2; PWWP: proline tryptophan tryptophan proline), CBL (TKB: Tyrosine Kinase Binding; RF: RING finger; PPP: Proline rich region; LZ: Leucine Zipper; UBA: Ubiquitin Associated), and NF1 (Sec: Sec14-like; PH: Pleckstrin Homology) proteins. Triangles represent nonsense mutations, circles represent frameshift mutations and squares represent missense mutations.

PV (HD-0880), a PMF (HD-0719) and a post-MF blast-phase (HD-1137), and mutations in *SUZ12* in two cases: a PV (HD-0716) and a blast-phase of post-ET MF (HD-1038) (Brecqueville et al., 2011a,b), (Table 1, Fig. 1).

By using array-comparative genomic hybridization (aCGH) we had found heterozygous 17q11 deletions containing the *NF1* gene in four MPN cases (Brecqueville et al., 2011b); we therefore studied *NF1* in our series; results were available for 36 MF cases, 5 blast-phases and 3 MPN/MDS. We found two *NF1* mutations in two PMF: c.3790G>A p.Glu1264Lys (Exon 28) (HD-1123) and c.8499T>G; p.Asp2833Lys (Exon 58) (HD-0726) (Table 1, Fig. 1, Supporting Information Fig. S2); these cases did not show any 17q11 deletion as seen by aCGH (data not shown).

Mutations in components of the splicing machinery were described in myeloid malignancies, especially myelodysplastic syndromes (Papaemmanuil et al., 2011; Stein et al., 2011; Yoshida et al., 2011; Visconte et al., 2012). They were more rarely found in *de novo* AMLs and MPNs (9.4%) (Yoshida et al., 2011; Lasho et al., 2012). The *SF3B1* gene was found mutated in three-quarters of refractory anemia with ring sideroblasts (RARS) and RARS associated with marked thrombocytosis (RARS-T). *SF3B1* encodes a subunit of a splicing factor (U2 snRNP) and histone acetyltransferase (STAGA) complexes. Because this gene is implicated in RARS-T, an MPN/MDS form, we analyzed this gene in our series. We found the same mutation within Exon 15 (c.2098A>G; p.Lys700-Glu), which represent the most common reported *SF3B1* mutation (Papaemmanuil et al., 2011; Visconte et al., 2012), in five patients: one ET (HD-0541), one post-ET MF (HD-0554), one blast-phase post-ET MF (HD-0646), one PMF (HD-1207), and one MPN/MDS (HD-0496) (a RARS associated with MF) (Table 1, Fig. 1).

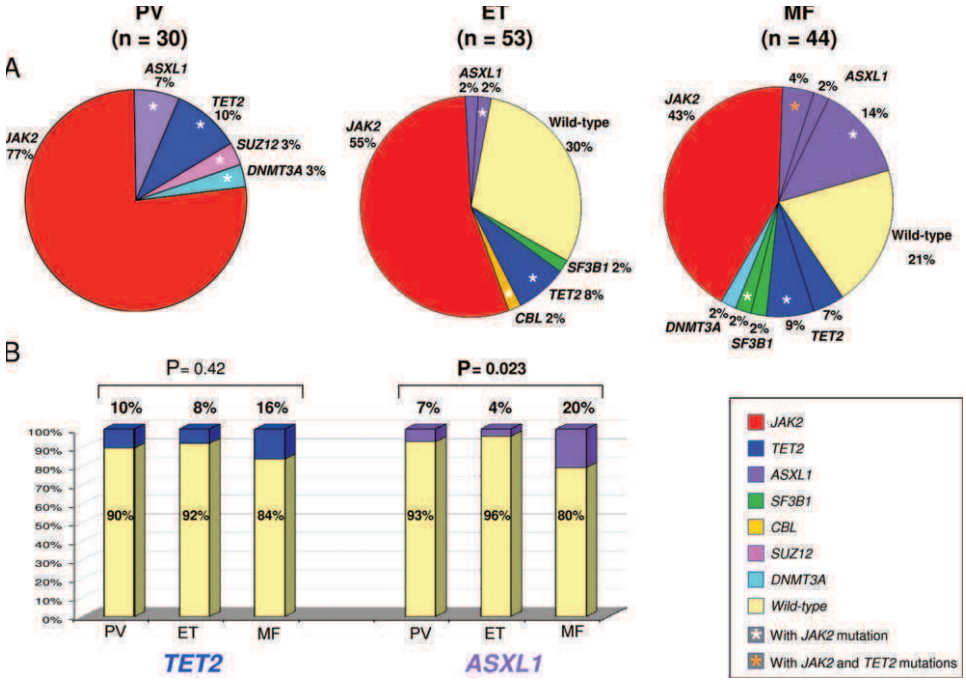

Figure 2. Gene mutations in 127 "classic" myeloproliferative neoplasms. A: Respective frequency of *ASXL1, CBL, DNMT3A, JAK2, SF3B1, SUZ12*, and *TET2* mutations. B: Distribution of *ASXL1* and *TET2* mutations in PV, ET, and MF cases. Among *ASXL1*-mutated MPNs, MF are significantly more mutated than PV or ET ($P = 0.023$). ET: essential thrombocythemia, MF: myelofibrosis, PV: polycythemia vera.

None of the 24 reactive cases was mutated in any of the 11 genes (including at the *ASXL1* 1934 nucleotide position).

Analysis of DNAs from buccal swabs and non-clonal CD3+ cells allowed the distinction between somatic mutations and nonacquired variants for a portion of cases. Two of the *ASXL1* (including the c.1934dupG; p.Gly646TrpfsX12) and seven of the *TET2* mutations were not found in any of the germ line DNAs tested (Table 1, Supporting Information Figs. S1, S2, and S3). Similarly, the c.3790G>A; p.Glu1264Lys and c.8499T>G; p.Asp2833Lys *NF1* mutations were not found in the buccal swabs (Table 1, Supporting Information Fig. S2). Even when using four different polymerases the c.1934dupG; p.Gly646TrpfsX12 *ASXL1* mutation was found in peripheral blood but not in buccal swab DNAs (Supporting Information Fig. S4). Finally, we found 24 missense mutations in our series, 4 in *ASXL1*, 19 in *TET2* and 1 in *CBL*; only 19 of them have been reported as single nucleotide polymorphisms in dbSNP (Supporting Information Table S3).

Patterns of Concomitantly Mutated Genes in MPNs

In the 127 classic MPNs studied, *ASXL1* was mutated in 11/95 JAK2V617F (12%) and 2/32 JAK2wt (5%) cases, and in 2/14 *TET2*-positive (14%), and 11/113 *TET2*-negative (10%) cases (Fig. 3). Reciprocally, 85% (11/13) and 15% (2/13) of *ASXL1*-mutated cases carried JAK2V617F and *TET2* mutations, respectively. Only two of the patients, a PMF (HD-1289) and a post-ET MF (HD-0601), carried a mutation in each of the three genes. The ET case (HD-0547) with a *CBL* mutation carried the JAK2V617F. The PMF case (HD-1138) mutated in *MPL* carried an *ASXL1* mutation. Interestingly, the same patient 36 months before (HD-0540) was negative for the *MPL* mutation (Supporting Information Fig. S1) but the *ASXL1* mutation was already present. The ET blast-phase mutated in *MPL* also carried a *TET2* mutation. Both NF1-mutated PMF cases (HD-0726 and HD-1123) had the JAK2V617F and HD-1123 carried also an *ASXL1* mutation. Among the three cases mutated in *DNMT3A*, only one (a PV) carried an associated mutation (JAK2V617F). Among the two cases mutated in *SUZ12*, the blast-phase post-ET MF (HD-1038) was also mutated in *TET2* and the PV case (HD-0716) carried the JAK2V617F. Interestingly, the four classic MPNs with *SF3B1* mutation did not carry any other mutation in the tested genes except the JAK2V617F in two cases (post-ET MF and blast-phase post-ET MF); we found an

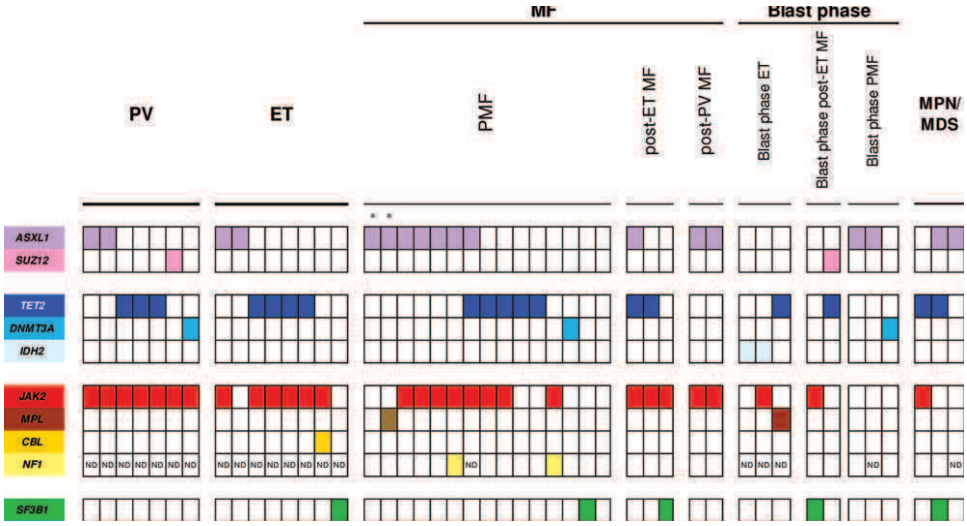

Figure 3. Patterns of concomitantly mutated genes in myeloproliferative neoplasms. Identified mutations in 11 genes in 149 samples are shown by colored squares. Only cases with at least one mutation (JAK2V617F is not taken into account) are shown. *: paired samples HD-0540/HD-1138, ET: essential thrombocythemia, MDS: myelodysplastic syndrome, MF: myelofibrosis, MPN: myeloproliferative neoplasm, ND: not determined, PMF: primary myelofibrosis, PV: polycythemia vera.

association of *ASXL1*, *TET2*, and *SF3B1* mutation only in the MPN/MDS case (HD-0496). Figure 3 summarizes these results. In 30% of the 53 ETs and 21% of the 44 MFs, no mutation was found (Fig. 2A).

Associations Between Mutations and Clinico-Biological Features of the 127 Classic MPNs

MPN cases with mutation in *ASXL1* (Table 2) were associated with an older age ($P = 0.008$) than cases without *ASXL1* mutation. *ASXL1*- and *TET2*-mutated patients had a tendency to be more frequently males than females ($P = 0.14$ with OR = 2.9 and $P = 0.18$ with OR = 2.1, respectively). The presence of an *ASXL1* mutation did not influence leukocyte count or platelet count but hemoglobin count was lower in *ASXL1*-mutated classic MPN cases ($P = 0.04$) (Table 2). We observed no association between the presence of mutant *ASXL1* or *TET2* and the individual thrombotic risk in ET and PV.

The DIPSS-plus score was available for 28 of our 30 PMF patients. PMF with *ASXL1* mutations showed a tendency to be included in the high-risk group (one intermediate-1 risk and five intermediate-2 risk; $P = 0.63$, OR = 2.8) (Table 3). The PMF patient (HD-0927) mutated in *ASXL1* and stratified as intermediate-1 risk presented constitutional symptoms such as splenomegaly and pruritus justifying cytoreductive therapy.

We evaluated the prognostic impact of *ASXL1* mutation in MF with available follow-up information ($n = 44$) (Table 3). Although the number of patients was small, we observed a difference ($P = 0.02$) in 5-year OS between patients with *ASXL1* mutation (56% 5-year OS) and patients without *ASXL1* mutation (87% 5-year OS) (Table 3, Fig. 4). This was not observed with *TET2* mutations (Fig. 4).

DISCUSSION

We found a high incidence of *ASXL1* mutation in myelofibrosis, including PMF (20%), post-PV MF (50%) and post-ET MF (10%), and a low incidence in PV (7%) and ET (4%). *ASXL1* mutations in MF have been described. A first study on MPN granulocytes reported 23 *ASXL1* mutations in 77 MF cases (30%) including 15/47 (32%) PMF and 11/30 (37%) post-ET/PV MF (Stein et al., 2011). A second study identified *ASXL1* mutations in 6/46 (13%) PMF, 5/22 (23%) post-PV/ET MF, and 2/11 (18%) MPN blast-phases in a series of bone marrow samples, and in 12% of 25 granulocyte PMF cases (Abdel-Wahab et al., 2010); however, this study did not take into account the p.Gly646TrpfsX12 mutation, which roughly accounts for half of *ASXL1* mutations (Boultwood et al., 2010; Chou et al., 2010; Fernandez-Mercado et al., 2010; Stein et al., 2011). A third study on blood granulocytes or bone marrow samples identified *ASXL1* mutations in 23/42 (55%) PMF and 5/23 (22%) post-ET/PV MF cases (Ricci et al., 2011). We found one *ASXL1* mutation in ET and two in PV. Only one case of

TABLE 2. Features of ASXL1-Mutated or -Unmutated Patients with PV, ET, or MF

		Total number	*ASXL1* *unmutated*	*ASXL1* *mutated*	*P*-value	Odds ratio
Classic MPN patients		127	114	13		
Older Age (y) mean (range)			63 (29–97)	74 (58–83)	**0.008**	
Sex	female	57	54 (47%)	3 (23%)	0.14	2.9
	male	70	60 (53%)	10 (77%)		
Leukocyte count ($\times 10^9$/L); median (range)			9.2 (1.9–120)	11.4 (2.1–42)	0.20	
Hemoglobin count ($g{\cdot}dL^{-1}$); median (range)			14.1 (7.2–20.4)	11.7 (9.2–21.3)	**0.04**	
Hematocrit count (%); median (range)			42.5 (20.2–63.1)	36.0 (27–66.4)	0.06	
Platelet count (x 10^9/L); median (range)			563 (5–2,175)	450 (108–1188)	0.27	
Diagnosis	PV	30	28 (25%)	2 (15%)		
	ET	53	51 (45%)	2 (15%)	**0.02**	
	MF	44	35 (31%)	9 (69%)		
PV						
Leukocyte count (x 10^9/L); median (range)		30	8.65 (2.3–26.5)	5.55 (4.7–6.4)	0.07	
Hemoglobin count ($g{\cdot}dL^{-1}$); median (range)		30	14.3 (11.1–17.8)	13.5 (11.9–15.1)	0.61	
Hematocrit count (%); median (range)		30	42.2 (36–51.7)	40.5 (35.7–45.4)	0.56	
Platelet count (x 10^9/L); median (range)		30	700 (411–2175)	648 (482–815)	0.59	
ET						
Leukocyte count (x 10^9/L); median (range)		53	8.5 (2.3–26.5)	5.4 (2.1–8.7)	0.32	
Hemoglobin count ($g{\cdot}dL^{-1}$); median (range)		53	14.1 (8.4–17.8)	11.8 (9.2–14.3)	0.38	
Hematocrit count (%); median (range)		53	42.3 (24.7–51.7)	32.9 (27–38.8)	0.07	
Platelet count (x 10^9/L); median (range)		53	709 (365–2175)	570 (450–691)	0.25	
MF						
Leukocyte count (x 10^9/L); median (range)		44	8.6 (1.9–120)	11.7 (4.6–42)	0.20	
Hemoglobin count ($g{\cdot}dL^{-1}$); median (range)		44	11.5 (7.2–17.8)	11 (9.4–13.7)	0.96	
Hematocrit count (%); median (range)		44	35 (20.2–52)	33.5 (29.7–45.6)	0.7	
Platelet count (x 10^9/L); median (range)		44	295 (5–890)	407 (108–1188)	0.27	
	PMF	30	24 (80%)	6 (20%)		
	post PV-MF	4	2 (50%)	2 (50%)	0.27	
	post ET-MF	10	9 (90%)	1 (10%)		

ET, essential thrombocythemia; MF, myelofibrosis; MPN, myeloproliferative neoplams; PMF, primary myelofibrosis; PV, polycythemia vera; y, years.

TABLE 3. DIPSS-Plus Risk Distribution in PMF and MF Outcomes Depending on *ASXL1* Mutation Status

		Total number	*ASXL1* unmutated	*ASXL1* mutated	*P*-value	Odds ratio
PMF patients		28	22	6		
DIPSS-plus risk *n* (%)	Low	3	3 (15%)	0	0.39	
	Intermediate-1	6	5 (25%)	1 (17%)		
	Intermediate-2	14	9 (45%)	5 (83%)		
	High	5	5 (25%)	0		
DIPSS-plus risk group *n* (%)	Low	9	8 (36%)	1 (17%)	0.63	2.8
	High	19	14 (64%)	5 (83%)		
MF patients		44	35	9		
Follow-up from initial MF diagnosis, months; median (range)		44	39.1 (0–210.2)	13.6 (0–85.4)		
5-years overall survival % [95CI]			87% [74–100]	56% [23-100]		
Deaths *n* (%)		9 (20%)	6 (17%)	3 (33%)		

DIPSS, dynamic international prognostic scoring system; MF, myelofibrosis; PMF, primary myelofibrosis.
For PMF, DIPSS-plus depends on age (>65 years), on hemoglobin (<10 $g{\cdot}dL^{-1}$), on leukocyte count (>25.10^9/L), on circulating blasts (≥1%), on platelet count (<100.10^9/L), on karyotype and on transfusion status.

ASXL1-mutated PV and one case of *ASXL1*-mutated ET have been described so far (Stein et al., 2011; Martinez-Aviles et al., 2011). In our series, among the two *ASXL1*-mutated PV cases, one (HD-0659) had the hematocrit elevated to 63%, subnormal EPO level and showed a JAK2V617F mutation with low allele burden (2%), which is unusual in PV. We found *ASXL1*

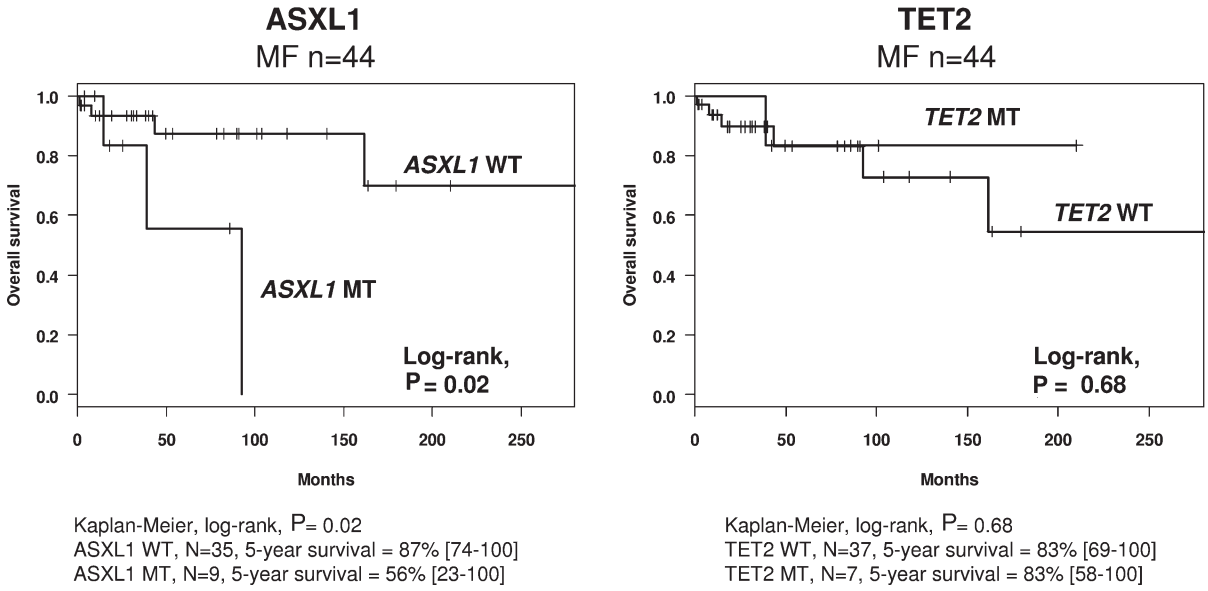

Figure 4. Survival analysis of myelofibrosis patients according to the *ASXL1* and *TET2* mutational status. Survival curves were defined with the Kaplan-Meier method and compared with the log-rank test. MF: myelofibrosis, MT: mutated, WT: wild-type.

mutations in 2/14 (14%) cases of PV/ET/MF blast-phases; a similar frequency was observed in a previous study (2/11) of MPN blast-phases (Abdel-Wahab et al., 2011b).

We noticed an association of mutations in genes involved in signaling pathways (*CBL, JAK2*, *MPL*, *NF1*) and mutations in genes involved in epigenetic regulations (*ASXL1*, *SUZ12*, *DNMT3A*, *IDH2*, *or TET2*). In our PV and ET cases, *ASXL1* and *TET2* mutations were mutually exclusive but this was not true in MF and MPN/MDS. This association is indeed frequent in MPN/MDS (especially chronic myelomonocytic leukemia—CMML) (Abdel-Wahab et al., 2011b). Another study did not find MPN cases with concomitant mutations in *ASXL1*, *JAK2*, and *TET2* genes (Abdel-Wahab et al., 2010) whereas another one described a single case of post-PV MF with mutations in these three genes (Abdel-Wahab et al., 2011b). A *SUZ12*-mutated case was associated with a *TET2* mutation whereas the three *DNMT3A*-mutated cases had neither *ASXL1* nor *TET2* mutation. For *DNMT3A*, *SF3B1*, and *SUZ12* the low mutational frequencies prevent to draw any more conclusions (Fig. 2).

We found the same incidence of *ASXL1* mutations in JAK2wt and JAK2V617F patients. For seven patients we were able to determine that the *ASXL1* mutation was present at the time of diagnosis. For the paired samples HD-0507/HD-1122, HD-0528/HD-1300, HD-0610/HD-0841, and HD-0555/HD-0866 obtained at different times of the disease (Supporting Information Table S1B) we found an elevation of the JAK2V617F allele burden but no apparition of an *ASXL1* mutation. In a previous study, with the exception of a single patient who acquired both *ASXL1* and *TET2* mutations, all patients with *ASXL1* mutation at leukemic transformation already had *ASXL1* mutation at the chronic MPN state (Abdel-Wahab et al., 2010). These observations suggest that *ASXL1* mutations may constitute early alterations in MPN oncogenesis and precede *JAK2* and *MPL* mutations (Carbuccia et al., 2009; Abdel-Wahab et al., 2010; Szpurka et al., 2010). The study of the HD-0540/HD-1138 pair strengthens this hypothesis (Fig. S1). Taken together our results suggest that alterations of epigenetic regulators cooperate with and may precede mutations in components of signaling pathways. The opposite is possible. In one JAK2V617F and *TET2* mutated PMF case (HD-0528) we observed the acquisition of a second *TET2* mutation during chronic phase (Fig. S1). This was not surprising but further document the fact that *TET2* mutations can occur at various time of the disease (Delhommeau et al., 2009; Abdel-Wahab et al., 2010). Genotyping of individual clones and functional studies will be necessary to precise the actual pathogenic cooperation between *ASXL1* mutation and other mutations such as *JAK2*, *IDH1/2*, and *TET2* in terms of fibrotic and leukemic transformation.

MPN cases with mutation in *ASXL1* were associated with older age and patients with *ASXL1* or

TET2 mutations tended to be more frequently males than females. A recent study showed an association between *ASXL1* lesions and older age and male sex (Stein et al., 2011). These data may be explained in part by higher level of mitotic recombination events in males than in females (Stein et al., 2010). The proportion of *ASXL1* mutations was higher in post-PV MF and post-ET MF (50 and 10%, respectively) than in PV and ET (7 and 4%, respectively), suggesting that the *ASXL1* status may help predict the risk of evolution of PV and ET to MF. Hematological features were not influenced by *ASXL1* mutation but the numbers of patients studied for each type were too low to allow firm conclusions.

In the 44 MF patients, the 5-year OS was shorter in *ASXL1*-mutated patients than in nonmutated patients. The presence of an *ASXL1* mutation could be an incentive to use an investigational drug therapy (pomalidomid, JAK2 inhibitors, mTOR inhibitors) as proposed in the management of intermediate-2 or high-risk disease (Tefferi, 2011). A previous survival analysis also showed that *ASXL1* mutations tended to be associated with worse survival of PMF ($P = 0.06$) (Abdel-Wahab et al., 2011b). A similar association of *ASXL1* mutations with aggressiveness has been found in MDS (Boultwood et al., 2010), in CMML (Gelsi-Boyer et al., 2010) and in *de novo* acute myeloid leukemia (Chou et al., 2010). *ASXL1* mutations are associated with secondary AML (Carbuccia et al., 2010; Rocquain et al., 2010). In primary cytogenetically normal AMLs, *ASXL1*-mutated older patients have an unfavorable outcome (Metzeler et al., 2011). In contrast, another study did not show any correlation between *ASXL1* mutations and accelerated transition rate from ET or PV to MF; however, MF patients with *ASXL1* lesions were more likely to have received anemia-directed therapy compared to those without lesions (Stein et al., 2011). In a recent study, *ASXL1*-wild type MF carried a higher percentage of JAK2V617F allele than *ASXL1*-mutated MF (Ricci et al., 2011). *ASXL1* alterations may lead to an increase in the self-renewal pathway in MPN progenitors through modifications of DNA and histone regulatory marks (Acquaviva et al., 2010); this may render the disease more aggressive.

One mutation in *CBL* was found in an ET patient. This finding is new. However, the patient presented with thrombocytosis only, without cytopenia, and with normal spleen size; there were rare immature granulocytes associated with rare dacryocytes on blood smears, suggesting MF apparition, as documented for one case in a previous study (Grand et al., 2009). We found *IDH2* mutations in two post-ET blast-phases but none in chronic-phase diseases, as previously described (Tefferi et al., 2010). Despite the fact that *TET2* and *IDH* mutations are exclusive and may intervene in the same methylation pathway, this suggests that their influence on MPN pathogenesis is different.

Three of the five *SF3B1* mutations were detected in patients with ET history and none in PV. This is in agreement with a pioneering study on *SF3B1* (Papaemmanuil et al., 2011), which described mutations of this gene in 6/189 ET cases and 6/136 PMF cases but not in 95 PV cases. Five other splicing factor genes were found mutated in around 10% of MPNs (not otherwise specified) (Yoshida et al., 2011). In another study, *SF3B1* mutations were found in 6.5% of PMF (10/155 cases), invariably associated with bone marrow ring sideroblasts (Lasho et al., 2012). *SF3B1* mutations have been described in 72% of patients with RARS-T (Visconte et al., 2012). Spliceosome mutations could represent a pathogenic link between RARS-T and MPN with thombocytosis and open a new field of investigation in the pathogenesis of MPN, essentially in ET and MF. Because *SF3B1*-mutated patients with RARS and RARS-T have thrombotic events (Visconte et al., 2012), it would be interesting to study *SF3B1* mutation status in large ET series to evaluate the individual thrombotic risk for mutated patients.

Deletions of the tumor suppressor gene *NF1* associated with a mutation in the remaining allele in half of the cases were recently described in 5% of myeloid malignancies (Haferlach et al., 2012); no mutation was found in MPN chronic-phases whereas one blast-phase was mutated (Haferlach et al., 2012). We described only two mutations in our series, in two PMF. These *NF1* mutations could lead to RAS activation pathway during disease evolution of MF.

The genetic complexity of non-CML MPNs is highlighted by the recent discovery of novel mutations in several genes including *ASXL1, CBL, DNMT3A* (Abdel-Wahab et al., 2011a; Brecqueville et al., 2011a; Stegelmann et al., 2011), *EZH2* (Ernst et al., 2010; Nikoloski et al., 2010), *IDH1/2, JAK2, LNK* (Oh et al., 2010; Pardanani et al., 2010b), *MPL*, splicing genes (Yoshida et al., 2011), *SUZ12* (Brecqueville et al., 2011b; Score et al., 2012) and *TET2*. High-throughput sequencing studies are likely to identify even more alterations in MPNs. Excluding *JAK2* and *MPL* mutations, the described mutations are not

MPN-specific. Alteration of the *ASXL1* gene is prominent in MF suggesting that *ASXL1* alteration may play a role in myelofibrosis physiopathology. Among the classic MPNs, MF seems to be a more complex molecular group than PV or ET. A stronger alteration of DNA and histone modifications could be associated with transition of PV or ET to MF.

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ARTICLE n°6

Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase.

Brecqueville M, Rey J, Devillier R, Guille A, Gillet R, Adélaide J, Gelsi-Boyer V, Arnoulet C, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D & Murati A.

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<u>Article 6</u> - Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase.

Afin d'affiner la caractérisation moléculaire des MF, nous avons étudié les MFP, les MF post-TE/PV et les LAM post-MF. Nous voulions établir un état des lieux des anomalies récurrentes dans les différents types de MF (primaire et secondaire), et étudier leur possible impact pronostique. Nous avons conjointement mené deux approches : la CGH-array et le séquençage. La CGH-array qui détecte les CNA (gains et délétions) a été réalisée sur 63 cas de MF en phase chronique (38 MFP, 14 MF post-PV, 11 MF post-TE) et sur 17 cas de transformation en LAM. Le séquençage nous a permis d'étudier le statut mutationnel de 23 gènes dans 68 cas de MF (39 PMF, 15 MF post-PV et 14 MF post-TE) et 17 cas de transformation en LAM. Parmi ces 23 gènes, 18 gènes (*ASXL1, CBL, DNMT3A, EZH2, IDH1/2, JAK2, KRAS, LNK, MPL, NF1, NRAS, PTPN11, SF3B1, SRSF2, SUZ12, TET2* et *TP53)* ont été sélectionnés pour leur implication dans la leucémogenèse ; et les 5 autres *(BMI1*, *PPP1R16B/TIMAP, RCOR1, SOCS2* et *TRPS1)* pour leurs altérations retrouvées en CGH-array et leurs potentielles implications dans la physiopathologie de la MF.

Ces travaux ont mis en évidence les éléments suivants :

Au sein des MF primaires (MFP) et secondaires (MF post-PV/TE)

- L'analyse génomique par CGH-array nous a permis de révéler des anomalies cryptiques non détectables au caryotype, soulignant l'intérêt certain d'utiliser cette technologie afin de « pointer » des gènes candidats.
- Nous avons identifié une forte instabilité chromosomique dans la MF. En effet, un peu plus de la moitié des patients (54%) atteints de MF étaient porteurs de CNA. Contrairement aux TE qui étaient altérés dans 2% des cas (1/42) selon nos résultats précédents (Bernard *et al.* 2009).
- Nous n'avons remarqué aucune différence de CNA en fonction du sexe, et du type de MF, suggérant que les MF primaires ou secondaires à une PV ou TE sont des pathologies très proches, voire semblables moléculairement.

- Parmi les 72 CNA détectées, la plupart (63/72) étaient des délétions, suggérant que les gènes impliqués dans la physiopathologie de la MF sont majoritairement des gènes suppresseurs de tumeurs.
- Les CNA les plus récurrentes étaient la del(20q) (n=14), la del(17q)(n=7), la del (7p) (n=5), la del(13q)(n=3) et les gains du 1q (n=3) et du 9p (n=3).
- La délétion 20q retrouvée dans 14 échantillons (22%) avait une taille moyenne de 16.0 Mb.
- Au sein de la délétion 20q, nous avons identifié deux régions communément délétées (CDR1 et CDR2). CDR1 s'étendait sur 0.3 Mb et comprenait environ une dizaine de gènes dont *PPP1R16B* et plusieurs *SNORNA*. CDR2 s'étendait sur 3.9 Mb et comprenait également des *SNORNA*. Les *SNORNA* sont des petits ARN non-codants qui participent à la régulation épigénétique de l'épissage.
- Aucune mutation n'a été identifiée au sein du gène *PPP1R16B* qui était systématiquement inclus dans la del(20q) et potentiellement intéressant dans la physiopathologie des MF puisque sa synthèse est inhibée par le TGFβ (Cao *et al.* 2002). L'absence de mutation suggère que l'haploinsuffisance de plusieurs gènes de cette région pourrait contribuer à la leucémogénèse. Une autre hypothèse serait que les *SNORNA* qui sont présents dans les deux CDR puissent jouer un rôle dans le développement de la maladie.
- Bien que la délétion 17q était de taille cryptique, les gènes *NF1* et *SUZ12* étaient la plupart du temps inclus.
- Au sein de la délétion 7p qui était le troisième CNA récurrente, nous avons identifié des délétions du gène *EZH2*, suggérant que son inactivation dans la MF pourrait se faire par mutation mais aussi par délétion.
- Nous avons identifié que les patients avec des del(12p),del (17q) et del(20q) avaient un temps de transformation aiguë (TTAT) plus faible. Ce qui est nouveau car dans les SMD et dans les formes dysplasiques de la LMMC, la délétion 20q isolée confère un pronostic favorable.

- Parmi les **23 gènes séquencés**, les gènes mutés de façon prédominante dans les MF étaient : *JAK2* (69%), *ASXL1* (26%), *TET2* (14%), *EZH2* (8%), *NF1* (6%) et *SRSF2* (6%).

- Dans la majorité des patients (88%), nous avons identifié la présence d'au moins une mutation.
- Pour la première fois, des mutations du gène *PTPN11* ont été identifiées dans les MF. Ce gène qui code pour une protéine régulatrice négative de la voie RAS/MAPK et JAK2/STAT, est également muté dans d'autres hémopathies myéloïdes (LMMC et LMMJ).
- Notre étude a permis de souligner que le spectre mutationnel des MF post-TE/PV est différent et plus complexe que celui des maladies initiales (TE et PV). De plus, il existe une répartition des mutations différente entre la MFP et les MF secondaires. En effet les MF post-TE/PV présentent une mutation au sein des gènes de l'épigénétique et sont toujours muté *JAK2* contrairement aux MFP qui peuvent avoir une mutation isolée de *ASXL1* ou *TET2*. Cette observation laisse supposer qu'il existe différentes voies pour aboutir à la MF. Dans les PV et TE, la présence de la mutation *JAK2* pourrait donner un avantage à la cellule pour l'acquisition de nouvelles anomalies responsable de l'évolution en MF.
- Les patients avec plus d'une mutation avaient une TTAT et une survie globale plus faible.
- Nous confirmons sur une série de 68 MF, ce qui avait été observé dans l'article 5, à savoir que la mutation *ASXL1* est de mauvais pronostic (p=0.02). Cet impact délétère sur la survie globale est renforcé par l'association des mutations de *JAK2* et d'*ASXL1* (p=0.02). Ces résultats soulèvent la question d'une orientation thérapeutique en fonction du statut mutationnel (allogreffe ou épidrogues).
- En analyse multivariée, nous avons également retrouvé, le mauvais pronostic des mutations d'*EZH2* et *SRSF2* indépendamment des autres mutations (Guglielmelli *et al.* 2011; Lasho *et al.* 2012; Vannucchi *et al.* 2013).

Analyse des transformations aiguë de MF primaire ou secondaire

Afin d'identifier quels sont les acteurs moléculaires impliqués dans la transformation de MF en phase aiguë, nous avons étudiés 17 LAM post-MF (4 transformations de MF post-PV, 7 transformation de MF post-TE, 5 transformation de MFP et 1 transformation de SMP inclassable MF). Pour cinq de ces patients, nous avons étudié les prélèvements en phase chronique et aiguë.

- **Parmi les 17 LAM post-MF**, nous avons détecté des CNA dans 82% de cas (14/17). Les anomalies les plus récurrentes étaient la délétion 12p13 détectée dans 5 cas associée le plus souvent à une altération du chromosome 7 (monosomie 7 ou délétion 7q) (3 cas/5), ainsi que la délétion 17q11 détectée dans 1 cas. A noter que la majorité de ces anomalies n'avaient pas été détectées au caryotype en raison de leur petite taille (moyenne de 4 Kb).
- **La délétion 12p comprenait** systématiquement les gènes *ETV6* et *CDKN1B* ; la délétion 7q comprenait le gène *EZH2 ;* et la délétion 17q11, le gène *NF1*.
- Les mutations les plus fréquentes étaient celles de *JAK2* (44%), *ASXL1* (25%), *TP53* (23%) et *EZH2* (22%) soulignant leurs fortes implications au cours de la leucémogenèse. Comparé aux formes chroniques, la fréquence des mutations dans les LAM, était plus faible pour *JAK2* , plus élevé pour *EZH2* et *TP53*. La fréquence des mutations d'*ASXL1* étaient par contre identique dans les phases chroniques et phases aiguës.
- **Parmi les 5 MF transformées en LAM**, trois patients avaient une altération du chromosome 7 (-7 ou délétion 7p) associée à la délétion 12p. Deux de ces patients présentaient déjà en phase chronique ces altérations, suggérant que celles-ci puissent jouer un rôle dans la leucémogenèse. La délétion 12p avait déjà été rapportée dans plusieurs études comme de mauvais pronostic dans les MF (Hussein *et al.* 2010; Tam *et al.* 2009; Tefferi *et al.* 2001; Caramazza *et al.* 2011) mais jamais associé au risque de transformation en LAM.

  De plus, une étude récente dans les SMD et LAM a révélé que chez les patients atteints de monosomie 7, une suppression supplémentaire d'*ETV6* est souvent présente renforçant l'idée que l'association de ces anomalies pourrait être de mauvais pronostic (Haferlach *et al.* 2012).
- De même, deux patients MFP ont acquis au cours de la progression de leur maladie une délétion 20q en plus des mutations déjà présentes (respectivement *ASXL1, JAK2, SRSF2* et *TET2, SRSF2*) suggérant que l'instabilité engendrée par les mutations et les CNA a entrainé le passage du SMP en phase chronique vers la LAM.

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Early Release Paper

Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase

by Mandy Brecqueville, Jérôme Rey, Raynier Devillier, Arnaud Guille, Rémi Gillet, José Adelaide, Véronique Gelsi-Boyer, Christine Arnoulet, Max Chaffanet, Marie-Joelle Mozziconacci, Norbert Vey, Daniel Birnbaum, and Anne Murati

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Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase.

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Running head: aCGH array and sequencing of myelofibrosis

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Abstract

Myelofibrosis is a myeloproliferative neoplasm that occurs *de novo* (primary myelofribrosis) or results from the progression of polycythemia vera or essential thrombocytemia (hereafter designated as secondary myelofibrosis or post-polycythemia vera/ essential thrombocytemia myelofibrosis). To progress in the understanding of myelofibrosis and to find molecular prognostic markers we studied 104 samples of primary and secondary myelofibrosis at chronic (N=68) and acute phases (N=12) from 80 patients, by using array-comparative genomic hybridization and sequencing of 23 genes (*ASXL1, BMI1, CBL*, *DNMT3A, EZH2, IDH1/2, JAK2*, *K/N-RAS*, *LNK*, *MPL, NF1, PPP1R16B, PTPN11, RCOR1, SF3B1, SOCS2, SRSF2, SUZ12, TET2, TP53, TRPS1*). We found copy number aberrations in 54% of samples, often involving genes with a known or potential role in leukemogenesis. We show that cases carrying a del(20q), del(17) or del(12p) evolve in acute myeloid leukemia (p=0.03). We found that 88% of the cases were mutated, mainly in signaling pathway (*JAK2* 69%, *NF1* 6%) and epigenetic genes (*ASXL1* 26%, *TET2* 14%, *EZH2* 8%). Overall survival was poor in patients with more than one mutation (p=0.001) and in patients with *JAK2*/*ASXL1* mutations (p=0.02). Our study highlights the heterogeneity of myelofibrosis, and points to several interesting copy number aberrations and genes with diagnostic and prognostic impact.

Keywords: myelofibrosis, myeloproliferative neoplasm, aCGH, del(20q), mutations, *ASXL1*.

Introduction

Myelofibrosis (MF) is a clonal hematopoietic stem cell disorder belonging to the non-chronic-myeloid-leukemia myeloproliferative neoplasms (MPN). MF is characterized by cytopenias and/or leukocytosis, leukoerythroblastosis in blood, progressive marrow fibrosis and extramedullary hematopoiesis with splenomegaly.[1] The disease can occur *de novo* as primary MF (PMF) or result from the progression of polycythemia vera (PV) or essential thrombocytemia (ET) (hereafter called post-PV/ET MF) with no clinical and histological characteristics differences.[2] MF has a poor prognosis; the median overall survival (OS) is 5 years.[3] Evolution to acute myeloid leukemia (AML) occurs in around 20% of patients.[3] OS is predicted by the International Prognostic Scoring System (IPSS),[3] dynamic-IPSS (DIPSS)[4] and DIPSS-plus system.[5] These scores help therapeutic choices. DIPSS-plus takes into account unfavorable karyotypic abnormalities such as +8, -7/7q-, i(17q), inv(3), -5/5q-, 12p- or 11q23 rearrangement. The karyotype is abnormal in around 35% of PMF cases;[6] the most frequent lesions are del(20q), del(13q) and abnormalities of chromosome 1.[7]

The discovery of *JAK2*[8] and *MPL*[9] mutations in 50% to 60% and 5% to 10% of patients, respectively, allowed a better understanding of MF pathogenesis. Mutations in *CBL,*[10] *TET2,*[11] *ASXL1,*[12] *IDH,*[13] *IKZF1,*[14] *LNK,*[15] *EZH2,*[16] *DNMT3A,*[17] *NF1,*[18] *SUZ12,*[18] *SF3B1,*[19] and *SRSF2*[20] genes have been described in MPNs including MF. MF seems to have more genetic alterations than ET and PV, for example more *ASXL1* mutations.[12,21–23] The prevalence of an abnormal karyotype is not different among the three MF subtypes (PMF and post-PV/ET MF)[24] but the difference in mutation frequency is not well established and the genetic events that trigger PMF and post-ET/PV MF remain unknown. Early studies had shown that *ASXL1,*[23]

EZH2,[25] *IDH*[26] and *SRSF2*[20] mutations have prognosis impact. A recent study of a cohort of 879 PMF patients has shown that transformation to leukemia is indeed influenced by *ASXL1, EZH2, SRSF2 and IDH* mutations, and that *ASXL1* mutations have an impact on survival independent of the DIPPS-plus score.[27]

We have studied here 80 MF cases by using array-comparative genomic hybridization (aCGH) and Sanger sequencing of 23 genes on 104 MF samples. We have compared the molecular abnormalities in primary, secondary and blast phase MF.

Methods

Patients

A total of 104 samples corresponding to 80 patients with MF were studied, including 68 cases at chronic phases at diagnosis (n=24) (**Supplemental Table 1A**) or during disease course (n=44) (**Supplemental Table 1B**) and 12 blast phases (BP)-MF (**Supplemental Table 1C** – in this table 5 other BP cases are evolution of 5 of the 68 chronic phases). Based on the World Health Organisation criteria,[28] MF at chronic phase comprised 39 PMF, 15 post-PV MF, 14 post-ET MF. For 19 of the 80 patients, two or more samples were obtained (at different times during chronic phase, or at chronic phase and at blast phase) allowing a preliminary study of its progression (**Supplemental Table 1B**). Prognostic impact for PMF was evaluated with IPSS,[3] DIPSS[4] and DIPSS-plus.[5] We obtained 25 paired normal DNAs for the 80 patients (buccal swabs and CD3+ cells). For all patients, median time for follow-up was 47 months (range: 2-207). Median time from diagnosis to sampling was 47 months

(range: 3-289). Samples and associated data were obtained from the IPC/CRCM Tumor Bank, that operates under authorization #AC-2007-33 granted by the French Ministry of Research. Prior to scientific use of samples and data, patients were appropriately informed and asked to consent in writing, in compliance with French and European regulations. The project was approved by the IPC Institutional Review Board (Comité d'Orientation Stratégique).

DNA extraction

DNAs from PB leukocytes (n=97), BM (n=7) and matched normal (n=25) samples were extracted following a described protocol.[23]

Array comparative genomic hybridization (aCGH)

Genomic imbalances were analyzed by using genome-wide, high-density 244K CGH Microarrays (Hu-244A, Agilent Technologies, Massy, France) as described in supplemental methods.[29]

Sanger sequencing

Genes were selected because of their known involvement in leukemogenesis or their alteration in the aCGH-study and studied by Sanger-sequencing of their exon-coding regions: *ASXL1, BMI1, CBL, DNMT3A*, *EZH2*, *IDH1/2*, *JAK2, KRAS*, *LNK*, *MPL*, *NF1*, *NRAS*, *PPP1R16B*, *PTPN11*, *RCOR1*, *SF3B1*, *SRSF2*, *SOCS2*, *SUZ12*, *TET2*, *TP53* and *TRPS1*. Primer sequences are described in **Supplemental Table 2A**. PCR amplifications of DNA were done as previously described.[23] PCR

program for *TET2* is described in **Supplemental Table 2B.** All mutations were confirmed on an independent PCR product.

Statistical analysis

Analyses were done on patients at diagnosis and/or during follow-up. Correlations between sample groups and clinico-biological data were calculated with the Chi2 and Fisher's exact tests for qualitative variables with discrete categories, and the Mann-Whitney *U*-test for continuous variables. For post-PV/ET MF statistical analyses were restricted to cases at MF phase. OS and time to acute transformation (TTAT) were calculated from the time of MF diagnosis. Death from any cause was defined relevant event for OS while the occurrence of acute transformation was considered as relevant event for TTAT. Patients without event at last contact were censored. Causes of death for MF included leukemic transformation, marrow failure, and complications from infections. Survival curves were defined with the Kaplan-Meier method and compared with the log-rank test. Two-tailed *p*-values <5% were considered as significant for all statistical tests.

Results

Copy number aberrations in myelofibrosis

We searched for copy number aberrations (CNA) in 63 MF samples, comprising 38 PMF, 14 post-PV MF and 11 post-ET MF (**Supplemental Table 1A**). Among the 34 samples (54%) that showed CNAs, 17 (50%) carried a single CNA, 6

(18%) two CNAs, and 11 (32%) three or more CNAs (**Table 1**). We identified 72 CNAs including 9 gains and 63 deletions (del) (**Figure 1**). We classified aCGH profiles into three types. Type 1 (45/72) comprised large CNAs (>4Mb) affecting chromosome arms: +1q, -5q, -7, +9p, -8q, -11q, -12p, -13q, -17q, -18p, -20q. Type 2 (27/72) showed CNAs that affected few or single genes such as deletions involving *CSMD2* (1p35.1), *TET2* (4q24), *MYB* (6q23.2), *CUX1* (7q22.1), *TRPS1* (8q24.11), *ETV6*, *CDKN1B/p27* (12p13), *SOCS2 (*12q22), *RCOR1* (14q32.33), *NF1* (17q11), genes, and gains involving *TPO* (2p25) and *SALL3* (18q23) genes (**Table 1**, **Supplemental Figure 1**). In type 3 profiles, observed in 46% (n=29) of patients, no CNA were detected.

We detected no difference in CNAs according to sex (p=0.50), or to the character of primary or secondary MF (p=0.44). Nevertheless, we identified CNAs in 58% of PMF (22/38), 71% of post-PV MF (10/14), and 18% of post-ET MF cases (2/11). Cases that carried at least one CNA (n=34) were associated with lower hemoglobin (p=0.001), lower hematocrit (p=0.0004), and the need for red cell transfusion (p=0.03). The DIPSS score is established only for primary MF; the presence of CNA was associated with intermediate-2/high risk DIPSS and DIPSS-plus scores (p=0.01 and p=0.03) (**Table 2**).

Recurrent alterations in myelofibrosis

We detected 34 recurrent aberrations (>3 times), in 20q (n=14), 17q (n=7), 7p (n=5), 9p (n=3), 13q (n=3), and 1q (n=3). Del(20q), found in 22% (14/63) of the cases, was the most recurrent alteration (41%, 14/34); it was identified by aCGH and karyotype in 13 cases (11 PMF and 2 post-PV MF) and in one post-PV MF by

karyotype only (clone with del(20q) was minority) (**Figures 1-2**). Del(20q) was isolated in half of the cases and associated with at least another CNA in the other half. The average size of the deletion was 16.0 Mb (range 4.3-28.3Mb) (**Table 1**). We distinguished two separate commonly-deleted regions (CDR1 and CDR2). CDR1 spanned 0.3 Mb and included around 10 genes including *PPP1R16B* and two small nucleolar RNA (*SNOR*) host genes (*SNORA71, SNHG11*). CDR2 spanned 3.9 Mb and comprised several potential leukemogenic genes: *STK4, SDC4, CD40, NCOA3, SULF2, ZFAS1* and several *SNORs* (**Figure 2, Supplemental Table 3**). Cases with del(20q) were associated with lower leucocyte count, hemoglobin, and hematocrit (p=0.01, p=0.03 and p=0.02 respectively) and the need for red cell transfusion (p=0.03). PMF cases with del(20q) were associated with intermediate-2/high risk DIPSS and DIPSS-plus scores (p=0.03 and p=0.04) (**Table 2**).

The second most recurrent CNA was del(17q), detected in 7 cases (3 post-PV MF and 4 PMF cases). Four del(17q) were microdeletions that spanned around 1.2 Mb, with the loss of few genes including *NF1* and *SUZ12* (**Figure 1, Table 1**). *NF1* deletions were found in 3 post-PV MF and 2 PMF. In one post-PV case (HD-1427_1656), this deletion was associated with del(1p) and gain (6q) as the patient got worse; in one post-PV MF (HD-1813_1836), it was associated with monosomy 7 and del(12p) and the disease evolved in AML; in a PMF (HD-0689), it was associated with del(4q24) and del(14q); the patient developed fatal evolution.

The third most recurrent CNA was del(7p) detected in 5 cases (3 post-PV MF, 1 post-ET MF and 1 PMF). Three del(7p) were part of monosomies 7, which affect several leukemogenic genes, such as *EZH2, CUX1* and *IKZF1*. Monosomy 7 was accompanied by a microdeletion in 12p, which encompassed *ETV6* and *CDKN1B/p27* genes, in two post-PV MF cases that both evolved in AML, and

associated with del(20q) in a patient with post-PV MF who deceased (**Figure 1, Table 1**).

Gene mutations in myelofibrosis

We studied the mutational status of 23 genes in 68 MF cases comprising 39 PMF, 15 post-PV MF and 14 post-ET MF (**Supplemental Table 4**). Eighteen genes (*ASXL1, CBL, DNMT3A, EZH2, IDH1/2, JAK2, KRAS, LNK, MPL, NF1, NRAS, PTPN11, SF3B1, SRSF2, SUZ12, TET2,* and *TP53)* were selected for their involvement in leukemogenesis, and five because they were found altered by the aCGH analysis (*BMI1*, *PPP1R16B/TIMAP, RCOR1, SOCS2,* and *TRPS1*). Frequently mutated genes were *JAK2* (69%), *ASXL1* (26%), *TET2* (14%), *EZH2* (8%), *NF1* (6%), and *SRSF2* (6%). All other studied genes were mutated in less than 5% of the cases (*CBL*, *DNMT3A*, *LNK*, *MPL, NRAS*, *PTPN11*, *SF3B1*, and *TP53*) or not mutated (*BMI1, IDH1/2, KRAS, PPP1R16B, RCOR1, SOCS2, SUZ12* and *TRPS1)* (**Figure 3A, Supplemental Figure 2**). We identified one new gene mutated in MF, *PTPN11,* which was also affected by deletions. In two PMF cases (HD-0777 and HD-1289), we found *PTPN11* missense mutations (c.1471C>T;p.Pro491Ser and c.1508G>A;p.Gly503Glu). These mutations, which affect the Protein Tyrosine Phosphatase domain (exon 13) were probably both acquired; p.Pro491Ser has been described as somatic in childhood acute leukemia[30] and we did not find p.Gly503Glu in the patient's buccal swab DNA. No acquired mutation was found in *TRPS1.*

A total of 57 cases (84%) were mutated in at least one of the studied genes. *JAK2*, *ASXL1*, *TET2*, *EZH2*, *DNMT3A*, *LNK* and *SF3B1* mutations were evenly found in PMF and secondary MF. *SF3B1* mutations were detected in post-ET MF (1/12,

8%) and PMF (2/36, 6%) and *SRSF2* mutations (4/38, 8%) in PMF patients only. We did not find any mutation in 35% of post-ET MF and 15% of PMF (**Figure 3A**).

Within the three MF subtypes, the majority of concomitant mutations implicated *JAK2* mutation and another gene mutation (**Figure 3A-B**). In secondary MF, mutations of genes involved in epigenetic regulation or in splicing were systematically associated with the JAK2V617F mutation, whereas in PMF these mutations could be isolated (**Figure 3A-B**). Mutations in *CBL, LNK, MPL, NF1, PTPN11* and *NRAS* were mutually exclusive but could occur with a *JAK2* mutation (**Figure 3B**). Mutations in *TET2* and *DNMT3A* were mutually exclusive, whereas *ASXL1*, *EZH2*, and *TET2* mutations could be concomitant and could co-occur with a *JAK2* mutation. Mutations in *SF3B1* and *SRSF2* were mutually exclusive, and could occur with a *JAK2* mutation (**Figure 3B**).

ASXL1, TET2 and *SRSF2* mutations were associated with older age (p=0.02, p=0.03 and p=0.05, respectively). Cases mutated in *ASXL1, EZH2* or *SRSF2* genes had an increased WBC count (p=0.01, p=0.009 and p=0.006 respectively) (**Table 3**). Platelet count was high in *SRSF2*-mutated cases (p=0.04) (**Table 3**). Patients with a need for red cell transfusion were preferentially *ASXL1*-mutated (p=0.04) (data not shown).

Alterations during disease course and blast phase transformation

To identify molecular markers associated with disease course, we studied 19 MF matched pairs (corresponding to patients with two or more samples during disease course) and 17 BP-MF (**Supplemental Table 1B**).

Evolution to MF. A PV case (HD-0842) was *JAK2*-, *ASXL1*- and *EZH2 mutated* without CNA; 24 months later, at post-PV MF stage (HD-1401), a del(8q) including *CSMD3* and *RAD21* genes appeared. An ET case (HD-0551) was JAK2V617F (15-30%) and carried a del(11q) (-0.1); 61 months later at MF stage (HD-1616), the JAK2V617F mutation and del(11q) increased (50-70% and -0.8, respectively) and a trisomy 9 was present (**Table 4**).

MF disease course. Between cases studied at diagnosis (n=24) or after the initial diagnosis (n=44) there was no difference in the overall number of mutations (p=0.70) and no difference in mutational frequencies for *ASXL1, TET2* and *EZH2* genes (**Supplemental Figure 3**). Indeed, several patients carried already *ASXL1*, *EZH2*, *JAK2*, *SF3B1*, *SRSF2* and *TET2* gene mutations at MF diagnosis. Only one PMF case (HD-0540) mutated in *ASXL1* acquired an additional *MPL* mutation 36 months later. Similarly, CNAs were analyzed according to whether MF cases were studied at diagnosis for 23 patients or later for 40 patients. Overall, there was again no difference between the two groups (p=0.76) (**Supplemental Figure 3**). Among the 19 pairs, 8 (42%) did not acquire any additional alteration during disease course. Additional CNAs or more visible CNAs were observed in few patients during disease course. A post-ET MF case (HD-1360), JAK2V617F-mutated with no CNA, acquired 8 months later a del(12p). Another post-ET MF (HD-0614) was not mutated in the genes studied and did not show any CNA, but the patient got worse during disease course (HD-1352) and acquired a complex karyotype including losses of 4q24 (*TET2*), 7p (*IKZF1, ETV1*), 12q23.1 (*SOCS2*). A post-PV MF (HD-0789) carried del(20q), *ASXL1* and *JAK2* mutations, and 37 months later, the patient (HD-1691) acquired a monosomy 7 and died (**Table 4**).

Blast phase MF transformation. Among MF transformed to AML (n=5), two post-PV MF (HD-1559 and HD-1813) at chronic stage harbored a monosomy 7 and a small del(12p) encompassing *ETV6* (not detectable on karyotype for HD-1559) (HD-1813 had also a del(17q11) encompassing *NF1* not detectable on karyotype). These two MF evolved rapidly in AML, keeping their CNAs. One PMF case (HD-0927), which was *JAK2*-, *ASXL1*-, *SRSF2*-mutated with no CNA, acquired during disease course (HD-1265, HD-1461) a del(20q) and evolved in AML (HD-1853) with a trisomy 8. Another PMF case (HD-0528) *JAK2*-, *TET2*- and S*RSF2*- mutated without CNA at diagnosis, relapsed 47 months later (HD-1300) despite engraftment, and acquired a del(20q), an additional *TET2* mutation with *JAK2* and *SRSF2* mutations increase; one year later at BP transformation, a del(6q) was added to del(20q) and the patient (HD-1611) died. Another post-ET MF (HD-1309) without CNA was not mutated in the studied genes; at leukemic transformation 14 months later (HD-1741), we detected a complex karyotype with several CNAs including del(7p), del(12p) (**Supplemental Figure 4**); the patient died rapidly (**Table 4**).

Among 17 BP-MF, we detected CNAs in 82% of the cases (14/17) (**Supplemental Table 5**). We identified 82% of type 1 CNAs (55/67) including large gains: +3q, +9p; and large deletions: -7q, -8q, -11q, -16q. Type 2 CNAs represented 18% (12/67) of CNAs and included deletions in 12p (*ETV6, CDKNA1B/p27*), 13q14.2 (*RB1*), 15q21.3 (*TCF12*), 17q11 (*NF1*), 18p11 (*PTPN2*), and 21q22 (*RUNX1*) (**Supplemental Figure 4 and Table 5**). Recurrent CNAs were del(12p), including *ETV6* and *CDKN1B/p27*, detected in 5 AMLs (3 post-PV/ET MF, one post-PMF and one post-MPN MF), del(7q), including *EZH2,* detected in 5 AMLs (5 post-PV/ET MF), del(20q) detected in 3 AMLs (one post-PV MF and 2 post-PMF), del(17q11), including *NF1,* detected in 2 AMLs (2 post-PV/ET). The most often mutated genes

were *JAK2* (44%, 4/9), *ASXL1* (25%, 3/12), *TP53* (23%, 3/13) and *EZH2* (22%, 2/9) (**Supplemental Table 6**).

Prognostic impact of CNAs and gene mutations

We studied TTAT according to CNAs and genes frequently mutated (>5%) (**Supplemental Table 7**). In univariate analysis, TTAT was not different between patients with and without CNAs (p=0.58). However, TTAT was decreased in patients with at least one CNA if it were del(20q) or del(17q) or del(12p) (p=0.03) (**Figure 4A**). TTAT was decreased in patients with more than one mutation (p=0.04) and in *SRSF2*- and *EZH2*-mutated patients compared to non-mutated patients (p=0.0002 and p=0.01) (**Supplemental Table 7**).

The 5-year OS was severely decreased in patients with more than one mutation (81% versus 47%, p=0.001) (**Figure 4B**), with at least one mutation in epigenetic-associated genes (*ASXL1, EZH2, TET2, DNMT3A)* (63% versus 73%, p=0.03) and with at least one mutation in a splicing-associated gene (*SF3B1*, *SRSF2*) (42% versus 68%, p=0.004). Patients who carried *ASXL1* or *EZH2* mutation had a poorer OS than patients not mutated in the two genes (p=0.02, p=0.003, respectively). This was not the case for *TET2* (p=0.59). *SRSF2*-mutated patients had a poor OS (p=0.01) (**supplemental Table 5**). In multivariate analyses, we included *ASXL1*, *EZH2* and *SRSF2* mutations as co-variates; *EZH2* and *SRSF2* mutations predicted poor OS (p=0.04 and p=0.007, respectively) (**Supplemental Table 5**). We found a significant interaction between *JAK2* and *ASXL1* status; cases mutated in both genes had a poor outcome (p=0.02) (**Figure 4C**).

Discussion

We studied 80 patients with MF. We recognized several alterations involved in MPN pathogenesis and identified new alterations, which may have a role in MF initiation and/or progression. Overall for 68 MF patients at chronic phase, 54% of cases had CNAs and 88% were mutated.

Multiple pathways affected in MF

Components of signaling pathways such as *JAK2,*[8] *CBL*[10] and *LNK*[15] were frequently mutated. We also detected deletions of *SOCS2*, whose product down-regulates the JAK/STAT pathway, and of *NF1*[23] whose product regulates the RAS pathway. We showed that *PTPN11* mutations, found in juvenile myelomonocytic leukemia, myelodysplastic syndrome (MDS) and AML[30] are also present in MF. The TGFβ is thought to play a role in MF pathogenesis.[31,32] *TRPS1* was deleted in a PMF and a post-ET MF (**Figure 1**), suggesting a role in MF development; it was shown that the loss of *TRPS1* enhances TGFβ signaling leading to renal fibrosis.[33] Signaling mutations were frequently associated with mutations in genes involved in epigenetic regulation.[34]

We found a high frequency *of ASXL1*, *TET2* and *EZH2* mutations. We detected several gains and deletions involving other epigenetic regulators, such as gain of *SALL3*, whose encoded product interacts with DNMT3A.[35] We found one deletion of polycomb *BMI1*,[36] whose loss in the mouse model causes pathological hematopoiesis similar to PMF[37] and one deletion of *RCOR1.*

The third cell process affected in PMF and post-ET MF was RNA splicing. Few studies have previously described *SF3B1* and *SRSF2* mutations in PMF cases.[19,20]

Overall, MF seems to be characterized by alterations in known leukemogenic genes but also by rare alterations in other genes. Whole sequencing of an MF genome has indeed showed the presence of non-recurrent mutations in novel genes.[38]

Primary and secondary MF

There was no difference of CNAs between primary and secondary MF; and gene mutations of *ASXL1*, *TET2* and *EZH2* were evenly distributed in PMF (40%), post-PV MF (45%) and post-ET MF (44%). However, in secondary MF, mutations were always associated with the JAK2V617F mutation; in contrast, in PMF, mutations and del(20q) could be found independently of the JAK2V617F.[39] These observations, which would have to be complemented by data on colony assays, suggest different molecular ways to MF. First, patients with post-ET/PV MF carry both an epigenetic mutation and JAK2V617F mutation whereas PMF can develop with a non-JAK2 mutation (**Supplemental Figure 5**). Second, in both primary and secondary MF we found cases with a JAK2V617F mutation alone. Third, some patients with PMF or post-ET MF did not have mutation in any of the studied genes; the proportion of post-ET MF without mutation and CNAs was similar to that found in ET;[23] here, whole genome sequencing could help define the mechanisms (private gene mutations, mutation in miRNAs or other non-coding sequences).

We did not find any *SF3B1* and *SRSF2* mutations in post-PV MF whereas *SRSF2* mutations were found in four cases of PMF only and were associated with higher platelet count. *SF3B1* mutations were found in PMF and post-ET MF. Mutations in *SF3B1* were also found in refractory anemia with ring sideroblasts and

marked thrombocytosis.[40] These data suggested a link between spliceosome mutations and megakaryocyte lineage proliferation.

Disease progression and prognosis in myelofibrosis

We identified chromosomal abnormalities, i.e. del(20q), del(17q) and del(12p), associated with poor TTAT. These recurrent CNAs are observed in other myeloid malignancies such as MDS and AML.[41,42] We found an association between del(20q) and intermediate-2 DIPPS-plus score, low leucocyte count, low hemoglobin level, and the need for red cell transfusion. Genes included in the del(20q), such as *L3MBTL1,* have been studied for their possible involvement in leukemogenesis. However, sequencing analyses of 20q genes did not detect any mutation,[43] suggesting that haploinsufficiency of several genes of this chromosomal region could contribute to leukemogenesis. We compared the two minimal CDRs we identified with other studies (**Supplemental Figure 6**).[39] *SNORNA* host genes were present in these two CDRs. *SNORNAs* are 60–300 nucleotide-long non coding RNAs that are excised from intron regions of pre-mRNAs, downregulated in leukemic cells, suggesting that they may have a role in cancer development.[44]

The use of aCGH allowed the identification of abnormalities not detectable on karyotype, in particular del(17q), and del(12p) associated or not to monosomy 7. Del(17q11) was the second most recurrent CNA. Several studies have described del(17q) encompassing *NF1* tumor suppressor in myeloid malignancies.[45] We identified *NF1* deletion in 5 cases and mutations in 2 PMF cases with no CNAs. These cases evolved in AML or the patients deceased, suggesting that *NF1* alterations may contribute to MF progression and poor outcome. Monosomy 7 or del (7q) were associated with del(12p13) in 3 cases. In MDS and AML, a recent study

reported that in patients with monosomy 7, an additional *ETV6* deletion is common.[46] Del(12p) was not systematically detected by karyotype whereas monosomy 7 was always found when present.[46] Alterations of 12p have been described in various hematologic malignancies such as acute lymphoblastic leukemia, AML, MDS and MPNs.[47] The smallest deleted region encompasses the *ETV6* transcription factor and *CDKN1B/p27* tumor suppressor genes, in 12p13. In myeloid malignancies, *ETV6* rearrangements are frequently associated with other genetic events.[48] Our data strengthen the idea that when the karyotype reveals a monosomy 7, aCGH, FISH or sequencing could help identify an associated del(12p); this information could be important for therapeutic decision because of the high risk of acute transformation.

We found that poor TTAT was associated with *SRSF2* and *EZH2* mutations, suggesting that these mutations are associated with disease progression and may represent an important event leading to AML. Our univariate analyses showed that *ASXL1*, *EZH2* and *SRSF2* mutations, but not *TET2* mutations, are associated with poor prognosis, in agreement with previous reports in MPNs,[23,25,27] MDS,[49] chronic myelomonocytic leukemia,[29] and AML.[50] A study of a 879 PMF cases showed that *ASXL1* mutations had prognostic relevance independent of the DIPPS-plus model.[27] Due to a low number of patients we found only a tendency for *ASXL1* mutations to predict OS in multivariate analysis. Interestingly, we identified an impact on OS when both *JAK2* and *ASXL1* were mutated. This combination of mutations may lead to specific disease phenotype (MPN) and worse prognosis (clonal amplification). We did not find any additional mutation in the studied genes during MF disease course except for *MPL* and *TET2* mutations and increased *JAK2* allele burden. According to previous studies[21,22] patients with *ASXL1* mutations present during MF course had already *ASXL1* mutation at diagnosis (in PMF and secondary MF cases). *ASXL1*

mutations may constitute early alterations in MPN oncogenesis and precede *JAK2* and *MPL* mutations.[12]

In conclusion, we did not find any mutational and CNA difference between the three MF subtypes. The same scoring system might be used in PMF and secondary MF but this should be assessed in a specific study. We identified CNAs with impact on TTAT including some that could not be described by karyotyping, suggesting that additional molecular analysis could help therapeutic decision. In agreement with a recent important article[27] our study showed that mutations in *ASXL1*, *EZH2*, *SRSF2* associated with del(20q), del(17q) and monosomy 7/del(12p) identify MF patients at risk of premature death or leukemic transformation. This may help therapeutic decision and the design of new therapeutic association between JAK2 inhibitors and epigenetic drugs according to mutational status.

Autorship and Disclosures

AM was the principal investigator for the paper and takes primary responsibility for the paper. MB performed the laboratory work for this study, with assistance of RG. JR, RD, VGB, CA, MJM, NV and AM recruited the patients, and collected the samples and the annotations. MJM performed the cytogenetic analyses. MB, RD and AG did the statistical analyses. MB performed the data analysis. MB and AG performed the generation of figures. MB, DB and AM coordinated the research and did interpretation of data. MB, DB and AM wrote the paper, and JA and MC provided assistance with aCGH and editing. The authors report no potential conflicts of interest.

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Table 1. Summary of all detected CNAs in 34 primary and secondary myelofibrosis

Diagnosis	Samples	Karyotype	CNA nb	Deletion/ Gain	Chr band	Max Range (bp)	Max Size (bp)	Type of CNA	Nb of genes	Log2 ratio	Genes
post-PV MF	HD-0758	ND	4	D	1p36.11	chr1:25570060-25663551	93 491	2	7	-0.5	*SYF2, C1orf63, RHD, RHCE, TMEM57, LDLRAP1*
				G	1q44	chr1:247419685-249224147	1 804 462	2	>50	+0.5	*BCL9, ETV3, MNDA*
				G	2p25.3	chr2:435421-754937	319 516	2	5	+0.5	*TPO*
				G	9p	chr9:163131-47212417	47 049 287	1	>50	+0.5	*JAK2, MLLT3*
post-PV MF	HD-1401_0842	46,XY,del(8)(q21q23)[7] /46,XY[19]	1	D	8q22.3-8q24.13	chr8:104231983-127127107	22 895 124	1	>30	-0.4	*CSMD3, RAD21, MYC*
post-PV MF	HD-1427_1656	46,XX,t(7;14)(q11;q24)[2] /46,XX[18]	3	D	1p35.1-1p34.3	chr1:33696472-35645300	1 948 828	2	>10	-0.9	*CSMD2, PHC2*
				G	6q22.31	chr6:123527545-124319262	791 717	2	3	+0.5	*CLVS2, TRDN, NKAIN2*
				D	17q11.2	chr17:29071112-30451370	1 380 258	2	5	-0.2	*NF1, SUZ12*
post-PV MF	HD-1537	46,XX[20]	1	D	17q11.2	chr17:29187397-30244809	1 057 412	2	5	-0.2	*NF1*
post-PV MF	HD-1559_1649	45,XY,-7[9]/ 7,XY,+9[3]/46,XY[8]	3	D	7	chr7:45130-159118566	159 073 436	1	total	-0.2	*EZH2, HOXA9, ZNFN1A1, EGFR, MLL3, IKZF1*
				G	9p	chr9:163131-47212417	47049287	1	>50	+0.5	*JAK2, MLLT3*
				D	12p13.31-12p12.3	chr12:7695666-15370548	7 674 882	1	>50	-0.2	*ETV6, CDKN1B*
post-PV MF	HD-1602	46,XX,der(15)t(1;15)(p11;q12)[11]/46,XX[9]	1	G	1q	chr1:143646652-249224147	105 577 495	1	total	+0.2	*HIF1-β, PBX1, RGS2, CR1*
post-PV MF	HD-1664	46,XX,del(20)(q11q13)[3] /46,XX[17]	1	D	20q11-20q13	-	-	1	-	-	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
post-PV MF	HD-1691_0789	45,XY,-7,del(20)(q11q13)[20]/46,XY[2]	2	D	7	chr7:45130-159118566	159 073 436	1	>100	-0.2	*EZH2, HOXA9, ZNFN1A1, EGFR, MLL3, IKZF1*
				D	20q12-20q13.31	chr20:40216156-54565054	143 48 898	1	>100	-0.2	*CDR2, PTPRT, SRSF6, L3MBTL1*
post-PV MF	HD-1813_1836	45,XY,-7[6]/45,sl,del(12)(p12p13)[3]/ 45,sdl1,add(3)(q2?1)[13]	3	D	7	chr:45130-159118566	159 073 436	1	total	-0.8	*EZH2, HOXA9, ZNFN1A1, EGFR, MLL3, IKZF1*
				D	12p13.2-12p12.3	chr:11743077-16162282	4 419 205	2	>10	-0.8	*ETV6, CDKN1B*
				D	17q11	chr:28926042-30195057	1 269 015	2	>5	-0.8	*NF1,SUZ12*
post-PV MF	HD-1824_0589	46,XX,del(20)(q11q13)[8]/48,XX,+8,+9 [3]/48,idem,der(9)(1;9)(q21;q12q)[2]/ 46,XX[7]	1	D	20q11.22-20q13.2	chr20:31891717-53143776	21 252 059	1	>50	-0.1	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
post-ET MF	HD-1264	45,XY,der(7;18)(q10;q10)[20]	2	D	7p22.3-7p11.2	chr7:45130-57278548	57 233 418	1	>100	-0.4	*ETV1, HDAC9, CBX3, IKZF1, JAZF1, IL6, ZNFN1A1, EGFR*
				D	18p11.32-18p11.21	chr18:14316-14130869	14 116 553	1	>50	-0.4	*L3MBTL4, PTPN2*
post-ET MF	HD-1616_0551	47,XY,+9[5]/46,XY[15]	1	D	11q22.3-11q23.3	chr11:106783511-114591737	7 808 226	1	>30	-0.8	*ANKK1, ARGHEF12, ETS1*
PMF	HD-0497_1307	46,XX,del(20)(q11q13)[6] /46,XX[3]	1	D	20q11-20q13.32	chr20:31373532-57649807	26 276 275	1	>50	-0.8	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-0586	45,XY,-13[18]/46,XY[2]	2	D	13q12.3-13q34	chr13:30330695-115105806	84 775 111	1	>100	-0.5	*CDX2*
				D	18p11.32-18p11.21	chr18:14316-14796024	14 781 708	1	>50	-0.5	*L3MBTL4, PTPN2*
PMF	HD-0648	46,XX[20]	1	D	14q11-14q12	chr14:20445941-29023459	8 577 518	1	>50	-1	*BCL2L2*
PMF	HD-0689	ND	3	D	4q24	chr4:105992420-106249015	256 595	2	2	-0.3	*TET2*
				D	14q11.2	chr14:23489983-25294985	1 805 002	2	>10	-0.3	*BCL2L2*
				D	17q11.2	chr17:29124299-30227273	1 102 974	2	3	-0.3	*NF1*
PMF	HD-0719	46,XY[20]	1	D	9q34.13-9q34.2	chr9:134453373-135867067	1 413 694	2	10	-1	*SETX*
PMF	HD-0728	46,XX,t(6;17)(p21-22;q11)[10]/46,XX[13]	7	D	6p22.3-6p22.1	chr6:16186392-27130840	43 317 232	1	>30	-0.9	*CDKL1*
				D	6p25.3-6p25.2	chr6:1490872-2821073	1 330 201	2	10	-0.9	*GMDS*
				D	14q32.31-14q32.33	chr14:102027999-106559103	4 531 104	1	>15	-0.9	*RCOR1*
				D	17p13.3-17p13.1	chr17:29169-8883422	8 854 253	1	>30	-0.9	*YWHAE, PAFAH1B1*
				D	17q11.2-17q21.33	chr17:28805330-48948882	20 143 552	1	>50	-0.9	*NF1, SUZ12*
				D	17q25.1-17q25.3	chr17:71663856-81124227	9 460 371	1	>10	-0.9	*SOCS7*
				D	22q11.1-22q13.33	chr22:16124218-51219009	35 094 791	1	>100	-0.2 to -0.9	*NF2, SOX10, SH3BP1*
PMF	HD-0759	46,XX,del(5)(q13q33),del(20) (q11q13)[19]/46,XX[1]	2	D	5q14.3-5q35.1	chr5:91751929-168088896	76 336 967	1	>50	-0.8	*CDC23, MADH5, IRF1, NPM1*
				D	20q11.21-20q13.2	chr20:30784003-50789021	20 005 018	1	>50	-0.8	*ASXL1, DNMT3B, CDR1, CDR2, TOP1, SRSF6, L3MBTL1*
PMF	HD-0777	nd	1	G	18q23	chr18:76690903-77153667	462 764	2	3	+1	*SALL3, ATP9B, NFATC1*
PMF	HD-1047	46,XY,del(20)(q11q13)[3]/46,idemadd(9)(p2?3)[17]	2	G	9p24.3-9p13.3	chr9:163131-34316631	34 153 500	1	>50	+0.5	*JAK2, MLLT3*
				D	20q11.23-20q13.13	chr20:35029598-49772864	14 743 266	1	>50	-1	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
				D	6q23.2-6q23.3	chr6:135507017-137366363	1 859 346	2	10	-0.8	*TNFAIP3*
PMF	HD-1095	ND	4	D	7p22.1	chr7:5227285-5794960	567 675	2	>5	-0.8	*FBXL18*
				D	10p12.31-10p12.2	chr10:21234380-23388380	2 154 000	2	>10	-0.8	*BMI1,COMMD3,COMMD3-BMI1*
				D	12q22	chr12:93329167-94430427	1 101 260	2	5	-0.8	*SOCS2*
PMF	HD-1100	46,XY,del(13)(q13q22-31)[6]46,sl,?del(17)(p12-13)[5]/44,sl,der(1)inv(1)(p32~34q23)?dup(1)(q31q32),-7,-16,?der(17)t(16;17)(p11-12;p12-13)[6]/44,sdl1,?del(17)(p12-13)[5] +der(17)t(16;17)(p11-12;p12-13)[6]/44,sdl1,?del(17)(p12-13)[5]	3	D	1p13.3-1p11.2	chr1:110645371-120693961	10 048 590	1	>30	-0.8	*NOTCH2, CD53*
				D	13q12.3-13q21.33	chr13:31686948-71733123	40 046 175	1	>50	-0.9	*RB1, CDX2*
				D	17q22-17q23.1	chr17:56125121-57161042	1 035 921	2	>10	-0.9	*RAD51C*
PMF	HD-1207	46,XY,ider(20)(q10) del(20)(q11q13)[20]	2	D	20p13-20p11.1	chr20:60747-25937994	25 877 247	1	>100	-0.5	*MACROD2*
				D	20q11.21-20q13.33	chr20:31930935-60222113	28 291 178	1	>100	-0.5	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-1265_0927	ND	1	D	20q11.21-20q13.32	chr20:38348387-53396700	15 048 313	1	>100	-0.2	*CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-1283	ND	1	D	20q11.21-20q13.32	chr20:31706596-57020553	25 313 957	1	>100	-0.9	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-1289	ND	1	D	20q11.23-20q13.2	chr20:37244953-52543027	15 298 074	1	>100	-0.9	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-1300_0528_1611	46, XY, del20(q11q13)[4]/46,XY[16]	1	D	20q11.22-20q13.12	chr20:33873527-47688996	13 815 469	1	>100	-0.2	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-1362	46,XX,dup(1)(q44q12), del(13)(q13q14)[20]	6	G	1q21.2-1q44	chr1:149041013-248808428	99 767 415	1	>100	+0.5	*SPTA1*
				D	8q23.3	chr8:116508962-117333595	824 633	2	1	-0.8	*TRPS1*
				D	13q14.2-13q21.1	chr13:47729733-57537767	9 808 034	1	>30	-0.9	*RB1*
				D	13q31.1-13q31.2	chr13:87547986-88697857	1 149 871	2	1	-0.9	*SLITRK5*
				D	18q21.2-18q21.33	chr18:52787524-60012588	7 225 064	1	>20	-0.8	*TCF4*
				D	20q11.21-20q12	chr20:31984856-38055012	6 070 156	1	>50	-0.4	*CDR1*
PMF	HD-1422	ND	1	D	17q23.1-17q23.2	chr17:57404219-58144817	740 598	2	>10	-0.8	*miR-21, TMEM49, TUBD1, RPS6KB1*
PMF	HD-1524	ND	1	D	11q14.1-11q24.2	chr11:81041293-127474614	46 433 321	1	>100	-1	*EED, ARGHEF12, ETS1*
PMF	HD-1538	ND	4	D	1p32.2-1p32.3	chr1:54184496-55619136	1 434 640	2	>20	-0.9	*GLIS1, DHC24, USP24, TTC4, TTC22*
				D	20q11.21-20q12	chr20:31219192-37630533	6 411 341	1	>50	-0.8	*CDR1*
				D	20q13.12-20q13.13	chr20:43752761-48061828	4 309 067	1	>20	-0.8	*CDR2*
				D	22q12.1	chr22:28674993-29966075	1 291 082	2	>10	-0.8	*NF2*
PMF	HD-1587	ND	3	D	6q21	chr6:107735547-110341820	2 606 273	2	>10	-0.2	*FOXO3*
				D	6q22.31-6q23.3	chr6:125619860-138310979	12 691 119	1	>30	-0.2	*MYB*
				D	20q11.22-20q13.13	chr20:33909838-47688996	13 779 158	1	>50	-0.2	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
PMF	HD-1654	46,XY,inv(11)(q12q22)[20]	1	D	7q22.1	chr7:101066625-102101201	1034576	2	>5	-0.4	*CUX1, SH2B2*

bp, base pairs; CDR, commonly deleted region; Chr, chromosome; CNA, copy number aberrations; D, deletion; G, gain; MF, myelofibrosis; nb, number; nd, not determined; PMF, primary myelofibrosis; post-ET MF, post-essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF.

Table 2. Features of MF cases with CNAs and 20q deletion

		Total number	CNA	no CNA	p	20q deleted	no 20q deleted	p
Myelofibrosis	n	63	34 (54%)	29 (46%)		14 (22%)	49 (78%)	
Sex	M	34	17	17	0.50	7	27	0.74
	F	29	17	12		7	22	
MF character	primary	38	22 (65%)	13 (45%)	0.44	11 (79%)	27 (55%)	0.11
	secondary	25	12 (35%)	16 (55%)		3 (21%)	22 (45%)	
Diagnosis	PMF	38	22 (65%)	16 (55%)	**0.02**	11 (79%)	27 (55%)	0.12
	post-PV MF	14	10 (29%)	4 (14%)		3 (21%)	11 (22.5%)	
	post-ET MF	11	2 (6%)	9 (31%)		0	11 (22.5%)	
Age (y) median (range)		69 (30-86)	70 (53-86)	66 (30-83)	0.23	67 (59-82)	68 (30-86)	0.76
Leukocyte count (x 10^9/L); median (range)		8.9 (1.3-120)	7.1 (1.3-120)	9.3 (1.9-48.8)	0.28	3.8 (1.3-35)	10.7 (1.9-120)	**0.01**
Hemoglobin count (g/dL); median (range)		11.4 (5.8-17.8)	9.8 (5.8-15.7)	12.0 (8.5-17.8)	**0.001**	9.4 (7.2-13.7)	11.6 (5.8-17.8)	**0.03**
Hematocrit count (%); median (range)		34.7 (18-52)	28.8 (18-51.9)	37.2 (24.8-52)	**0.0004**	26.9 (20.2-40.8)	35.0 (18-52)	**0.02**
Platelet count (x 10^9/L); median (range)		256 (5-1188)	150 (5-890)	274 (36-1188)	0.12	106 (5-890)	273 (26-1188)	0.05
Need red cell transfusion	no	40	17	23	**0.03**	5	35	**0.03**
	yes	19	14	5		7	12	
Circulating blasts	no	23	12	11	0.97	6	17	0.48
	yes	38	20	18		7	31	
IPSS category (PMF)	low/inter-1	13	5	8	0.14	2	11	0.13
	inter-2/high	20	13	7		8	12	
DIPSS category (PMF)	low/inter-1	13	4	9	**0.01**	1	12	**0.03**
	inter-2/high	23	17	6		10	13	
DIPSSplus category (PMF)	low/inter-1	12	4	8	**0.03**	1	11	**0.04**
	inter2/high	24	17	7		10	14	

CNA, copy number aberrations; DIPSS, dynamic international prognostic scoring system ; F, female; Inter, intermediate; IPSS, international prognostic scoring system; M, male; MF, myelofibrosis; PMF, primary myelofibrosis; post-ET MF, post-essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF; y, years. For PMF, IPSS, DIPSS and DIPPS plus scores depend on age (>65 years), on hemoglobin (< 10 g/dL), on leukocyte count (> 25.10^9/L), on circulating blasts (> or = 1%), on platelet count (< 100.10^9/L), on presence of constitutional symproms (weight loss, night sweats, fever). For DIPPSplus score, karyotype and transfusion status must be added to DIPSS. IPSS score was calculated at MF diagnosis whereas DIPPS and DIPPSplus score were calculated at sampling.

Table 3. Features of MF cases with gene mutations

Genes		*JAK2*			*LNK*			*PTPN11*			Signaling (*CBL, JAK2, LNK, MPL, NF1, NRAS, PTPN11*)			*ASXL1*			*EZH2*			*TET2*			Epigenetic (*ASXL1, EZH2, DNMT3A, TET2*)			*SRSF2*			*SF3B1*			Splicing (*SRSF2, SF3B1*)		
Status		**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**	**mut**	**wt**	**p**
MF	n	47	21		3	62		2	61		49	8		17	49		5	58		9	55		27	36		4	62		3	58		7	53	
Sex	M	24	13	0.44	2	34	0.41	1	33	0.08	26	7	0.15	8	28	0.47	3	31	0.78	6	30	0.50	15	19	0.83	3	27	0.12	1	34	0.39	4	25	0.61
	F	23	8		1	28		1	28		24	1		9	21		2	27		3	25		12	17		0	23		2	24		2	20	
MF	primary	24	16	**0.06**	1	37	0.61	2	36	0.51	27	4	1	11	29	0.77	3	35	1	5	32	1	18	20	0.44	3	30	0.28	2	35	1	5	28	0.41
	secondary	23	5		3	35		0	25		23	4		6	21		2	23		4	23		9	16		0	20		1	23		1	17	
Diag	PMF	24	16		1	37		2	36		27	4		11	28		3	35		5	32		18	20		3	30		2	35		5	28	
	post-PV MF	14	0	**0.009**	1	12	0.66	0	13	0.51	14	0	0.07	4	9	0.53	1	12	1	2	11	0.98	6	7	0.38	0	10	0.38	0	12	0.62	0	9	0.46
	post-ET MF	9	5		1	13		0	12		9	4		2	12		1	11		2	12		3	9		0	10		1	11		1	8	
Age (y) median (range)		70 (30-89)	65 (32-86)	0.51	80 (73-82)	66 (30-89)	**0.05**	80.5 (75-86)	66 (30-89)	0.06	69.5 (30-89)	64.5 (32-75)	0.21	71 (57-83)	65 (30-89)	**0.02**	73 (66-86)	66 (30-89)	0.09	75 (55-86)	66 (30-89)	**0.03**	73 (39-86)	64 (30-89)	**0.002**	75 (55-85)	66 (30-89)	**0.27**	64 (59-79)	70 (30-89)	0.83	75 (59-85)	69 (30-86)	0.21
Leukocyte count (x 10^9/L); median (range)		11.5 (1.3-48.8)	7.4 (2.6-120)	0.22	8.6 (4.1-20.7)	11.4 (1.3-120)	0.74	32.8 (11.4 - 54.2)	11.2 (1.3-120)	0.26	13 (1.3-120)	7 (2.6-15.2)	0.11	12.8 (4.6-76)	8.45 (1.3-120)	**0.01**	35.4 (8.6-76)	11.0 (1.3-120)	**0.009**	20.7 (2.4-54.2)	9.85 (1.3-120)	0.12	12.8 (2-120)	6.4 (1.3-35.1)	**0.004**	35.7 (27.3-120)	9.3 (1.3-76)	**0.006**	8.3 (2.6-12)	11.4 (1.3-120)	0.37	19.65 (2.6-120)	9.85 (1.3-76)	0.20
Hemoglobin count (g/dL); median (range)		11.5 (7.6-17.8)	11.5 (5.8-14.5)	0.28	9.2 (8.7-15)	11.8 (5.8-17.8)	0.61	10.1 (7.7-12.5)	11.7 (5.8-17.8)	0.47	11.5 (5.8-17.8)	12.1 (7.2-14.5)	0.92	12 (5.8-14.2)	11.5 (7.2-17.8)	0.98	8.7 (5.8-13.4)	11.7 (7.2-17.8)	0.18	12.5 (7.6-15)	11.55 (5.8-17.8)	0.56	12 (5.8-15)	11.5 (7.2-17.8)	0.83	10.9 (8.6-13.2)	11.5 (5.8-17.8)	0.71	10.2 (7.2-13.2)	11.8 (5.8-17.8)	0.34	10.2 (7.2-13.2)	11.65 (5.8-15.7)	0.22
Hematocrit count (%); median (range)		33.3 (14.3-52)	34.8 (18-44.3)	0.60	25.6 (14.3 - 27.1)	35.8 (14.3-52)	**0.04**	31.2 (25.2 - 37.1)	35 (14.3-52)	0.69	33.5 (14.3-52)	35 (20.2-44.3)	0.87	34.65 (18-47)	35 (14.3-52)	0.79	25.4 (18-41.2)	35 (14.3-52)	0.19	35.3 (14.3-44.3)	35 (14.3-52)	0.59	33.5 (14.3-47)	35 (14.3-52)	0.62	34 (26.0-38.6)	35 (14.3-51.9)	0.89	30.95 (20.2-41.7)	35 (14.3-52)	0.77	32 (20.2-41.7)	35 (18-51.9)	0.52
Platelet count (x 10^9/L); median (range)		300 (39-1188)	266 (5-890)	0.77	361 (101-386)	302 (5-1188)	1	425 (88-761)	300 (5-1188)	0.78	315 (26-1188)	301.5 (5-758)	1	334 (26-1188)	284.5 (5-890)	0.47	315 (26-761)	297 (5-1188)	0.87	315 (88-761)	308.5 (5-1188)	0.61	315 (26-1188)	269 (5-890)	0.68	568.5 (295-1188)	272 (5-890)	**0.04**	77 (5-540)	302 (26-1188)	0.25	509.5 (5-1188)	296 (26-890)	0.41

Diag, diagnosis; DIPSS, dynamic international prognostic scoring system ; F, female ; Inter, intermediate ; IPSS, international prognostic scoring system ; M, male ; MF, myelofibrosis ; mut, mutated ; PMF, primary myelofibrosis ; post-ET MF, post-essential thrombocythemia MF ; post-PV MF, post-polycythemia vera MF ; wt, wild-type ; y, years. For PMF, IPSS, DIPSS and DIPPSplus scores depend on age (>65 years), on hemoglobin (< 10 g/dL), on leukocyte count (> 25.10^9/L), on circulating blasts (> or = 1%), on platelet count (< 100.10^9/L), on presence of constitutional symptoms (weight loss, night sweats, fever). For DIPPSplus score, karyotype and transfusion status must be added to DIPSS. IPSS score was calculated at MF diagnosis whereas DIPPS and DIPPSplus score were calculated at sampling.

Table 4. Molecular data of 19 matched pairs of MF

A.Evolution to MF

Diagnosis	Samples	M	Add	Karyotype	aCGH	12p	17q	20q	*TET2*	*ASXL1*	*EZH2*	*JAK2*	*MPL*	*SF3B1*	*SRSF2*
PV	HD-0842_1401	24	yes	ND	No CNA					x	x	50-60			
post-PV MF	HD-1401_0842			46,XY,del(8)(q21q23)[7]/46,XY[19]	del 8q22.3-8q24.13(**-0.4**)					x	x	50-60			
ET	HD-0551_1616	61	yes	ND	del 11q22.3-11q23.3(**-0.1**)							15-30			
post-ET MF	HD-1616_0551			47,XY,+9[5]/46,XY[15]	gain 9 (+0.1), del 11q22.3-11q23.3(**-0.8**)							50-70			

B.MF disease course

Diagnosis	Samples	M	Add	Karyotype	aCGH	12p	17q	20q	*TET2*	*ASXL1*	*EZH2*	*JAK2*	*MPL*	*SF3B1*	*SRSF2*
PMF	HD-1412_1495	2	no	ND	No CNA									x	
PMF	HD-1495_1412			46,XY[2]	No CNA									x	
post-ET MF	HD-0725_1380	28	no	46,XY[20]	No CNA				xx			40			
post-ET MF	HD-1380_0725			46,XY[20]	No CNA				xx			50-60			
PMF	HD-0497_1307	44	no	46,XX,del(20)(q11q13)[6]/46,XX[3]	del 20q11-q13.32(**-0.8**)			del	x			20-30			
PMF	HD-1307_0497			ND	del 20q11-q13.32(**-1**)			del	x			ND			
PMF	HD-0607_1130	32	no	46,XY[20]	No CNA										
PMF	HD-1130_0607			46,XY[20]	No CNA							ND			
PMF	HD-0679_1291	26	no	46,XY[20]	No CNA							5-10			
PMF	HD-1291_0679			ND	No CNA						ND	5-10			
PMF	HD-0717_1244	23	no	46,XY[20]	No CNA				x						
PMF	HD-1244_0717			ND	No CNA				x		ND				
post-PV MF	HD-0589_1824	62	no	48,XX,+8,+9[3]/46,XX,del(20)(q11q13)[2]/46,XX[15]	No CNA			del				50-70			
post-PV MF	HD-1824_0589			46,XX,del(20)(q11q13)[8]/48,XX,+8,+9[3]/48,idem, der(9)t(1;9)(q21;q12)[2]/46,XX[7]	del 20q11.22-q13.2(**-0.1**)			del				50-70			
post-PV MF	HD-1427_1656	9	no	46,XX,t(7;14)(q11;q24)[2]/46,XX[18]	del 1p35.1-1p34.3(-0.8), gain 6q22.31(+0.5), del 17q11.2(-0.2)		del								
post-PV MF	HD-1656_1427			46,XX[20]	del 1p35.1-1p34.3(**-1**), gain 6q22.31(+0.5), del 17q11.2(**-0.5**)		del		ND		ND		ND	ND	
post-PV MF	HD-0789_1691	37	yes	46, XY, del(20)(q11q13)[4]/46,XY[16]	No CNA			del		x	ND	x			
post-PV MF	HD-1691_0789			45,XY,-7,del(20)(q11q13)[20]/46,XY[2]	del 7(**-0.2**), del 20q12-q13.31(**-0.2**)			del	ND	x	ND	ND		ND	
post-ET MF	HD-1360_1605	8	yes	ND	No CNA							30-50			
post-ET MF	HD-1605_1360			ND	del 12p13.2 (**-0.9**)	del						30-50			
post-ET MF	HD-0614_1112_1352	29	yes	46,XY[22]	No CNA										
post-ET MF	HD-1112_0614_1352			ND	No CNA										
post-ET MF	HD-1352_0614_1112	9		46,XY,del(7)(q22q36)[2] /46,XY,add(4)(q21-22),-12,-13,+der(?)t(?;4)(?;q21-22),+mar[3]/44,XY,add(4),der(6)t(4;6)(q21-22;p2?3),-7,-12,-15,-16,+2mar[3]	del 4p15.31(**-0.4**), del 4q24 (**-0.4**), de l6p22.1-p25.3(**-0.2**), del 7p12.1-7p22.3(**-0.2**), del 12q23.1(**-0.2**), del 13q14.3(**-0.2**)				del			ND		ND	
PMF	HD-0540_1138_1398	36	yes	46,XY[20]	No CNA					x					
PMF	HD-1138_0540_1398	9		ND	No CNA					x			x		
PMF	HD-1398_0540_1138			ND	No CNA					x			x		

C. Blast phase MF transformation

Diagnosis	Samples	M	Add	Karyotype	aCGH	12p	17q	20q	*TET2*	*ASXL1*	*EZH2*	*JAK2*	*MPL*	*SF3B1*	*SRSF2*
post-PV MF	HD-1559_1649	4	no	45,XY,-7[9]/47,XY,+9[3]/46,XY[8]	dell 7(**-0.2**), gain 9p(**+0.5**), del 12p12.3-p13.31(**-0.9**)	del						x			
BP post-PV MF	HD-1649_1559			45,XY,-7[20]	del 7(**-0.2**), del 12p12.3-p13.31(**-0.2**)	del			ND		ND	ND		ND	
post-PV MF	HD-1813_1836	1	no	45,XY,-7[6]/45,sl,del(12)(p12p13)[3] /45,sdl1,add(3)(q2?1)[13]	del 7 (-0.8), del 12p (-0.8), del 17q11 (-0.8)	del			ND		ND	x		ND	
BP post-PV MF	HD-1836_1813			45,XY,-7[6]/45,sl,del(12)(p12p13)[3] /45,sdl1,add(3)(q2?1)[13]	ND	del	ND	ND	ND		ND	x	ND	ND	
PMF	HD-0927_1265_1461_1853	13	yes	ND	No CNA					x		40-50			x
PMF	HD-1265_0927_1461_1853			ND	del 20q11-q13.32(-0.2)			del		x		30-50			x
PMF	HD-1461_0927_1265_1853	8		ND	del 20q11-q13.32(-0.1)			del		x	ND	30-50		ND	x
BP PMF	HD-1853_0927_1265_1461	15		46,XY,+8[16]/46,XY[6]	trisomy 8 (+0.2)	ND	ND	ND			ND	x	ND	ND	x
PMF	HD-0528_1300_1611	47	yes	46, XY[20]	No CNA				x			75			x
PMF	HD-1300_0528_1611			46, XY, del20(q11q13)[4]/46,XY[16]	No CNA			del	xx		ND	80-90			x
BP PMF	HD-1611_1300_0528	12		46,XY,del(20)(q11q13)[12]/46,XY,inv(3)(q21q26)[4]/ 46,idem,del(6)(q2?1q2?6)[3]/46,XY[1]	del 6q12-q24.1(**-0,2**), del 20q11.22-q13.12(**-0,2**)			del	xx			ND			x
post-ET MF	HD-1309_1741	14	yes	46,XX[20]	No CNA									ND	
BP post-ET MF	HD-1741_1309			46,XX,-3,der(5)t(3;5)(q13;q21), del(7)(q21q36),t(9;17)(q11;q11),+21[11]	del 3p25.2-p26.3 (**-0.8**), del3 p22.1-p25.1(**-0.8**), del 3p12.1-p21.2(**-0.8**), del 5q14.1-q35.2(**-0.8**), del 7q21.11-q35(**-0.8**), del 9p12-p13.3(**-0.8**), del 12p12.3-p13.2(**-0.8**), del 17p12-p13.1(**-0.8**), gain 21(**+0.5**)	del			ND		ND	ND		ND	

For each CGH data, log2 ratio of the gain or loss is in parenthesis. The number of crosses corresponds to the number of mutations identified. Add , additional alterations (CNAs and mutations); BP, blast phase; CNA, copy number aberrations; del, deletion; M, months; MF, myelofibrosis; ND, not determined; PMF, primary MF; post-ET MF, post- essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF

Figures legends

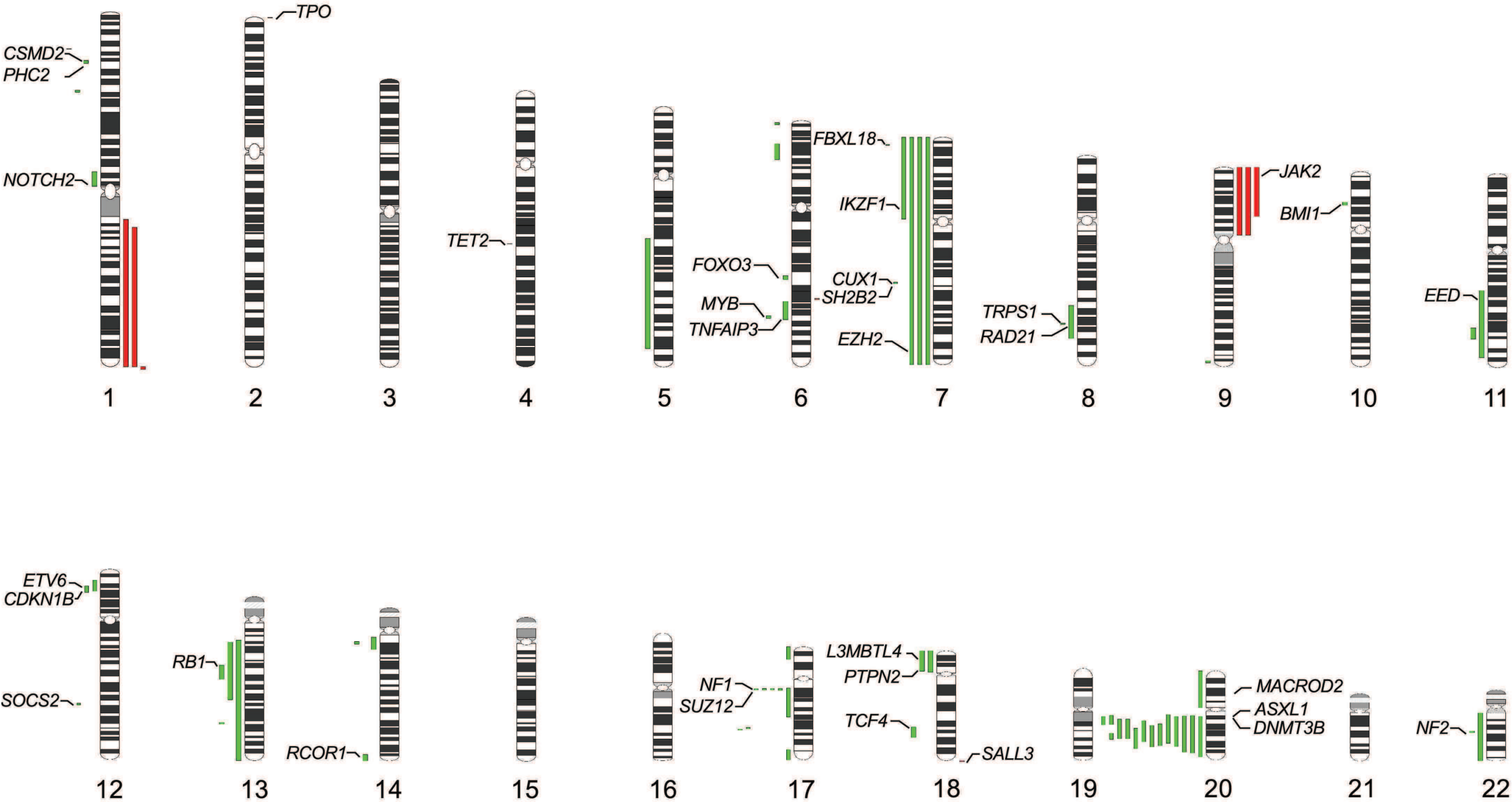
Figure 1. Karyoview of copy number aberrations (CNAs) detected in 63 patients with myelofibrosis by using array-comparative genomic hybridization. The bars depict the physical positions and the size of aberration: green bars indicate deletions and red bars indicate gains. Some genes known or suspected to play a role in leukemogenesis are indicated when included in the shown CNAs.

Figure 2. aCGH profiles of recurrent del(20q). Del(20q) is a recurrent CNA detected in 13 cases by a aCGH. Two separate commonly-deleted regions (CDR), CDR1 and CDR2, were identified, with a respective size of 0.3 Mb (HD-1289;HD-1538) and 3.9 Mb (HD-1538;HD-1587). Genes lost in CDR1 and CRD2 are shown in **supplemental Table 3**.

Figure 3. Frequency and distribution of gene mutations. A: Circos plot showing frequencies of gene mutations in MF, in PV and ET according to a previous study,[23] in PMF, in post-PV MF and post-ET MF. The ribbon representing the splicing pathway is composed of *SF3B1* and *SRSF2* mutations; that representing the signaling pathway is composed of *CBL*, *LNK*, *MPL*, *NF1*, *NRAS*, and *PTPN11* mutations. **B**: Patterns of concomitantly mutated genes and CNAs in myelofibrosis**.** Identified mutations are shown by colored squares and CNAs seen by aCGH are shown by colored squares with *. For DIPSS scores, in PMF, green and red squares represent low/intermediate-1 and intermediate-2/high risk.
+: gain, -: deletion, C: *CBL*, DNMT: *DNMT3A*, ET: essential thrombocythemia, M: *MPL*, MF: myelofibrosis, N: *NF1*, PTP: *PTPN11*, PMF: primary myelofibrosis, PV: polycythemia vera, R: *NRAS*, SUZ: *SUZ12*.

Figure 4. Kaplan-Meier estimates. A: Time to Acute Transformation (TTAT) according to deletions: del(12p), del(17q), del(20q). **B**: Overall Survival (OS) according to more than one mutation. **C**: OS according to *JAK2*/*ASXL1* combined mutations. del: deletion, mt: mutated, wt: wild-type.

Figure 1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TPO
TET2
FBXL18
IKZF1
FOXO3
CUX1
SH2B2
MYB
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TRPS1
RAD21
JAK2
BMI1
EED
1
2
3
4
5
6
7
8
9
10
11
ETV6
CDKN1B
SOCS2
RB1
RCOR1
NF1
SUZ12
L3MBTL4
PTPN2
TCF4
SALL3
MACROD2
ASXL1
DNMT3B
NF2
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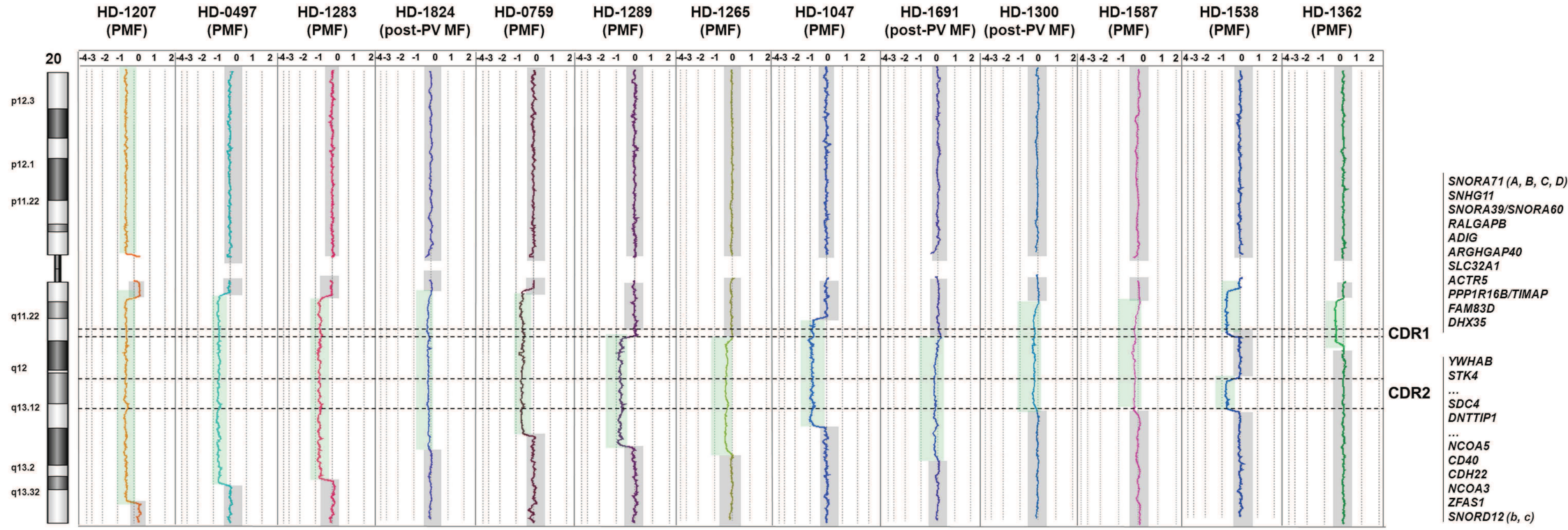
22

Figure 2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HD-0497 (PMF)
HD-1283 (PMF)
HD-1824 (post-PV MF)
HD-0759 (PMF)
HD-1289 (PMF)
HD-1265 (PMF)
HD-1047 (PMF)
HD-1691 (post-PV MF)
HD-1300 (post-PV MF)
HD-1587 (PMF)
HD-1538 (PMF)
HD-1362 (PMF)
20
p12.3
p12.1
p11.22
q11.22
q12
q13.12
q13.2
q13.32
CDR1
CDR2
SNORA71 (A, B, C, D)
SNHG11
SNORA39/SNORA60
RALGAPB
ADIG
ARGHGAP40
SLC32A1
ACTR5
PPP1R16B/TIMAP
FAM83D
DHX35
YWHAB
STK4
...
SDC4
DNTTIP1
...
NCOA5
CD40
CDH22
NCOA3
ZFAS1
SNORD12 (b, c)

Figure 3

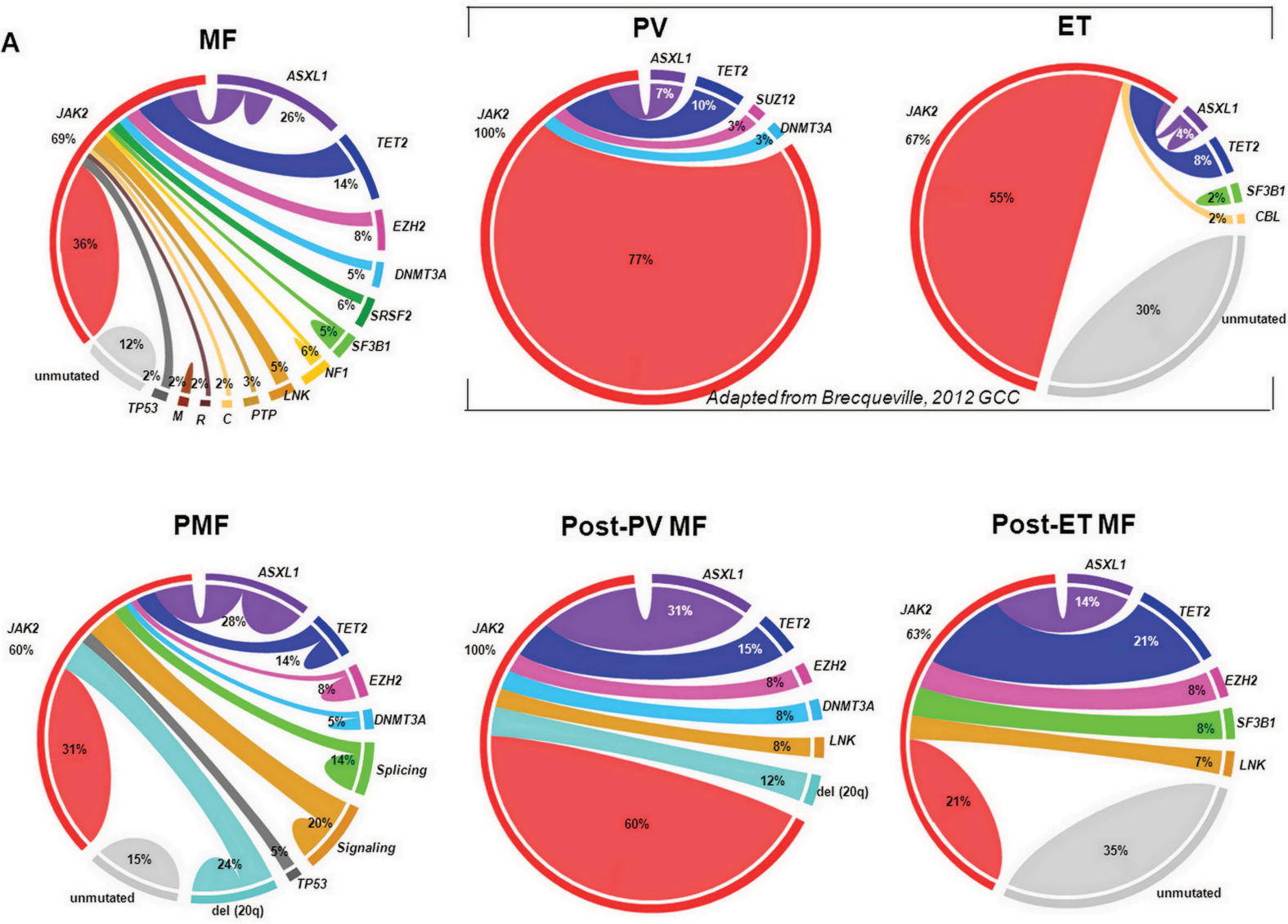
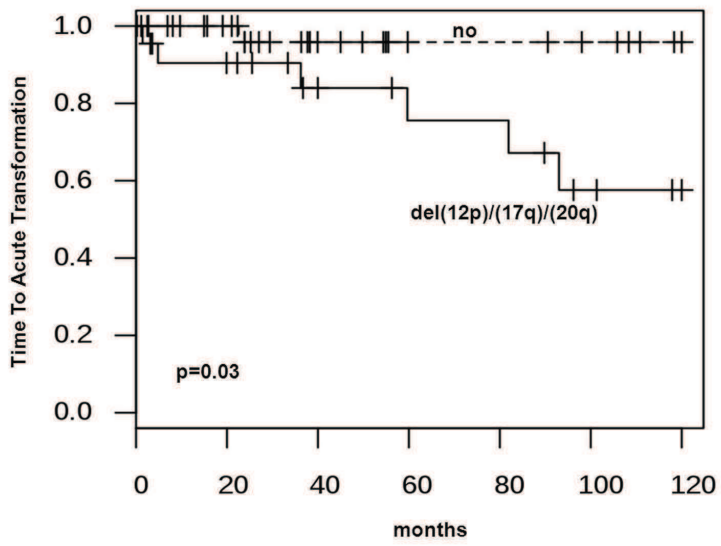
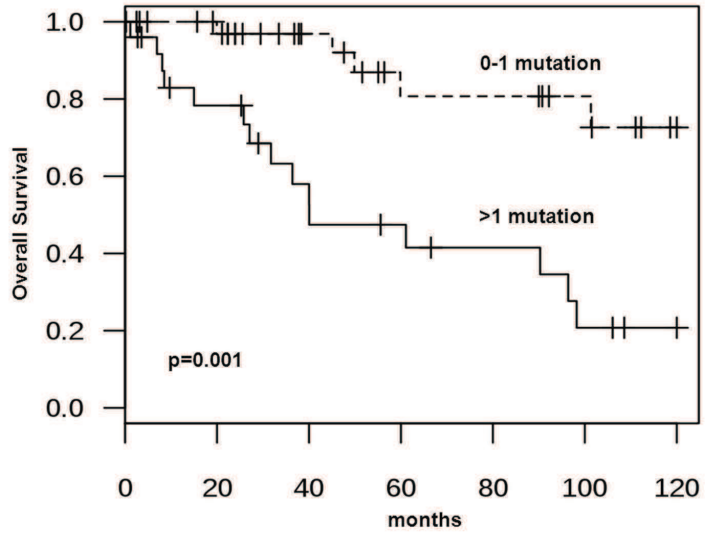

A
MF
ASXL1
26%
TET2
14%
EZH2
8%
DNMT3A
5%
SRSF2
6%
SF3B1
5%
NF1
6%
LNK
5%
PTP
3%
C
2%
R
2%
M
2%
TP53
2%
unmutated
12%
JAK2
69%
36%
PV
ASXL1
7%
TET2
10%
SUZ12
3%
DNMT3A
3%
JAK2
100%
77%
ET
ASXL1
4%
TET2
8%
SF3B1
2%
CBL
2%
JAK2
67%
55%
30%
unmutated
Adapted from Brecqueville, 2012 GCC
PMF
ASXL1
28%
TET2
14%
EZH2
8%
DNMT3A
5%
Splicing
14%
Signaling
20%
TP53
5%
del (20q)
24%
unmutated
15%
JAK2
60%
31%
Post-PV MF
ASXL1
31%
TET2
15%
EZH2
8%
DNMT3A
8%
LNK
8%
del (20q)
12%
JAK2
100%
60%
Post-ET MF
ASXL1
14%
TET2
21%
EZH2
8%
SF3B1
8%
LNK
7%
JAK2
63%
21%
35%
unmutated
B
PMF
post-ET MF
post-PV MF
ASXL1
Polycomb
DNA methylation
JAK2
Signaling
Splicing
TP53
20q deletion
CNA
DIPSS score
Deaths

Figure 4

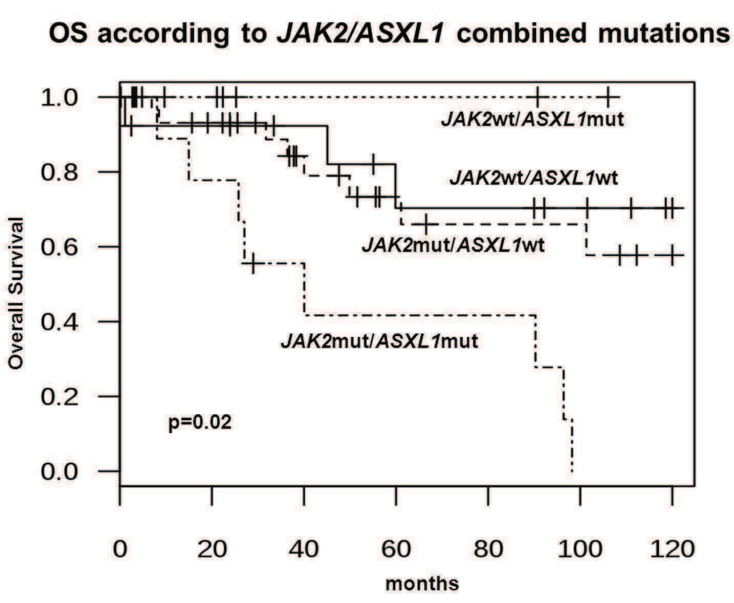
A


TTAT according to deletions:
del(12p), del(17q), del(20q)
Time To Acute Transformation
1.0
0.8
0.6
0.4
0.2
0.0
no
del(12p)/(17q)/(20q)
p=0.03
0
20
40
60
80
100
120
months

B


OS according to more than one mutation
Overall Survival
1.0
0.8
0.6
0.4
0.2
0.0
0-1 mutation
>1 mutation
p=0.001
0
20
40
60
80
100
120
months

C


OS according to JAK2/ASXL1 combined mutations
Overall Survival
1.0
0.8
0.6
0.4
0.2
0.0
JAK2wt/ASXL1mut
JAK2wt/ASXL1wt
JAK2mut/ASXL1wt
JAK2mut/ASXL1mut
p=0.02
0
20
40
60
80
100
120
months

Supplemental information

Supplemental Tables

Supplemental Table 1. Clinical and biological characteristics of 104 MF samples studied by aCGH and DNA sequencing.

Supplemental Table 2. Sequencing primers.

Supplemental Table 3. List of genes located in the two 20q commonly deleted regions (CDR1 and CDR2).

Supplemental Table 4. Gene mutations of 68 MF cases with *TET2, IDH1/2, DNMT3A, ASXL1, EZH2, SUZ12, BMI1, RCOR1, JAK2, MPL, CBL, SOCS2, LNK, NF1, KRAS, NRAS, PTPN11, PPPP1R16B, TRPS1, SF3B1, SRSF2* and *TP53* mutations

Supplemental Table 5. Summary of all detected CNAs in 14 blast phase MF.

Supplemental Table 6. Gene mutations of 17 blast phase MF cases with *TET2, IDH1/2, DNMT3A, ASXL1, EZH2, SUZ12, BMI1, RCOR1, JAK2, MPL, CBL, SOCS2, LNK, NF1, KRAS, NRAS, PTPN11, PPPP1R16B, TRPS1, SF3B1, SRSF2* and *TP53* mutations.

Supplemental Table 7. Prognostic impact of CNAs and gene (>5%) on Time to Acute Transformation (TTAT) and Overall Survival (OS). Univariate (log-rank tests) and multivariate (Cox-models) analyses.

Supplemental Table 1. Clinical and biological characteristics of 104 MF samples studied by aCGH and DNA sequencing

A. Clinical and biological characteristics of 68 chronic MPNs

Samples	Sex/Age	Diagnosis	PB/ BM	IPSS	DIPSS	DIPSS plus	Time from diagnosis to sampling (m)	Karyotype	Leukocyte count (x 10^9/L)	Hemoglobin level (g/dL)	Hematocrit (%)	Platelet count (x 10^9/L)	RCT need	Ci blast cells	Symptoms	JAK2 V617F %	Previous Therapies	aCGH
HD-0577	F/57	post-PV MF	PB	NA	NA	NA	<1	46,XX[20]	27.5	14.7	47	255	no	no	satisfactory	80-90	HU	X
HD-0758	F/80	post-PV MF	PB	NA	NA	NA	<1	ND	10.4	9.7	29.9	132	no	yes	infectious health	50	ND	X
HD-1302	M/69	post-PV MF	PB	NA	NA	NA	<1	46,XY[20]	14.3	12.8	39.5	297	no	yes	no functional symptoms	80-90	HU	X
HD-1401_0842	M/67	post-PV MF	PB	NA	NA	NA	<1	46,XY,del(8)(q21q23)[7]/46,XY[19]	31.1	13.4	41.2	714	no	yes	weight loss	50-60	HU, antiplatelet therapy	X
HD-1426	M/82	post-PV MF	PB	NA	NA	NA	60	46,XY[20]	20.7	15	14.3	361	no	yes	asthenia and fatigue	80-90	X, PA, antiplatelet therapy	no
HD-1427_1656	F/71	post-PV MF	PB	NA	NA	NA	45	46,XX,t(7;14)(q11;q24)[2]/46,XX[18]	16.6	15.7	51.9	363	no	no	night sweats	80-90	M	X
HD-1480	F/64	post-PV MF	PB	NA	NA	NA	31	46,XX[20]	15.2	11.8	36.2	631	no	no	no functional symptoms	75	HU, X, antiplatelet therapy	X
HD-1537	F/70	post-PV MF	PB	NA	NA	NA	21	46,XX[20]	3.1	10.7	33.1	165	no	yes	no functioNAl symptoms	80	HU, EPO	X
HD-1559_1649	M/63	post-PV MF	BM	NA	NA	NA	<1	45,XY,-7[9]/47,XY,+9[3]/46,XY[8]	2	9.8	ND	39	no	no	asthenia and fatigue	30-50	antiplatelet therapy	X
HD-1570bis	F/55	post-PV MF	PB	NA	NA	NA	11	46,XX[20]	3.7	11	ND	85	no	no	satisfactory	80-90	none	X
HD-1602	F/72	post-PV MF	PB	NA	NA	NA	<1	46,XX,der(15)t(1;15)(p11;q12)[11]/46,XX[9]	24.6	12.4	ND	600	no	no	night sweats	80-90	HU, P, X	X
HD-1664	F/60	post-PV MF	PB	NA	NA	NA	14	46,XX,del(20)(q11q13)[3]/46,XX[17]	2.5	10.1	ND	81	no	yes	satisfactory	2	none	X
HD-1691_0789	M/69	post-PV MF	BM	NA	NA	NA	36	45,XY,-7,del(20)(q11q13)[20]/46,XY[2]	13.3	13.7	ND	60	no	no	night sweats	mutated	anti-JAK1/2	X
HD-1813_1836	M/77	post-PV MF	PB	NA	NA	NA	<1	45,XY,-7[6]/45,sl,del(12)(p12p13)[3]/ 45,sdl1,add(3)(q2?1)[13]	4.6	11.5	34.7	58	yes	yes	ND	2-5	none	X
HD-1824_0589	F/70	post-PV MF	PB	NA	NA	NA	117	46,XX,del(20)(q11q13)[8]/48,XX,+8,+9[3]/48,idem, der(9)t(1;9)(q21;q12q)[2]/46,XX[7]	2.5	8.8	26.9	71	no	yes	satisfactory	50-70	none	X
HD-0554	F/79	post-ET MF	PB	NA	NA	NA	<1	46,XX[20]	8.3	10.2	ND	540	no	no	asthenia and fatigue	30-50	P, HU, EPO, A	X
HD-0598	F/71	post-ET MF	PB	NA	NA	NA	<1	46,XX[20]	6.4	10.7	31.4	758	no	no	asthenia and fatigue	wt	HU, P, X	X
HD-0599	M/80	post-ET MF	PB	NA	NA	NA	3	ND	4.1	9.2	27.1	386	yes	no	no functional symptoms	30-40	HU, EPO	no
HD-0601	M/76	post-ET MF	PB	NA	NA	NA	<1	46,XY[20]	11	11	33.5	407	no	yes	asthenia and fatigue	60-70	HU, EPO, antiplatelet therapy, X	X
HD-0614_1112_1352	M/76	post-ET MF	PB	NA	NA	NA	139	46,XY[22]	4.5	11.2	35	269	no	yes	satisfactory	wt	EPO, antiplatelet	X

																		therapy	
HD-0725_1380	M/74	post-ET MF	PB	NA	NA	NA	11	46,XY[20]	35.4	12.6	37.6	315	no	yes	satisfactory	40	HU	X
HD-0983	M/32	post-ET MF	PB	NA	NA	NA	<1	ND	15.2	12.2	36.8	419	no	yes	satisfactory	wt	X	X
HD-1264	M/72	post-ET MF	BM	NA	NA	NA	<1	45,XY,der(7;18)(q10;q10)[20]	4	12	35	150	no	yes	satisfactory	wt	B, antiplatelet therapy	X
HD-1309_1741	F/36	post-ET MF	PB	NA	NA	NA	73	46,XX[20]	12.2	9.9	ND	750	no	yes	weight loss	wt	HU, A, P, T	X
HD-1360_1605	M/67	post-ET MF	PB	NA	NA	NA	12	ND	5.8	11	32.8	392	no	yes	no functional symptoms	30-50	HU, X, antiplatelet therapy	X
HD-1376	M/76	post-ET MF	PB	NA	NA	NA	9	46,XY[20]	27.2	7.3	21.9	49	no	yes	satisfactory	50-70	HU, antiplatelet therapy	X
HD-1462	F/63	post-ET MF	PB	NA	NA	NA	<1	46,XX[20]	13.2	11.5	ND	351	no	no	none	75	antiplatelet therapy	no
HD-1569	F/54	post-ET MF	PB	NA	NA	NA	47	46,XX[20]	31.8	12.5	37.7	317	no	yes	asthenia and fatigue	50-60	C, PA	no
HD-1616_0551	M/71	post-ET MF	PB	NA	NA	NA	<1	47,XY,+9[5]/46,XY[15]	11.4	11.4	34.2	376	no	no	ND	50-70	C, EPO	X
HD-0497_1307	F/82	PMF	PB	inter-2	inter-2	inter-2	160	46,XX,del(20)(q11q13)[6]/46,XX[3]	2.4	7.6	21.2	205	yes	no	satisfactory	20-30	T	X
HD-0586	M/62	PMF	PB	inter-2	inter-2	high	31	45,XY,-13[18]/46,XY[2]	3.2	7.8	23.8	83	yes	yes	satisfactory	wt	HU, T	X
HD-0607_1130	M/51	PMF	PB	low	inter-1	inter-1	70	46,XY[20]	7.6	13.3	39	471	no	yes	satisfactory	wt	HU, antiplatelet therapy	X
HD-0616	M/54	PMF	PB	low	low	low	11	46,XY[4]	14.3	17.8	52	257	no	no	satisfactory	35-40	none	X
HD-0648	F/60	PMF	PB	ND	inter-1	inter-1	129	46,XX[20]	20.3	11.5	35	112	no	yes	satisfactory	80-90	HU, EPO	X
HD-0679_1291	M/31	PMF	PB	low	low	low	<1	46,XY[20]	5.3	15.3	44.9	208	no	no	satisfactory	5-10	none	X
HD-0683	M/62	PMF	PB	inter-2	inter-2	inter-2	<1	ND	1.9	8.5	24.8	101	yes	yes	ND	10	none	X
HD-0689	F/74	PMF	PB	ND	inter-2	inter-2	298	ND	6	7.6	22.2	167	yes	yes	asthenia aND fatigue	80-90	HU, T	X
HD-0717_1244	M/65	PMF	PB	inter-2	inter-1	inter-1	70	46,XY[20]	9.3	14.5	44.3	191	no	yes	satisfactory	wt	none	X
HD-0719	M/85	PMF	PB	inter-1	inter-1	inter-1	<1	46,XY[20]	120	8.6	26	479	no	yes	fever	wt	none	X
HD-0728	F/54	PMF	PB	inter-2	low	low	<1	46,XX,t(6;17)(p21-22;q11)[10]/46,XX[13]	35.1	13.2	ND	691	no	no	satisfactory	70-80	none	X
HD-0759	F/63	PMF	PB	inter-2	inter-2	high	3	46,XX,del(5)(q13q33),del(20)(q11q13)[19]/46,XX[1]	4.1	8	23	77	yes	yes	asthenia and fatigue	50-75	EPO	X
HD-0777	M/86	PMF	PB	high	inter-2	inter-2	<1	ND	54.2	7.7	25.2	761	yes	no	asthenia and fatigue	wt	A, antiplatelet therapy	X
HD-0862	M/83	PMF	PB	high	high	high	4	46,XY[20]	4.6	9.7	29.7	302	yes	yes	weight loss	12-30	EPO	X
HD-1047	M/65	PMF	PB	inter-2	inter-2	inter-2	<1	46,XY,del(20)(q11q13)[3]/46,idem,add(9)(p2?3)[17]	26.8	13.6	40.8	467	no	yes	satisfactory	50-75	none	X
HD-1095	M/64	PMF	PB	ND	ND	ND	<1	ND	ND	ND	ND	ND	ND	ND	ND	50-70	ND	X
HD-1098	M/75	PMF	PB	ND	ND	ND	<1	ND	9.3	13.4	40.4	143	ND	ND	ND	wt	ND	X
HD-1100	M/73	PMF	PB	high	high	high	80	46,XY,del(13)(q13q22-31)[6]/46,sl,?del(17)(p12-13)[5]/44,sl,der(1)inv(1)(p32~34q23)?dup(1)(q31q32),-7,-16,?der(17)t(16;17)(p11-12;p12-13)[6]/44,sdl1,?del(17)(p12-13)[5] +der(17)t(16;17)(p11-12;p12-13)[6]/44,sdl1,?del(17)(p12-13)[5]	11.7	10.6	32.2	645	yes	yes	no functioNAl symptoms	31-50	HU, antiplatelet therapy	X
HD-1111	F/84	PMF	PB	inter-2	inter-1	inter-1	101	46,XX[20]	20.7	15	14.3	361	no	yes	asthenia and fatigue	80-90	HU, P, T, C	no
HD-1123	F/80	PMF	PB	inter-2	inter-2	inter-2	<1	46,XX[20]	27.3	10.2	32	1188	no	no	asthenia and fatigue	50-70	none	X
HD-1138_0540_1398	M/61	PMF	PB	inter-2	inter-1	inter-1	82	ND	27	12.0	36.2	378	no	yes	satisfactory	wt	none	X

HD-1207	M/64	PMF	PB	inter-1	inter-2	high	28	46,XY,ider(20)(q10)del(20)(q11q13)[20]	2.6	7.2	20.2	5	yes	yes	asthenia and fatigue	wt	T	X
HD-1208	M/74	PMF	PB	inter-2	high	high	3	48,XY,+8,+19[19]/46,XY[1]	8.6	8.7	25.6	101	yes	yes	fever	2	C	X
HD-1227	F/70	PMF	PB	low	inter-1	inter-2	82	48, XX +8,+9[7]/46,XX[13]	5.6	14.2	43.7	60	no	yes	asthenia and fatigue	2-5	EPO	X
HD-1265_0927_1461_1853	M/72	PMF	PB	high	inter-1	inter-1	13	ND	7.1	11.6	34.8	147	no	no	satisfactory	30-50	HU	X
HD-1283	M/75	PMF	PB	inter-2	inter-2	inter-2	84	ND	5	9.7	25	890	yes	no	satisfactory	wt	C, EPO, T, HU	X
HD-1289	M/75	PMF	PB	inter-1	inter-2	high	79	ND	11.4	12.5	37.1	88	no	yes	night sweats	mutated	HU, antiplatelet therapy	X
HD-1300_0528_1611	M/59	PMF	PB	inter-2	inter-2	inter-2	47	46, XY, del 20(q11q13)[4]/46,XY[16]	41.1	8.8	27.3	98	no	ND	satisfactory	80-90	graft therapy	X
HD-1362	F/67	PMF	PB	ND	inter-2	inter-2	195	46,XX,dup(1)(q44q12),del(13)(q13q14)[20]	1.3	9.8	29.7	123	yes	yes	satisfactory	80-90	E	X
HD-1412_1495	M/52	PMF	PB	ND	inter-1	inter-1	33	46,XY[2]	12	13.2	41.7	77	no	yes	satisfactory	wt	EPO, HU	X
HD-1422	F/58	PMF	PB	low	inter-1	inter-1	79	ND	12.8	12.2	ND	351	no	yes	satisfactory	wt	EPO	X
HD-1439	M/71	PMF	PB	low	inter-2	inter-2	89	46,XY,del(7)(q22q36)[2]/46,XY[21]	48.8	14.2	47	271	no	yes	satisfactory	50-75	HU	X
HD-1524bis	F/70	PMF	PB	inter-2	high	high	18	ND	76	5.8	18	26	yes	yes	weight loss	wt	HU, C	X
HD-1538	F/77	PMF	PB	high	inter-2	inter-2	<1	ND	3.4	9.7	28.8	366	no	yes	satisfactory	30-40	none	X
HD-1572bis	F/66	PMF	PB	inter-2	inter-1	inter-1	6	46,XX,t(12;17)(q12;q21)[15]/46,XX[5]	7.4	11.6	ND	228	no	yes	satisfactory	wt	HU	X
HD-1587	F/60	PMF	PB	inter-2	inter-2	inter-2	87	ND	3.5	9.2	ND	266	yes	yes	asthenia and fatigue	wt	C, EPO	X
HD-1606	F/40	PMF	BM	low	low	low	108	46,XX[20]	6.5	13.6	41.1	274	no	no	satisfactory	12-30	HU, antiplatelet therapy	X
HD-1617	F/82	PMF	PB	inter-1	inter-1	inter-2	18	46,XX[20]	6.5	12	35.8	36	yes	yes	satisfactory	wt	C	X
HD-1654	M/65	PMF	PB	low	inter-2	high	54	46,XY,inv(11)(q12q22)[c20]	4.2	7.6	22.2	38	yes	yes	asthenia and fatigue	wt	T	X

B. Clinical and biological characteristics of 19 matched pairs of MF

Samples	Sex/ Age	Diagnosis	PB/ BM	IPSS	DIPSS	DIPSSplus	Time from diagnosis to sampling (m)	Karyotype	Leukocyte count (x 10^9/L)	Hemoglobin level (g/dL)	Hematocrit (%)	Platelet count (x 10^9/L)	RCT need	Ci blast cells	Symptoms	JAK2 V617F %	Previous Therapies	aCGH
HD-0842_1401	M/64	PV	PB	NA	NA	NA	NA	ND	6.3	21.3	66.4	269	no	no	satisfactory	50-60	HU, antiplatelet therapy	X
HD-1401_0842	M/67	post-PV MF	PB	NA	NA	NA	<1	46,XY,del(8)(q21q23)[7]/46,XY[19]	31.1	13.4	41.2	714	no	yes	weight loss	50-60	HU, antiplatelet therapy	X

HD-0551_1616	M/66	ET	PB	NA	NA	NA	NA	ND	8.3	13.6	39.9	399	no	no	satisfactory	15-30	HU, antiplatelet therapy	X
HD-1616_0551	M/71	post-ET MF	PB	NA	NA	NA	<1	47,XY,+9[5]/46,XY[15]	11.4	11.4	34.2	373	no	no	ND	50-70	C, EPO	X
HD-1412_1495	M/52	PMF	PB	high	inter-1	inter-1	34	46,XY[2]	12	13.2	41.7	77	no	yes	satisfactory	wt	EPO, HU	X
HD-1495_1412	M/59	PMF	PB	inter-2	inter-2	NA	36	46,XY[2]	2.7	9.3	28.4	49	ND	yes	infectious disease	wt	EPO	X
HD-0725_1380	M/74	post-ET MF	PB	NA	NA	NA	11	46,XY[20]	19.2	12.6	37.6	321	no	yes	satisfactory	40	HU	X
HD-1380_0725	M/76	post-ET MF	PB	NA	NA	NA	40	46,XY[20]	35.4	12.6	37.6	315	no	yes	satisfactory	50-70	antiplatelet therapy	X
HD-0497_1307	F/82	PMF	PB	inter-2	inter-2	inter-2	160	46,XX,del(20)(q11q13)[6]/46,XX[3]	2.4	7.6	21.2	205	yes	no	satisfactory	20-30	T	X
HD-1307_0497	M/86	PMF	PB	NA	NA	NA	204	ND	3.2	11.8	34.7	64	yes	ND	asthenia and fatigue	ND	none	X
HD-0607_1130	M/51	PMF	PB	low	inter-1	inter-1	70	46,XY[20]	7.6	13.3	39	471	no	yes	satisfactory	wt	HU, antiplatelet therapy	X
HD-1130_0607	M/53	PMF	PB	low	inter-1	inter-1	102	46,XY[20]	9.5	14.5	42.7	474	no	yes	satisfactory	wt	HU, antiplatelet therapy	X
HD-0679_1291	M/31	PMF	PB	low	low	low	<1	46,XY[20]	5.3	15.3	44.9	208	no	no	satisfactory	5-10	none	X
HD-1291_0679	M/33	PMF	PB	low	low	low	26	ND	5	15.8	46.2	171	no	no	satisfactory	5-10	antiplatelet therapy	X
HD-0717_1244	M/65	PMF	PB	inter-2	inter-1	inter-1	71	46,XY[20]	9.3	14.5	44.3	191	no	yes	satisfactory	wt	none	X
HD-1244_0717	M/67	PMF	PB	inter-2	inter-1	inter-1	94	ND	6.5	13.3	40.9	115	no	yes	satisfactory	wt	none	X
HD-0589_1824	F/65	post-PV MF	PB	NA	NA	NA	55	48,XX,+8,+9[3]/46,XX,del(20)(q11q13)[2]/46,XX[15]	5.8	13.4	41.4	188	no	no	no functional symptoms	50-70	none	X
HD-1824_0589	F/70	post-PV MF	PB	NA	NA	NA	117	46,XX,del(20)(q11q13)[8]/48,XX,+8,+9[3]/48,idem, der(9)t(1;9)(q21;q12q)[2]/46,XX[7]	2.5	8.8	26.9	71	no	yes	satisfactory	50-70	none	X
HD-0789_1691	M/66	post-PV MF	PB	NA	NA	NA	<1	46,XY,del(20)(q11q13)[4]/46,XY[16]	20.3	13.7	45.7	360	ND	ND	ND	80-90	HU	X
HD-1691_0789	M/69	post-PV MF	BM	NA	NA	NA	36	45,XY,-7,del(20)(q11q13)[20]/46,XY[2]	13.3	13.7	ND	60	ND	ND	night sweats	ND	anti-JAK1/2	X
HD-1427_1656	F/71	PMF	PB	inter-2	inter-1	inter-1	45	46,XX,t(7;14)(q11;q24)[2]/46,XX[18]	16.6	15.7	51.9	363	no	no	weight loss	80-90	M	X
HD-1656_1427	F/72	PMF	PB	high	inter-2	inter-2	54	46,XX[21]	8.2	14	41.7	310	no	yes	night sweats	ND	antiplatelet therapy	X
HD-1360_1605	M/44	post-ET MF	PB	NA	NA	NA	12	ND	5.8	11	32.8	392	no	yes	no functional symptoms	30-50	X, antiplatelet therapy	X
HD-1605_1360	M/45	post-ET MF	BM	NA	NA	NA	20	ND	7.5	12	ND	547	no	ND	no functional symptoms	30-50	PA, antiplatelet therapy	X
HD-0614_1112_1352	M/76	post-ET MF	PB	NA	NA	NA	140	46,XY[22]	4.5	11.2	35	269	no	yes	satisfactory	wt	EPO, antiplatelet therapy	X
HD-1112_0614_1352	M/77	post-ET MF	PB	NA	NA	NA	170	ND	4.3	7.8	22.8	78	yes	yes	asthenia and fatigue	wt	EPO, antiplatelet	X

																	therapy	
HD-1352_0614_1112	M/78	post-ET MF	PB	NA	NA	NA	178	46,XY,del(7)(q22q36)[2]/46,XY,add(4)(q21-22),-12,-13,+der(?)t(?;4)(?;q21-22),+mar[3]/44,XY,add(4),der(6)t(4;6)(q21-22;p2?3),-7,-12,-15,-16,+2mar[3]	3.5	ND	23.4	159	yes	yes	asthenia aND fatigue	wt	EPO, antiplatelet therapy	X
HD-0540_1138_1398	M/58	PMF	PB	inter-1	ND	ND	46	46,XY[20]	11.5	12.3	ND	334	no	ND	satisfactory	wt	none	X
HD-1138_0540_1398	M/61	PMF	PB	inter-2	inter-1	inter-1	82	ND	27	12.0	36.2	378	no	yes	satisfactory	wt	none	X
HD-1398_0540_1138	M/62	PMF	PB	inter-2	inter-1	inter-1	91	ND	27.3	12.6	38	315	no	yes	satisfactory	wt	none	X
HD-0927_1265_1461_1853	M/72	PMF	PB	high	high	inter-2	<1	ND	42	11.7	36	658	no	yes	weight loss	40-50	none	X
HD-1265_0927_1461_1853	M/72	PMF	PB	high	inter-1	inter-1	13	ND	7.1	11.6	34.8	147	no	no	satisfactory	30-50	HU	X
HD-1461_0927_1265_1853	M/73	PMF	PB	high	inter-1	inter-1	20	ND	9.7	10.8	ND	328	no	no	satisfactory	30-50	HU	X
HD-1853_0927_1265_1461	M/75	BP-PMF	PB	NA	NA	NA	1	46,XY, +8[16]/46,XY[6]	5.1	10.2	32.6	168	yes	yes	asthenia and fatigue	ND	HU	X
HD-1559_1649	M/63	post-PV MF	BM	NA	NA	NA	<1	45,XY,-7[9]/47,XY,+9[3]/46,XY[8]	2.2	8.2	ND	25	ND	no	satisfactory	30-50	antiplatelet therapy	X
HD-1649_1559	M/63	BP post-PV MF	PB	NA	NA	NA	4	45,XY,-7[20]	73	10	ND	27	yes	yes	asthenia and fatigue	ND	C, T	X
HD-1813_1836	M/77	post-PV MF	PB	NA	NA	NA	<1	45,XY,-7[6]/45,sl,del(12)(p12p13)[3]/45,sdl1,add(3)(q2?1)[13]	4.6	11.5	34.7	58	yes	yes	ND	2-5	none	X
HD-1836_1813	M/77	BP post-PV MF	PB	NA	NA	NA	1	45,XY,-7[6]/45,sl,del(12)(p12p13)[3]/45,sdl1,add(3)(q2?1)[13]	1.5	8.4	24.2	13	yes	yes	ND	2-5	none	X
HD-0528_1300_1611	M/56	PMF	PB	inter-2	inter-1	inter-1	<1	46,XY[20]	29.4	13.2	38.6	295	no	yes	satisfactory	75	none	X
HD-1300_0528_1611	M/59	PMF	PB	inter-2	inter-2	inter-2	47	46, XY, del 20(q11q13)[4]/46,XY[16]	41.1	8.8	27.3	98	no	ND	satisfactory	80-90	graft therapy	X
HD-1611_0528_1300	M/60	BP-PMF	PB	NA	NA	NA	59	46,XY,del(20)(q11q13)[12]/46,XY,inv(3)(q21q26)[4]/46,idem,del(6)(q2?1q2?6)[3]/46,XY[1]	6.5	12	35.8	36	no	yes	asthenia aND fatigue	ND	M	X
HD-1309_1741	F/36	post-ET MF	PB	NA	NA	NA	73	46,XX[20]	16.2	9.9	ND	750	ND	yes	asthenia and fatigue	wt	none	X
HD-1741_1309	F/37	BP post-ET MF	PB	NA	NA	NA	87	46,XX,-3,der(5)t(3;5)(q13;q21),del(7)(q21q36),t(9;17)(q11;q11),+21[20]	77.3	9.6	ND	469	no	yes	asthenia and fatigue	wt	HU	X

C. Clinical and biological characteristics of 17 blast phase MF

Samples	Sex/ Age	Diagnosis	PB / BM	Time from diagnosis to sampling (m)	Karyotype	Leukocyte count (x 10^9/L)	Hemoglobin level (g/dL)	Hematocrit (%)	Platelet count (x 10^9/L)	JAK2 V617F %	Previous Therapies	aCGH
HD-0492	M/67	BP post-PV MF	PB	20	47,XY,+8[17]/48,idem,+9[3]	17.0	10.0	36.0	44	80-100	HU	X
HD-1517	M/89	BP post-PV MF	BM	ND	46,XY[20]	15.1	10.4	30.5	94	wt	ND	X
HD-1649_1559	M/63	BP post-PV MF	PB	4	45,XY,-7[20]	73	10	ND	27	ND	C, T	X
HD-1836_1813	M/77	BP post-PV MF	PB	<1	ND	1.5	8.4	24.2	13	2-5	none	X
HD-0535	M/68	BP post-ET MF	PB	153	46,XY,t(3;12)(q26-27;q13-14)[20]	10.3	11.5	ND	661	wt	HU	X
HD-0536	M/53	BP post-ET MF	PB	<1	46,XY,add(15)(q25-26)[5]/46XY[19]	5.0	13.1	39.2	535	wt	none	X
HD-0646	F/61	BP post-ET MF	PB	15	46,XX,inv(3)(q21q26)[3]/46,idem,der(16)t(1;16)(q11;q11)[17]	5.2	1.7	33.0	315	5-10	HU	X
HD-0856	F/57	BP post-ET MF	PB	15	46,XX,del(7)(q22q36),del(8)(q22q24)[18]/47,idem,+8[2]	7.7	9.2	29.0	1052	wt	HU	X
HD-1167	F/68	BP post-ET MF	PB	12	49,XX,add(3)(p14),del(5)(q31q35),+8,+8,+9,?del(11)(p11p13),-15,add(16)(p12),+mar[20]	15	8	23.7	117	wt	HU, A	X
HD-1387	M/60	BP post-ET MF	PB	15	ND	ND	ND	ND	ND	ND	ND	X
HD-1741_1309	F/37	BP post-ET MF	PB	87	46,XX,-3,der(5)t(3;5)(q13;q21),del(7)(q21q36),t(9;17)(q11;q11),+21[20]	77.3	9.6	ND	469	wt	HU	X
HD-0655	M/76	BP-PMF	PB	26	46,XY,inv(16)(p13q22)[20]	6.6	8.1	24.0	25	30-50	none	X
HD-1611_0528_1300	M/60	BP-PMF	PB	59	46,XY,del(20)(q11q13)[12]/46,XY,inv(3)(q21q26)[4]/46,idem,del(6)(q2?1q2?6)[3]/46,XY[1]	6.5	12	35.8	36	ND	M	X
HD-0635	M/76	BP-PMF	PB	26	46,XY,inv(16)(p13q22)[20]	6.6	8.1	24.0	25	30-50	none	X
HD-1847	M/79	BP-PMF	PB	<1	ND	24.4	11.6	36.2	164	mutated	HU	X
HD-1853_0927_1265_1461	M/75	BP-PMF	PB	<1	46,XY, +8[16]/46,XY[6]	5.1	10.2	32.6	168	ND	HU	X
HD-1137	M/82	BP post-MPN MF	PB	3	44,XY,add(1)(q4?2),add(3)(p14),del(5)(q23q34),der(9)t(9;10)(q23;q22),-10,?dic(15;20)(q12;p12),add(17)(p11)[19]/46,XY,del(12)(p11p13)[1]	106	9.4	30	46	wt	EPO, C	X

MF samples are classified according to the WHO criteria.

aCGH, array comparative genomic hybridization; A, anagrelid; B, busulfan; BM, bone-marrow; C, corticoid; Ci blasts, circulating blasts; DIPSS, dynamic international prognostic scoring system; DIPSSplus, dynamic international prognostic scoring system plus; E, eposin; F, female; HU, hydroxyurea; IPSS, international prognostic scoring system; m, month; M, male; ME, melphalan; MF, myelofibrosis; NA, not applicable; ND, not determined; P, pipobroman; PA, peginterferon alfa-2a; PB, peripheral blood; PMF, primary myelofibrosis; post-ET MF, post-essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF; RCT need, red cell transfusion need; T, thalidomide; wt, wild-type; y, years. For PMF, IPSS, DIPSS and DIPPSplus scores depend on age (>65 years), on hemoglobin (< 10 g/dL), on leukocyte count (> 25.10^9/L), on circulating blasts (> or = 1%), on platelet count (< 100.10^9/L), on presence of constitutional symproms (weight loss, night sweats, fever). For DIPPSplus score, karyotype and transfusion status must be added to DIPSS. IPSS score was calculated at MF diagnosis whereas DIPPS and DIPPSplus score were calculated at sampling.

Supplemental Table 2. Sequencing primers

A List of primers used for PCR and sequencing

Genes	Forward primers	Sequence 5' to 3'	Reverse primers	Sequence 5' to 3'	annealing (°C)
ASXL1	ASXL1_EX12_F1	AGGTCAGATCACCCAGTCAGTT	ASXL1_EX12_R1	TAGCCCATCTGTGAGTCCAACTGT	55
	ASXL1_EX12_F2	AGAGGACCTGCCTTCTCTGAGAAA	ASXL1_EX12_R2	TTCGATGGGATGGGTATCCAATGC	55
	ASXL1_EX12_F3	ACTTGAAAACCAAGGCTCTCGT	ASXL1_EX12_R3	GCAACCATCCCATCTGTCCTTGTA	55
	ASXL1_EX12_F4	GGTGGACAAGGATGAGAAACCCAA	ASXL1_EX12_R4	TGTCCTGTGACATAGCACGGACTT	55
	ASXL1_EX12_F5	TGGATTCCAAAGAGCAGTTCTCTTC	ASXL1_EX12_R5	CATGACAAAGGGCATCCCTTCCAA	55
	ASXL1_EX12_F6	ACAGGAAAGCTACTGGGCATAGTC	ASXL1_EX12_R1	CAAGAGTGCTCCTGCCTAAAGAGT	55
BMI1	BMI1-F2-3	TGATTACTAGATGATCTCCATTCTTG	BMI1-R2-3	AAATAAAGAGGGTTGCCTTCAG	55
	BMI1_F4-5-6-7	TTGAAAGGCACACTTCTTTTG	BMI1_R4-5-6-7	CTAAAGGGCAACACAATCCC	61
	BMI1_F8-9	GGTCACCTCCAATTTTGTTTG	BMI1_R8-9	TGTATTTCAATGGAAGTGGACC	55
	BMI1_F10	GAGAAGGGTAAGTAGCATATCTGTTG	BMI1_R10	AACAAACTATGGCCCAATGC	55
CBL	CBL_F8	ACCCAGACTAGATGCTTTCTG	CBL_R8	AGGCCACCCCTTGTATCAGT	58
	CBL_F9	TTCAGATGCATCTGTTACTATCT	CBL_R9	AGTGTTTTACGGCTTTAGAAGACA	58
DNMT3A	DNMT3A_F15	TGTAAAACGACGGCCAGTTTTCCATTCCAGGTAGCACACC	DNMT3A_R15	CAGGAAACAGCTATGACCAGGCTCCTAGACCCACACACC	60
	DNMT3A_F16	TGTAAAACGACGGCCAGTAGGGTGTGTGGGTCTAGGAGC	DNMT3A_R16	CAGGAAACAGCTATGACCGCTGTGAAGCTAACCATCATTTCG	60
	DNMT3A_F17	TGTAAAACGACGGCCAGTGACTTGGGCCTACAGCTGACC	DNMT3A_R17	CAGGAAACAGCTATGACCAAATGAAAGGAGGCAAGGGC	60
	DNMT3A_F18	TGTAAAACGACGGCCAGTATAGGACAGTGGTGTGGCTCG	DNMT3A_R18	CAGGAAACAGCTATGACCTTCTTCCTGTCTGCCTCTGTCC	60
	DNMT3A_F19	TGTAAAACGACGGCCAGTGACAGCTATTCCCGATGACCC	DNMT3A_R19	CAGGAAACAGCTATGACCTGCAGATGAGACAGGATGAAGC	60
	DNMT3A_F20	TGTAAAACGACGGCCAGTGCCGGCGCTGTTTCATGC	DNMT3A_R20	CAGGAAACAGCTATGACCCCACTATGGGTCATCCCACCTGC	60
	DNMT3A_F21	TGTAAAACGACGGCCAGTCCTTCCCGCTGTTATCCAGG	DNMT3A_R21	CAGGAAACAGCTATGACCCATCCTGCCCTTCCTTCTCC	60
	DNMT3A_F22	TGTAAAACGACGGCCAGTTGGCATATTTGGTAGACGCATGAC	DNMT3A_R22	CAGGAAACAGCTATGACCTGGGAAATGCTTGATAAAACCCAC	60
	DNMT3A_F23	TGTAAAACGACGGCCAGTGTGTGGTTAGACGGCTTCCG	DNMT3A_R23	CAGGAAACAGCTATGACCCCCATGTCCCTTACACACACG	60
EZH2	EZH2_F2	GGTGATCATATTCAGGCTGG	EZH2_F2	AAACTTATTGAACTTAGGAGGGG	60
	EZH2_F3	GACACCCTGAGGTCAATGAT	EZH2_F3	ATCATTGACCTCAGGGTGTC	60
	EZH2_R4	GGCTACAGCTTAAGGTTGTCCT	EZH2_R4	CTGTCTTGATTCACCTTGACAAT	60
	EZH2_R5	AAATCTGGAGAACTGGGTAAAGAC	EZH2_R5	TCATGCCCTATATGCTTCATAAAC	60
	EZH2_R6	AGGCTATGCCTGTTTTGTCC	EZH2_R6	AAAAGAGAAAGAAGAAACTAAGCCC	60
	EZH2_R7	CTGACTGGCATTCCACAGAC	EZH2_R7	AAGTGTAGTGGCTCATCCGC	60
	EZH2_R8	CATCAAAAGTAACACATGGAAACC	EZH2_R8	TTGTAATAAATGATAGCACTCTCCAAG	60
	EZH2_R9	TCCATTAATTGACTTTTCCAGTG	EZH2_R9	ACCTCCACCAAAGTGCAAAG	60
	EZH2_R10	TTCTCTTCCATCAAAATGAGTTTTAG	EZH2_R10	TCCTCACAACACGAACTTTCAC	60
	EZH2_R11	GAGTTGTCCTCATCTTTTCGC	EZH2_R11	CCAAGAATTTTCTTTGTTTGGAC	60
	EZH2_F12	AAGAATGGTTTGCCTAAATAAGAC	EZH2_F12	CTTGCCTGCAGTGTCTATC	60

	EZH2_F13	TCTTGGCTTTAACGCATTCC	EZH2_F13	CAAATTGGTTTAACATACAGAAGGC	60
	EZH2_F14	TGATCGTTTCCATCTCCCTG	EZH2_F14	AGGGAGTGCTCCCATGTTC	60
	EZH2_F15	GAGAGTCAGTGAGATGCCCAG	EZH2_F15	TTTGCCCCAGCTAAATCATC	60
	EZH2_F16	TTTTTGATGATGTGATTGTGTTTT	EZH2_F16	TGGCAATTCATTTCCAATCA	60
	EZH2_F17	TTCTGTCAGGCTTGATCACC	EZH2_F17	CTCGTTTCTGAACACTCGGC	60
	EZH2_F18-19	AGGCAAACCCTGAAGAACTG	EZH2_F18-19	TTCCAATTCTCACGTCAAAGGTA	60
	EZH2_F20	CCGTCTTCATGCTCACTGAC	EZH2_F20	AAAAACCCTCCTTTGTCCAGA	60
IDH1	IDH_F4	GGATGCTGCAGAAGCTATAA	IDH_R4	CATGCAAAATCACATTATTGCC	53.6
IDH2	IDH_F4	CTGTGTTGTTGCTTGGGGTT	IDH_R4	GGGGTGAAGACCATTTTGAA	56.9
JAK2	JAK2_F12	CTCCTCTTTGGAGCAATTCA	JAK2_R12	GAGAACTTGGGAGTTGCATA	55
KRAS	KRAS_F2	GCCATTTGTCCGTCATCTTTGGAG	KRAS_R2	TGCATGGCATTAGCAAAGACTCA	55
	KRAS_F3	AGGAAGGAAAATTTGGTGTAGTGGA	KRAS_R3	AGAAGCAATGCCCTCTCAAGAGAC	54
	KRAS_F4a	CTTGCACATGGCTTTCCCAGTA	KRAS_R4a	AGTGGTTGCCACCTTGTTACCT	54
	KRAS_F4b	CCTGTACACATGAAGCCATCGT	KRAS_R4b	CTAACAGTCTGCATGGAGCAGGAA	54
LNK	LNK_F2a	CACCCACGTGTCTTTCAGC	LNK_R2a	TCCAGGGCAGGAACTTCT	62
	LNK_F2b	CCCTGCTCCTTCCAGCAC	LNK_R2b	CTGGAAAGCCATCACACCTC	63
	LNK_F3-4	GGGACTCCTGGGGAGACTAT	LNK_R3-4	TGCATCTCTGCTTCTGTGCT	62
	LNK_F5-6	ACCACCTTTGCTGCTACCAC	LNK_R5-6	TGTCCCTCAGGACCCTGAA	62
	LNK_F7	AACTTTCAGGGGATAGCCAAG	LNK_R7	GATACCTGTACCCCTGGGTCT	62
	LNK_F8	TCTGTGTCCTGTCAGCACTTG	LNK_R8	TCTGGAAGGAAGGAAAGATCA	61
MPL	MPL_F10	CCGAAGTCTGACCCTTTTTG	MPL_R10	ACAGAGCGAACCAAGAATGC	60
NF1	NF1_F1	TAGTGGGGAGAGCGACCA	NF1_R1	TCCCCTCACCTACTCTGTCC	60
	NF1_F2	ATCATATATTGGTGTTTGAGATGC	NF1_R2	ACAGTTACTTGTGCTCACTGAATC	60
	NF1_F3	TGTGTGTTGATTGGTAGCAGAA	NF1_R3	GGACTGTCCTCTTGGTCCAC	60
	NF1_F4	TTTTGTTCTGTGTGTGTGTTTGA	NF1_R4	AAAACCTCATTTCCATTAACTTTTT	60
	NF1_F5	GAGATCCTCCTCCCTTAGCC	NF1_R5	GCCACCCTTGAGAGATCAAA	60
	NF1_F6-7	TTAGGTGTCTTTACCTTTCATTGC	NF1_R6-7	CAGTATTTCCTATTTGACACCAGTTG	55
	NF1_F8	CAGAATGCATTTGTGTAGTTGC	NF1_R8	AAGTCCATCAAACAAAGAAACC	60
	NF1_F9	AAATTATGAAATTGAAAACCACAAA	NF1_R9	TCAGTCCATTTAGGCTGATGAA	55
	NF1_F9_seq	CAAATATAAATTATGCATT			
	NF1_F10-11	GTGTGGGTAATGTGTTGATGTT	NF1_R10-11	ACGCAAAGAAAAGAAAGAAAAA	55
	NF1_F10-11_seq	TGTGTTGATGTTATTACATG			
	NF1_F12	GAAATCATGGTGTGTGTTTGC	NF1_R12	TGAAGGACCCATTCAATTCTC	60
	NF1_F13	TGGATAGCTATTATCCTGAGTCTTATG	NF1_R13	GCTAAACCCAATTAACTTAGTGTGA	60
	NF1_F13_seq	CTGAGTCTTATGTCTGATACC			
	NF1_F14	TCTTCCTCCTTCTAATCTCTCTCG	NF1_R14	GTTACAATCTTTCCTTCAAGAACAT	55
	NF1_F15	TGTTTGAGTGAGTCTTCTCTTTGTC	NF1_R15	CATAAAACCTTTGGAAGTGTAAGTTTT	60
	NF1_F16	TGCATTAGGTTATTGATGATGC	NF1_R16	TTGGAATGGTAATGGTGAGAGA	60
	NF1_F17	CCTCTTGGTTGTCAGTGCTTC	NF1_R17	TCATTCAGAAAACAAACAGAGCA	60
	NF1_F18	TTTATACATAAAATTACCCAAGTTGC	NF1_R18	TCAGTCTGCCTCAAAGCACA	60
	NF1_F19-20	TTTGGGTGGAGCTTATCAGG	NF1_R19-20	TGTTTACTTTACTGAGCGACTCTTG	60

	NF1_F21	TTGGATAAAGCATAATTTGTCAAGTC	NF1_R21	TTAAATAATCTGAAAGAAAAAGGCTTA	55
	NF1_F22-23	GGTCTGTCTTCTGGGCATTG	NF1_R22-23	TCCTTTCTACCAATAACCGCATA	60
	NF1_F24	AAGGTGTGTGTGTGGCTTCA	NF1_R24	GTTATAACTCTCACAGTAAAACCCACT	60
	NF1_F25	GAGGGGAAGTGAAAGAACTTGA	NF1_R25	GGGGGCTTTATTTGCTTTTT	60
	NF1_F26	CCATTCACACCATGCACATA	NF1_R26	TGTGGACAAACAGATGCAAA	60
	NF1_F27	TGGCATGTAAGAGAAGCAAAAA	NF1_R27	TGCCAACCACTTCCCTACAG	60
	NF1_F28-29	AACTTTGGGTTTACATTTTTGCTACT	NF1_R28-29	GCCTTGCTTCATGCAGTGT	60
	NF1_F30	GTCTACACGTTGCACTTGGC	NF1_R30	AGACAATTCCAACAATTCTCAATG	60
	NF1_F31	TCCATTTGTGTTACATTTTATGGTG	NF1_R31	CACAAATTGCCTTTGGGATAA	60
	NF1_F32-33	GCAAAGTTTGACCTTTGAACTCT	NF1_R32-33	TGGATTTATGTGAAACCGAAAA	60
	NF1_F34	TCTGGGTGTATCTGGTGTTGA	NF1_R34	TCTAAATTTAAACGGAGAGTGTTCA	60
	NF1_F35	CCTGAGGTCTTTTTGGTGCT	NF1_R35	ACATCTTTCCTCATAGTCTTCTATCAT	60
	NF1_F36	CTCAGTAGACAACATAAAGCCTCA	NF1_R36	CCCTGCTATATGCTCCCAAG	60
	NF1_F37	TGAATCCAGACTTTGAAGAATTG	NF1_R37	ACCTTGACATCCCAAAGTGC	60
	NF1_F38	GGTTGGTTTCTGGAGCCTTTT	NF1_R38	AGCAACAAACCCCAAATCAA	60
	NF1_F39	AGCCTCACAGTGCTCTTATGG	NF1_R39	GGTGGATTTCTCAATGTGGC	60
	NF1_F40	CAGGCCTGATTCTAGGTAATAGTC	NF1_R40	GGGTGAGTGTCTACATGCTTTC	60
	NF1_F41-42	TTGATTAGGCTGTTCCAATGAA	NF1_R41-42	GCAACTTGGTGTTAGAGCACA	60
	NF1_F43	GAGGTTTGATTTAGGGAACATGA	NF1_R43	CCAATAACACAGTCCATGCAA	55
	NF1_F44-45	TTGCATGGACTGTGTTATTGG	NF1_R44-45	TTTCATTGACCTCAAATTTAAACG	55
	NF1_F46	TTCCTGAATTCATTCCGAGATT	NF1_R46	TTCAACACTGATACCCAAAATGA	55
	NF1_F47	TTCTCAGTCCAGCTAACAGTGTC	NF1_R47	TTTATGAGCTGGTAAGGAAATATACTC	60
	NF1_F48	TCAAATTGAAAGGATTACTTATCTTG	NF1_R48	TGCATTTAAAGTAAGACATAAGGGC	60
	NF1_F49	ATGGCTATTCTTGGAAAGTAGGA	NF1_R49	TGTGAACTTTCTGCTCTGCC	60
	NF1_F50	TGCACATTTAACAGGTACTATGCTC	NF1_R50	CCATGTTACCTTCTAGATTACTCTGC	60
	NF1_F51	TGATTGCTGTTGTTAGGAAATAGG	NF1_R51	AAATGGCATCAAAAACTTTGC	60
	NF1_F52-53	CCAGGGATGTATTAGAGCTTTC	NF1_R52-53	GCTGAATTGTTATCTGTTTGGG	60
	NF1_F54	CAGGCGCTGCTTCTTACTG	NF1_R54	CCTCTTTGCAACCAGTGC	60
	NF1_F55-56	GCACATTATTCTGGGGAATG	NF1_R55-56	TGAACAGAAACCTTCATTTTGC	60
	NF1_F57	GTCTTGTGCATGGCTTTCAG	NF1_R57	TCAACCTTGGAATATCATGTGG	60
	NF1_F58	TGATTGTTTCCTAGAATGTGTCC	NF1_R58	TGCAGCATTACAACATGGG	60
NRAS	N-RAS_F1	TGTAGATGTGGCTCGCCAAT	N-RAS_R1	TGATCCGACAAGTGAGAGAC	55
	N-RAS_F2	GGCAATAGCATTGCATTCCC	N-RAS_R2	TCCCTAGTGTGGTAACCTCA	55
PPP1R16B	PPP16R1B_F2	CACAGGCCACACCATGAG	PPP16R1B_R2	GCCCACACGTCTACTGCAC	66.5
	PPP16R1B_F3	GGGAGTTGTTACTACATTCTTCATAGG	PPP16R1B_R3	CTCATTCCCTCCTCCCTGAG	57
	PPP16R1B_F4	CTGGATTCAAACAGGCCTTC	PPP16R1B_R4	AGAAAGAAGGAAGTTGGAAAAAGA	57
	PPP16R1B_F5	ACAGTAGGATTGCAGGCGAG	PPP16R1B_R5	GCCGATGCGTAGCTAATCTG	57
	PPP16R1B_F6	TGATCTATGGGCATGTCTGC	PPP16R1B_R6	TTCTTGCGGAGGTCCAAG	57
	PPP16R1B_F7	GGATGCTGTGGGAGTTGG	PPP16R1B_R7	GAGCTATATTCAACACCTTAAGCCT	57
	PPP16R1B_F8	TGGGTCACATCCTAAGAGGC	PPP16R1B_R8	CCATCTATCTATCTAGACACCAATCCA	57
	PPP16R1B_F9-10	CATGCCCTGAAAGATGCTTG	PPP16R1B_R9-10	GTTTAAGGGCAGTGCCTGGC	57

	PPP16R1B_F11	TGTACGGAAATTCATAGATTGGG	PPP16R1B_R11	AAGCAGAGCCCACCTCTCC	65
PTPN11	PTPN11_F3	CGACGTGGAAGATGAGATCTGA	PTPN11_R3	CAGTCACAAGCCTTTGGAGTCAG	60
	PTPN11_F13	CAACACTGTAGCCATTGCAACA	PTPN11_R13	CGTATCCAAGAGGCCTAGCAAG	60
RCOR1	RCOR1_F3	TTTCAGCAGACTCTCGCCTC	RCOR1_R3	CAACAGAGCCAGAATCCATC	63
	RCOR1_F4	GCAGACCATAGGGTGTACTCTG	RCOR1_R4	GAAGAAACACTGCTACTCAATACAAAG	55
	RCOR1_F5	GCTGGTCTCATTTCCATTTTC	RCOR1_R5	CAAGTATGCTGCAAAAGAGAGTTAG	55
	RCOR1_F6	CCGGCCTTAAACATTTGATT	RCOR1_R6	CTAAGAGCCCCAGATACACA	55
	RCOR1_F7	AAATCAATTTATCGTTTGTATTCGG	RCOR1_R7	TGCTGCTTTCCACATAGCAC	55
	RCOR1_F8	TGTTTTGGTGCCATGTTAAGG	RCOR1_R8	ATGCTCCTTTGAGATTTGCG	55
	RCOR1_F9-10	TCTCAGAATTCAGCTACAACAGATG	RCOR1_R9-10	AAGCAAATGCGTCAACCTTC	55
	RCOR1_F11	TCTTATCTGGATAAAGAGGTAAGTGTC	RCOR1_R11	TTTAGAAACTGGAGTATCAAGCG	55
	RCOR1_F12	TTGCTGGCTACCTTCTCTTTTC	RCOR1_R12	CACCTAGCCATCTGCATCAC	55
SF3B1	SF3B1_F15-16	TGTGAACATATTCTGCAGTTTGG	SF3B1_R15-16	AAACAAATCAAACAGTATTCGTGTAAC	56.9
SRSF2	SRSF2_F1-2	AGACGCCATTTCCCCAG	SRSF2_R1-2	TTCGAGAAGTACGGGCG	55
SOCS2	SOCS2_F2	CGTTTTGGGATTCGCACTGACTTC	SOCS2_R2	TATTCTTTGCTTCCCACCTCGACC	55
	SOCS2_F3	CCGGTAAACTTTCGCTCACA	SOCS2_F3	CCAGAGCACTCAAGTTTTGGTTC	53
SUZ12	SUZ12_F10	TTAAGGCAAATCCACATTGAC	SUZ12_R10	AAAGCACAAGACCTAACTCTGC	55
	SUZ12_F11	TCCTGCGATCATCAGTTGAG	SUZ12_R11	AGTGGTGGCATGTGCTTG	60
	SUZ12_F12	TTTGACAGTGTCTGCCTTGG	SUZ12_R12	TTGGTTGAGGATTTTGTGAGTC	55
	SUZ12_F12_seq	TGGGGTACAAGAGATTTCTTAACAC			
	SUZ12_F13	AAATTGGAGGGATATTTAGGATAGG	SUZ12_R13	TCATCTGGCTCCTTTGTTCC	55
	SUZ12_F14	TCCTAGTCGTGAGGTTAGATGG	SUZ12_R14	ATTTTAAAACCTGTACATTTCCG	55
	SUZ12_F15	GAAATGTTGCCACTTTGCTG	SUZ12_R15	CATTGGAATAGAATAAAATTTGGG	55
	SUZ12_F16	TGGTTATCACTGTTGCATTTGAC	SUZ12_R16	CATAATTCTTAGAGGATGAATTCCC	55
TET2	TET2_F3-1	TGAACTTCCCACATTAGCTGGT	TET2_R3-1	GAAACTGTAGCACCATTAGGCATT	variable
	TET2_F3-1_seq	GATAGAAATAAACACATTTT			variable
	TET2_F3-2	CAAAAGGCTAATGGAGAAAGACGTA	TET2_R3-2	GCAGAAAAGGAATCCTTAGTGAACA	variable
	TET2_F3-3	GCCAGTAAACTAGCTGCAATGCTAA	TET2_R3-3	TGCCTCATTACGTTTTAGATGGG	variable
	TET2_F3-4	GACCAATGTCAGAACACCTCAA	TET2_R3-4	TTGATTTTGAATACTGATTTTCACCA	variable
	TET2_F3-5	TTGCAACATAAGCCTCATAAACAG	TET2_R3-5	ATTGGCCTGTGCATCTGACTAT	variable
	TET2_F3-6	GCAACTTGCTCAGCAAAGGTACT	TET2_R3-6	TGCTGCCAGACTCAAGATTTAAAA	variable
	TET2_F4	ATACTACATATAATACATTCTAATTCCCTCACTG	TET2_R4	TGTTTACTGCTTTGTGTGTGAAGG	variable
	TET2_F5	CATTTCTCAGGATGTGGTCATAGAAT	TET2_R5	CCCAATTCTCAGGGTCAGATTTA	variable
	TET2_F6	AGACTTATGTATCTTTCATCTAGCTCTGG	TET2_R6	ACTCTCTTCCTTTCAACCAAAGATT	variable
	TET2_F7	ATGCCACAGCTTAATACAGAGTTAGAT	TET2_R7	TGTCATATTGTTCACTTCATCTAAGCTAAT	variable
	TET2_F8	GATGCTTTATTTAGTAATAAAGGCACCA	TET2_R8	TTCAACAATTAAGAGGAAAAGTTAGAATAATATTT	variable
	TET2_F9	TGTCATTCCATTTTGTTTCTGGATA	TET2_R9	AAATTACCCAGTCTTGCATATGTCTT	variable
	TET2_F10	CTGGATCAACTAGGCCACCAAC	TET2_R10	CCAAAATTAACAATGTTCATTTTACAATAAGAG	variable
	TET2_F11-1	GCTCTTATCTTTGCTTAATGGGTGT	TET2_R11-1	TGTACATTTGGTCTAATGGTACAACTG	variable
	TET2_F11-2	AATGGAAACCTATCAGTGGACAAC	TET2_R11-2	TATATATCTGTTGTAAGGCCCTGTGA	variable
TP53	TP53_F5-6	TTCCTCTTCCTACAGTACTC	TP53_R5-6	AGTTGCAAACCAGACCTCAG	55

	TP53_F7-8	AGGTTGGCTCTGACTGTACC	TP53_R7-8	CTTGTCCTGCTTGCTTACCTC	55
TRPS1	TRPS1_F2	TGCAGATGTACAGATCAGCTCTC	TRPS1_R2	AGCCACCCTCAAGTTATTATCTC	62
	TRPS1_F3.1	AAAGAATAAGTAATAGGGAGGGTAAAC	TRPS1_R3.1	TTGACAATTGGCTTGACCAC	64
	TRPS1_F3.2	GACCCTCAAGATATGGCCTG	TRPS1_R3.2	GGCTACCTGTCTGGTACTGGG	64
	TRPS1_F4.1	GCCTCTCAAAGAGTAGCTGTTG	TRPS1_R4.1	TGATAATGACGGAGAAGTGGC	64
	TRPS1_F4.2	AGTCAGGCGGCCTTAATCC	TRPS1_R4.2	CTCTCAACAATTCCCGGTTC	64
	TRPS1_F5	GTTAAGAATATTGCATTTCTTTCTACC	TRPS1_R5	GAGACAGATCATTAAGTTTCACTTCAC	62
	TRPS1_F6	TGACCTCATTATGGGCAGTG	TRPS1_R6	ACTGCAAGCCAGGGAATGG	57
	TRPS1_F7.1	AGCAGAAAGCCTATCCCTGC	TRPS1_R7.1	GATTCCCAGGAACGGAGA	57
	TRPS1_F7.2	ATTATTCACCACCAGGCAGC	TRPS1_R7.2	TGTTGGATAAGGCAGGCTCT	66.5

B PCR program of *TET2*

Time	Temperature (°C)	cycles number
15''	94	1
20''	94	2
20''	56	
depending amplicon lenght	72	
20''	94	2
20''	54	
depending amplicon	72	
20''	94	2
20''	52	
depending amplicon lenght	72	
20''	94	37
20''	50	
depending amplicon lenght	72	
10'	72	1

', minutes; '', secondes; F, forward; R, reverse.

Supplemental Table 3. List of genes located in the two 20q commonly deleted regions (CDR1 and CDR2).

CDR1	Functions
LOC388796	uncharacterized
SNORA71A	small nucleolar RNA, H/ACA box 71A
SNORA71B	small nucleolar RNA, H/ACA box 71B
SNORA71C	small nucleolar RNA, H/ACA box 71C
SNORA71D	small nucleolar RNA, H/ACA box 71D
SNHG11	member of the non-protein-coding multiple snoRNA host gene family, two snoRNAs are derived from the introns of this host gene
SNORA30	snoRNAs are derived from the introns of SNHG11
SNORA60	snoRNAs are derived from the introns of SNHG11
RALGAPB	Ral GTPase activating protein, beta subunit (non-catalytic)
MIR548O2	microRNA
RPS3P2	ribosomal protein S3 pseudogene 2
ADIG/SMAF1	adipocyte differentiation
ARHGAP40	Rho GTPase activating protein 40
LOC391247	INS complex subunit 2 (Psf2 homolog) pseudogene
SLC32A1	member of amino acid/polyamine transporter family II
ACTR5	in yeast, DNA double strand break, chromatin remodelling
PPP1R16B	synthesis of the encoded protein is inhibited by transforming growth factor beta-1 mRNAoverexpressed in hematopoietic cells
FAM83D	family with sequence similarity 83, member D
DHX35	member of the DEAD box protein family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division
NPM1P19	nucleophosmin 1 (nucleolar phosphoprotein B23, numatrin) pseudogene 19
LOC339568	uncharacterized

CDR2	Functions
YWHAB	interaction with RAF1 and CDC25 phosphatases, suggesting that it may play a role in linking mitogenic signaling and the cell cycle machinery
PABPC1L	poly(A) binding protein, cytoplasmic 1-like
TOMM34	chaperone-like activity, binding the mature portion of unfolded proteins and aiding their import into mitochondria
STK4	cytoplasmic kinase that is structurally similar to the yeast Ste20p kinase, which acts upstream of the stress-induced mitogen-activated protein kinase cascade
KCNS1	belongs to the S subfamily of the potassium channel family
WFDC5	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC12	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
PI3	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
SEMG1	predominant protein in semen
SEMG2	predominant protein in semen
SLPI	protects epithelial tissues from serine proteases
MATN4	member of von Willebrand factor A domain containing protein family

RBPJL	recombination signal binding protein for immunoglobulin kappa J region-like
SDC4	a syndecan-4/CXCR4 complex expressed on human primary lymphocytes and macrophages and HeLa cell line binds the CXC chemokine stromal cell-derived factor-1 (SDF-1)
SYS1	nonsense-mediated mRNA decay
SYS1-DBNDD2	nonsense-mediated mRNA decay
TP53TG5	TP53 target 5
PIGT	chosphatidylinositol glycan anchor biosynthesis, class T
AK127953	uncharacterized
WFDC2	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC6	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
SPINLW1-WFDC6	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC8	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC9	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC11	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC10B	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC13	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
WFDC3	member of the WAP-type four-disulfide core (WFDC) domain family , contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor
DNTTIP1	deoxynucleotidyltransferase, terminal, interacting protein 1
UBE2C	destruction of mitotic cyclins and for cell cycle progression
TNNC2	regulation of striated muscle contraction
SNX21	members of this family contain a phox (PX) domain, which is a phosphoinositide binding domain, and are involved in intracellular trafficking
ACOT8	peroxisomal thioesterase that appears to be involved more in the oxidation of fatty acids rather than in their formatio
ZSWIM3	zinc finger, SWIM-type containing 3
ZSWIM1	zinc finger, SWIM-type containing 1
C20orf165	uncharacterized
NEURL2	non-protein coding RNA due to the presence of an upstream open reading frame (uORF)
CTSA	glycoprotein which associates with lysosomal enzymes beta-galactosidase and neuraminidase to form a complex of high molecular weight multimers
PLTP	lipid transfer proteins
AK097925	uncharacterized
PCIF1	PDX1 C-terminal inhibiting factor 1
ZNF335	transcriptional activation by ligand-bound nuclear hormone receptors
MMP9	proteins of the matrix metalloproteinase
SLC12A5	an integral membrane K-Cl cotransporter
NCOA5	coregulator for the alpha and beta estrogen receptors and the orphan nuclear receptor NR1D2
CD40	member of the TNF-receptor superfamily
CDH22	cadherin 22, type 2
SLC35C2	solute carrier family 35, member C2
ELMO2	phagocytosis of apoptotic cells and in cell migration
ZNF334	zinc finger protein 334
C20orf123	uncharacterized
SLC13A3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3
TP53RK	TP53 regulating kinase

SLC2A10	regulation of glucose homeostasis
BC065739	uncharacterized
EYA2	role in eye development
ZMYND8	receptor for activated C-kinase (RACK) protein
NCOA3	uclear receptor coactivator that interacts with nuclear hormone receptors to enhance their transcriptional activator functions
SULF2	cell signaling
AK129540	uncharacterized
LOC284749	uncharacterized
PREX1	guanine nucleotide exchange factor for the RHO family of small GTP-binding proteins (RACs)
ARFGEF2	play an important role in intracellular vesicular trafficking
CSE1L	poptosis and in cell proliferation
STAU1	member of the family of double-stranded RNA (dsRNA)-binding proteins involved in the transport and/or localization of mRNAs to different subcellular compartments and/or organelles
DDX27	member of the DEAD box protein family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division
ZNFX1	zinc finger, NFX1-type containing 1
ZFAS1	potential marker for breast cancer
SNORD12	small nucleolar RNA, C/D box 12
SNORD12B	small nucleolar RNA, C/D box 12B
SNORD12C	small nucleolar RNA, C/D box 12C
KCNB1	potassium voltage-gated channel
PTGIS	member of the cytochrome P450 superfamily of enzymes
B4GALT5	membrane-bound glycoproteins

Supplemental Table 4.

Gene mutations of 68 MF cases with TET2, IDH1/2, DNMT3A, ASXL1, EZH2, SUZ12, BMI1, RCOR1, JAK2, MPL, CBL, SOCS2, LNK, NF1, KRAS, NRAS, PTPN11, PPP1R16B, TRPS1, SF3B1, SRSF2 and TP53 mutations.

Diagnosis	Samples	*TET2* (E3-11)	*IDH1/2* (E4)	*DNMT3A* (E15-23)	*ASXL1* (E12)	*EZH2* (E2-20)	*SUZ12* (E10-16)	*BMI1* (E1-10)	*RCOR1* (E3-12)	*JAK2* (E12 ;14)	*MPL* (E10)	*CBL* (E8-9)	*SOCS2* (E1-2)	*LNK* (E2-8)	*NF1* (E1-58)	*KRAS* (E2-4)	*NRAS* (E1-2)	*PTPN11* (E3;13)	*PPP1R16B* (2-11)	*TRPS1* (E2-7)	*SF3B1* (E15-16)	*SRSF2* (E2)	*TP53* (E5-8)
post-PV MF	HD-0577	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
post-PV MF	HD-0758	wt	wt	wt	**c.1774C>T ;p.Q592X**	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
post-PV MF	HD-1302	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
post-PV MF	HD-1401_0842	wt	wt	wt	**c.2324T>A ;p.L775X$^{N}_{C}$**	**c.446T>G ;p.L149R$^{N}_{C}$**	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	ND	wt	wt
post-PV MF	HD-1426	**c.4462A>T p.K1488X**	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	**c.639C>A;p.S213R**	ND	wt	wt	wt	ND	ND	wt	ND	wt
post-PV MF	HD-1427_1656	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-PV MF	HD-1480	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
post-PV MF	HD-1537	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-PV MF	HD-1559_1649	wt	wt	**c.2711C>T ;p.P904L**	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
post-PV MF	HD-1570bis	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	ND	ND	ND	wt	wt	wt
post-PV MF	HD-1602	**c.1526C>A;p.S509X and c.3594_3594+1G>C**	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	ND	ND	ND	wt	wt	wt
post-PV MF	HD-1664	ND	ND	ND	ND	ND	ND	ND	ND	**p.V617F**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	wt	ND
post-PV MF	HD-1691_0789	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	ND	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	ND	ND	ND	wt	ND	wt
post-PV MF	HD-1813_1836	ND	ND	ND	ND	ND	ND	ND	ND	**p.V617F**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	wt	ND
post-PV MF	HD-1824_0589	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	ND	wt
post-ET MF	HD-0554	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	**c.2098A>G;p.K700E**	wt	wt
post-ET MF	HD-0598	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-ET MF	HD-0599	wt	wt	wt	wt	ND	wt	wt	wt	**p.V617F**	wt	wt	wt	**c.1336A>G;p.I446V**	wt	wt	wt	ND	wt	wt	wt	wt	wt

post-ET MF	HD-0601	**c.5693C>T;p.S1898F**	wt	wt	**c.1888-1909del;p.H630PfsX66**	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
post-ET MF	HD-0614_1112_1352	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-ET MF	HD-0725_1380	**c.3820C>T;p.Q1274X[NC] aND c.4960C>T;p.Q1654X**	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-ET MF	HD-0983	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-ET MF	HD-1264	wt	wt	wt	wt	wt	wt	ND	wt	wt	wt	wt	ND	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
post-ET MF	HD-1309_1741	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	ND	wt	wt
post-ET MF	HD-1360_1605	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
post-ET MF	HD-1376	**c.5618T>C;p.I1873T aND c.5326delT;p.S1776LfsX44**	wt	wt	**c.1934dupG;p.G646WfsX12**	**c.2192A>G;p.Y731C**	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
post-ET MF	HD-1462	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
post-ET MF	HD-1569	wt	wt	wt	wt	ND	wt	ND	ND	**p.V617F**	ND	wt	ND	wt	ND	ND	wt	ND	ND	ND	ND	wt	wt
post-ET MF	HD-1616_0551	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-0497_1307	**c.1996_1997delGA;p.D666PfsX14[NC]**	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0586	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0607_1130	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0616	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0648	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-0679_1291	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-0683	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-0689	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0717_1244	**c.1972delC;p.H658TfsX42**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0719	wt	wt	**c.2644G>A;p.R882S**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	**c.35G>A;p.G12D**	wt	ND	ND	wt	**c.284_307del;p.P95R**	wt
PMF	HD-0728	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-0759	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	wt	ND	wt	wt	**c.665C>T;p.P222L**

PMF	HD-0777	**c.5602C>T;p.H1868Y**	wt	wt	wt	**c.2050C>T;p.R684C**	wt	wt	wt	wt	wt	wt	wt	wt	ND	wt	wt	**c.1471C>T;p.P491S**	ND	ND	wt	wt	wt
PMF	HD-0862	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1047	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1095	wt	wt	wt	wt	wt	wt	wt	ND	**p.V617F**	wt	wt	wt	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1098	wt	wt	wt	wt	ND	wt	wt	ND	wt	wt	wt	wt	wt	ND	wt	wt	ND	wt	wt	wt	ND	wt
PMF	HD-1100	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	**c.734G>A;p.G245D**
PMF	HD-1111	wt	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-1123	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	**c.3790G>A;p.E1264K**^{NC}	wt	wt	wt	ND	ND	wt	**c.283C>G;p.P95A**	wt
PMF	HD-1138_0540_1398	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	wt	wt	wt	**c.1543T>A;p.W515R**^{NC}	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1207	wt	wt	wt	wt	wt	wt	ND	wt	wt	wt	wt	ND	wt	wt	wt	wt	wt	ND	ND	**c.2098A>G;p.K700E**	wt	wt
PMF	HD-1208	wt	wt	wt	wt	**c.2050C>T;p.R684C**	wt	ND	wt	**p.V617F**	wt	wt	ND	**c.764_765delinsG;p.S255X aND c.1566dupC;E523RfsX23**	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1227	wt	wt	wt	wt	wt	wt	ND	wt	**p.V617F**	wt	wt	ND	wt	**c.8499T>G;p.D2833K**^{NC}	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1265_0927_1461_1853	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	**c.284C>A;p.P95H**	wt
PMF	HD-1283	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1289	**c.481_482insT;p.K161IfsX10**^{NC}	wt	wt	**c.1934dupG;p.G646WfsX12**^{NC}	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	**c.1508G>A;p.G503E**^{NC}	ND	ND	wt	wt	wt
PMF	HD-1300_0528_1611	**c.4062delA;p.A1355HfsX8**^{NC} **aND c.1648C>T;p.Arg550X**^{NC}	wt	wt	wt	wt	wt	wt	wt	**p.V617F**	wt	wt	wt	wt	wt	wt	wt	wt	ND	ND	wt	**c.284C>A;p.P95H**	wt
PMF	HD-1362	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1412_1495	wt	wt	wt	wt	wt	wt	ND	ND	wt	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	**c.2111T>G;p.I704S**	wt	wt
PMF	HD-1422	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	wt	wt	ND	ND	wt	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-1439	ND	wt	wt	**c.2077C>T;p.R693X**	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1524bis	wt	wt	wt	**c.1934dupG;p.G646WfsX12**	**c.2036T>A;p.V679E**	wt	ND	ND	wt	wt	**c.1301T>C;p.F434S**	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt

PMF	HD-1538	wt	wt	wt	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt
PMF	HD-1572bis	wt	wt	wt	**c.1934dupG;p.G646WfsX12 aND c.2418_2423delinsG;p.V807CfsX13**	wt	wt	ND	ND	wt	wt	wt	ND	wt	ND	wt	ND	ND	ND	ND	ND	wt	wt
PMF	HD-1587	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt	ND	ND	ND	ND	ND	wt	ND	ND	ND	wt	ND
PMF	HD-1606	wt	wt	**c.2431_2433del;p.D811K**	wt	wt	wt	ND	ND	**p.V617F**	wt	wt	ND	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt
PMF	HD-1617	wt	wt	wt	**c.1860insGGCG p.L622CfsX14**	wt	wt	ND	ND	wt	wt	wt	ND	wt	ND	wt	wt	wt	ND	ND	wt	wt	wt
PMF	HD-1654	ND	ND	ND	ND	ND	ND	ND	ND	wt	wt	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	wt	ND

References for nucleotide and protein sequences
TET2, NM_001127208.1, NP_001120680.1; IDH1, NM_005896.2, NP_005887.2; IDH2, NM_002168.2, NP_002159.2; DNMT3A, NM_022552.3, NP_072046.2; ASXL1, NM_015338.5, NP_056153.2; EZH2, NM_004456.4, NP_004447.2; SUZ12, NM_015355.2, NP_056170.2; BMI1, NM_005180.8, NP_005171.4;
RCOR1, NM_015156.3, NP_055971.2; JAK2, NM_004972.3, NP_004963.1; MPL, NM_005373.2, NP_005364.1; CBL, NM_005188.2, NP_005179.2; SOCS2, NM_001270470.1, NP_001257399.1; NF1, NM_001042492.2, NP_001035957.1; LNK, NM_005475.2, NP_005466.1; KRAS, NM_004985.3, NP_004976.2 and NM_033360.2, NP_203524.1; NRAS, NM_002524.4, NP_002515.1; PTPN11, NM_002834.3, NP_002825.3; PPP1R16B, NM_015568.2, NP_056383.1; TRPS1, NM_014112.2, NP_054831.2; SF3B1, NM_012433.2, NP_036565.2; SRSF2, NM_003016.4, NP_003007.2 TP53, NM_000546.5, NP_000537.3.

MF, myelofibrosis; NC, not constitutional mutation; ND, not determined; PMF, primary myelofibrosis; Post-ET MF, post-essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF; wt, wild-type.

Supplemental Table 5. Summary of all detected CNAs in 14 blast phase MF

Diagnosis	Samples	Karyotype	CNA nb	Deletion/Gain	Chr baND	Max range (bp)	Max size (bp)	Type of CNA	Nb of genes	Log2 ratio	Genes
Blast phase post-PV MF	HD-0492	47 XY,+8[17]/48,idem,+9[3]	1	G	8p23.3-8q24.3	chr8:73810-146294098	146 220 288	1	>200	+0.6	
Blast phase post-PV MF	HD-1517	46,XY[20]	1	D	21q22.11-21q22.12	chr21:34863258-36788958	1 925 700	2	>10	-0.2	*RUNX1*
Blast phase post-PV MF	HD-1649_1559	45,XY,-7[20]	2	D	7	chr7:45130-159118566	159 073 436	1	>250	-1	*EZH2, HOXA9, ZNFN1A1, EGFR, MLL3, IKZF1*
				D	12p12.3-12p13.31	chr12:7695666-15359104	7 663 438	1	>50	-1	*ETV6, CDKN1B, p27*
Blast phase post-PV MF	HD-1691_0789	45,XY,-7,del(20)(q11q13)[20]/46,XY[2]	2	D	7	chr7:45130-159118566	159 073 436	1	>200	-0.2	*EZH2, HOXA9, ZNFN1A1, EGFR, MLL3, IKZF1*
				D	20q12-20q13.2	chr20:40216156-54565054	14 348 898	1	>100	-0.2	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
Blast phase post-PV MF	HD-1836_1813	45,XY,-7[6]/45,sl,del(12)(p12p13)[3]/ 45,sdl1,add(3)(q2?1)[13]	3	D	7	chr:45130-159118566	159 073 436	1	total	-0.8	*EZH2, HOXA9, ZNFN1A1, EGFR, MLL3, IKZF1*
				D	12p13.2-12p12.3	chr:11743077-16162282	4 419 205	2	>10	-0.8	*ETV6, CDKN1B*
				D	17q11	chr:28926042-30195057	1 269 015	2	>5	-0.8	*NF1,SUZ12*
Blast phase post-ET MF	HD-0535	46,XY,t(3;12)(q26-27;q13-14)[20]	5	G	2p25.3-2p14	chr2:30341-66555302	66 524 961	1	>100	+0.4	
				D	2p13.2-2p13.1	chr2:73007094-73763847	756 753	2	9	-0.4	*FBX041*
				D	3q29	chr3:193435136-194860954	1 425 818	2	8	-0.9	*HES1*
				D	6p25.3-6p21.32	chr6:152634-32624845	32 472 211	1	>50	-0.6	*PBX2, DEK*
				D	17q11.1-17q21.33	chr17:25291809-48544433	23 252 624	1	>10	-0.9	*NF1, SUZ12*
Blast phase post-ET MF	HD-0856	46,XX,del(7)(q22q36),del(8)(q22q24)[18]/47,idem,+8[2]	3	G	3q26.2-3q29	chr3:168672810-197895228	29 222 418	1	>50	+0.5	*EVI1*
				D	7q21.2-7q36	chr7:92389957-159118566	66 728 609	1	>50	-0.8	*EZH2, CAV1, MLL3*
				D	8q23.3-q24.22	chr8:113604482-137231948	23 627 466	1	>50	-0.8	*RAD21, MYC*
Blast phase post-ET MF	HD-1167	49,XX,add(3)(p14),del(5)(q31q35),+8,+8 ,+9,?del(11)(p11p13),- 15,add(16)(p12),+mar[20]	13	G	8p23.3-8q24.3	chr8:73810-146294098	146 220 288	1	>200	+1	
				G	9p24.3-9q34.3	chr9:163131-141122114	140 958 983	1	>200	+0.5	*JAK2*
				G	13q31.1-13q34	chr13:83076377-115064121	31 987 744	1	>50	+0.5	
				G	17q23.3-q24.2	chr17:62111720-65955949	3 844 229	2	>20	+0.5	
				D	3p22.3-3p11	chr3:32434406-90309067	57 874 661	1	>30	-1	
				G	4p16.3-4p11	chr4:1589458-49581867	47992409	1	>100	+0.5	
				D	5q11.2	chr5:55877912-56107654	229 742	2	8	-1	
				D	5q11.2	chr5:56201487-57921887	1 720 400	2	5	-1	
				D	5q12.3-5q13.2	chr5:66508627-70587018	4 078 391	1	>10	-1	
				D	5q23.3-5q35.3	chr5:130551687-180712263	50 160 576	1	>100	-1	
				D	11p15.5-11p11.2	chr11:192372-47869001	47 676 629	1	>100	-1	
				D	15q21.3	chr15:56395780-57217890	822 110	2	6	-0.8	*TCF12*
				D	18p11.21	chr18:11649170-12999918	1 350 748	2	>10	-0.8	*PTPN2*
Blast phase post-ET MF	HD-1387	ND	6	G	1q21.1-1q44	chr1:143532171-249224147	105 691 976	1	>100	-0,5	
				G	3q21.3-3q29	chr3:128592775-197895228	69 302 453	1	>100	+0,2	*GATA2, SHOX2*
				D	7q11.21-7q36.3	chr7:61831840-159118566	97 286 726	1	>100	-0,2	*EZH2, CAV1, MLL3*
				G	9p24.3-9p11.2	chr9:163131-47212417	47 049 286	1	>100	+0,5	*JAK2, MLLT3*
				G	9q12-9q34.3	chr9:65632517-141122114	75 489 597	1	>100	+0.2	
				D	11q14.2-11q23.3	chr11:85800206-117441022	31 640 816	1	>50	-0,8	*EED, ARGHEF12, ETS1*
Blast phase post-ET MF	HD-1741_1309	46,XX,- 3,der(5)t(3;5)(q13;q21),del(7)(q21q36),t(9;17)(q11;q11),+21[11]	10	D	3p25.2-3p26.3	chr3:64066-11023254	10 959 188	1	>20	-0.8	*RAD18, EDEM1*
				D	3p26.1-3p25.3	chr3:7300772-11023254	37 22 482	1	>30	-0.8	
				D	3p22.1-3p25.1	chr5:79057767-180712263	101 654 496	1	>30	-0.8	*TGFBR2, FBXL2*
				D	3p12.1-3p21.2	chr3:50719155-88428270	37 709 115	1	>100	-0.8	*DOCK3, FHIT*
				D	5q14.1-3q35.2	chr5:79057767-180712263	101 654 496	1	>200	-0.8	
				D	7q21.11-7q35	chr7:80855869-149015317	68 159 448	1	>200	-0.8	
				D	9p12-9p13.3	chr9:32955146-39254388	6 299 242	1	>50	-0.8	*SNOR12B, PAX5*
				D	12p12.3-12p13.2	chr12:10336209-13445554	3 109 345	2	>20	-0.8	*ETV6, CDKN1B, p27*
				D	17p12-17p13.1	chr17:3792543-12133847	8 341 304	1	>50	-0.8	
				G	21	chr21:10874759-48090317	37 215 558	1	>200	+0.5	*RUNX1*
Blast phase PMF	HD-1611_0528_1300	46,XY,del(20)(q11q13)[12]/ 46,XY,inv(3)(q21q26)[4]/ 46,idem,del(6)(q2?1q2?6)[3]/46,XY[1]	2	D	6q12-6q24.1	chr6:66438112-115733789	49 295 677	1	>200	-0.2	*TNFAIP3*
				D	20q11.22-20q13.12	chr20:33894460-47723303	13 828 843	1	>100	-0.2	*CDR1, CDR2, TOP1, PTPRT, SRSF6, L3MBTL1*
Blast phase PMF	HD-1847	ND	2	D	12p13.2	chr12:11743077-14796474	3 053 397	2	>10	-0.8	*ETV6, CDKN1B*
				D	13q14.11-13q14.3	chr13:41303677-53419331	12 115 654	1	>20	-0.5	*RB1*
Blast phase PMF	HD-1853_0927_1265 _1461	46, XY, +8[16]/46,XY[6]	1	G	8	chr8:73810-146294098	146 220 288	1	>250	+0.2	
Blast phase post-MPN MF	HD-1137	44,XY,add(1)(q4?2),add(3)(p14),del(5)(q 23q34),der(9)t(9;10)(q23;q22),- 10,?dic(15;20)(q12;p12),add(17)(p11)[1 9]/46,XY,del(12)(p11p13)[1]	16	D	3p22.1-3p14.2	chr3:41897844-62216929	20 319 085	1	>50	-0.4	*FHIT*
				D	5q15-5q21.3	chr5:96081976-106522034	10 440 058	1	>10	-0.4	
				D	5q22.2-5q23.2	chr5:111541767-123042265	11 500 498	1	>10	-0.4	
				D	5q23.2-5q32	chr5:125057622-147179938	22 122 316	1	>50	-0.4	
				D	5q34-5q35.3	chr5:167579682-180712263	13 132 581	1	>50	-0.4	
				D	9q33.3-9q34.3	chr9:127413137-140513193	13 100 056	1	>20	-0.4	
				D	10q22.3-10q23.33	chr10:77474006-94633158	17 159 152	1	>20	-0.4	
				D	10q23.33-10q24.32	chr10:95591726-103584723	7 992 997	1	>20	-0.4	
				D	10q24.32-10q25	chr10:104190680-109625441	5 434 761	1	>10	-0.4	
				D	12p11.23-12p13.31	chr12:10357913-27227777	16 869 864	1	>20	-0.4	*ETV6, CDKN1B, p27*
				D	15q13.3-15q21.1	chr15:32928004-47656834	14 728 830	1	>50	-0.4	
				D	17p13.3-17p11.2	chr17:29169-18562710	18 533 541	1	>50	-0.4	
				D	20p11.23-20p12.1	chr20:11982565-18353006	6 370 441	1	>20	-0.4	*MACROD2*
				D	20p13-20p12.3	chr20:60747-6769229	6 708 482	1	>20	-0.4	
				D	20p12.2-20p11.23	chr20:11982565-18353006	6 370 441	1	>20	-0.4	
				G	21q22.13-21q22.3	chr21:39096247-48090317	8 994 070	1	>50	+0.2	

bp, base pairs; CDR, commonly deleted region; Chr, chromosome; CNA, copy number aberrations; D, deletion; G, gain; MF, myelofibrosis; nb, number; ND, not determined; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; post-ET MF, post-essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF.

Supplemental Table 6. Gene mutations of 17 blast phase MF cases with TET2, IDH1/2, DNMT3A, ASXL1, EZH2, SUZ12, BMI1, RCOR1, JAK2, MPL, CBL, SOCS2, LNK, NF1, KRAS, NRAS, PTPN11, PPP1R16B, TRPS1, SF3B1, SRSF2 and TP53 mutations.

Diagnosis	Samples	*TET2* (E3-11)	*IDH1/2* (E4)	*DNMT3A* (E15-23)	*ASXL1* (E12)	*EZH2* (E2-20)	*SUZ12* (E10-16)	*BMI1* (E1-10)	*RCOR1* (3-12)	*JAK2* (E12;14)	*MPL* (E10)	*CBL* (E8-9)	*SOCS2* (E1-2)	*LNK* (E2-8)	*NF1* (E1-58)	*KRAS* (E2-4)	*NRAS* (E1-2)	*PTPN11* (E3;13)	*PPP1R16B* (2-11)	*TRPS1* (E2-7)	*SF3B1* (E15-16)	*SRSF2* (E2)	*TP53* (E5-8)
BP post-PV MF	HD-0492	wt	wt	wt	wt	ND	wt	wt	wt	**80-100**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
BP post-PV MF	HD-1517	ND	ND	ND	**c.1934dupG;p.G646WfsX12**	**c.2077A>T p.N692Y**	ND	ND	ND	ND	wt	ND	ND	ND	ND	ND	ND	wt	wt	wt	ND	wt	wt
BP post-PV MF	HD-1649_1559	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	wt	ND
BP post-PV MF	HD-1836_1813	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BP post-ET MF	HD-0535	wt	wt	wt	**c.2285dupT; p.L762FfsX12**	ND	wt	wt	wt	wt	wt	wt	wt	ND	wt	ND	ND	ND	ND	ND	wt	ND	**c.868C>T p.R290C**
BP post-ET MF	HD-0536	wt	wt	wt	**c.1934dupG; p.G646WfsX12**	**c.1712G>A p.C571Y**	wt	wt	ND	wt	wt	wt	wt	ND	ND	ND	ND	wt	ND	ND	wt	wt	wt
BP post-ET MF	HD-0646	wt	wt	wt	wt	wt	wt	wt	ND	**5-10**	wt	wt	wt	ND	ND	ND	ND	wt	wt	wt	c.**2098A>G; p.K700E**	wt	wt
BP post-ET MF	HD-0856	wt	wt	wt	wt	wt	wt	wt	ND	wt	wt	wt	wt	ND	ND	ND	ND	wt	wt	wt	wt	wt	wt
BP post-ET MF	HD-1167	wt	wt	wt	wt	wt	wt	ND	wt	wt	wt	wt	ND	wt	wt	wt	wt	wt	wt	wt	wt	wt	**c.722C>T p.S241F**
BP post-ET MF	HD-1387	ND	ND	ND	wt	wt	ND	ND	ND	ND	ND	ND	ND	**c.494C>G p.T165S c.544T>C p.F182L**	ND	ND	ND	wt	**c.1606A>C p.K473Q**	wt	ND	wt	wt
BP post-ET MF	HD-1741_1309	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	wt	wt
BP-PMF	HD-0655	wt	wt	wt	wt	wt	wt	wt	ND	**30-50**	wt	wt	wt	ND	ND	ND	ND	wt	wt	wt	wt	ND	wt
BP-PMF	HD-1611_0528_1300	**c.4062delA; p.A1355HfsX8[NC] c.1648C>T p.R550X**	wt	wt	wt	wt	wt	ND	ND	ND	wt	wt	ND	wt	ND	wt	wt	ND	wt	wt	wt	**c.284C>A p.P95H**	wt
BP-PMF	HD-0635	wt	wt	wt	wt	ND	wt	ND	ND	**30-50**	wt	wt	ND	ND	ND	ND	ND	ND	ND	ND	wt	wt	wt
BP-PMF	HD-1847	ND	ND	ND	ND	ND	ND	ND	ND	mutated	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BP-PMF	HD-11853_0927_1265_1461	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BP post-MPN MF	HD-1137	wt	wt	**c.2644G>A ;p.A882S**	wt	wt	wt	ND	wt	wt	wt	wt	ND	wt	wt	wt	wt	wt	ND	ND	wt	wt	**intron 5+1G>A**

References for nucleotide and protein sequences
TET2, NM_001127208.1, NP_001120680.1; IDH1, NM_005896.2, NP_005887.2; IDH2, NM_002168.2, NP_002159.2; DNMT3A, NM_022552.3, NP_072046.2; ASXL1, NM_015338.5, NP_056153.2; EZH2, NM_004456.4, NP_004447.2; SUZ12, NM_015355.2, NP_056170.2; BMI1, NM_005180.8, NP_005171.4;
RCOR1, NM_015156.3, NP_055971.2; JAK2, NM_004972.3, NP_004963.1; MPL, NM_005373.2, NP_005364.1; CBL, NM_005188.2, NP_005179.2; SOCS2, NM_001270470.1, NP_001257399.1; NF1, NM_001042492.2, NP_001035957.1; LNK, NM_005475.2, NP_005466.1; KRAS, NM_004985.3, NP_004976.2 and NM_033360.2, NP_203524.1; NRAS, NM_002524.4, NP_002515.1; PTPN11, NM_002834.3, NP_002825.3; PPP1R16B, NM_015568.2, NP_056383.1; TRPS1, NM_014112.2, NP_054831.2; SF3B1, NM_012433.2, NP_036565.2; SRSF2, NM_003016.4, NP_003007.2 TP53, NM_000546.5, NP_000537.3.

BP, Blast phase; MF, myelofibrosis; NC, not constitutional mutation; ND, not determined; PMF, primary myelofibrosis; Post-ET MF, post-essential thrombocythemia MF; post-PV MF, post-polycythemia vera MF; wt, wild-type.

Supplemental Table 7. Prognostic impact of CNAs and gene mutations (>5%) on Time To Acute Transformation (TTAT) and Overall Survival (OS)

Univariate (log-rang tests) and multivariate (Cox models) analyses

		TTAT-UNIVARIATE				OS-UNIVARIATE				OS-MULTIVARIATE		
	Alterations	**n at risk**	**5 years TTAT %**	**95% CI**	**p**	**n at risk**	**5 years OS %**	**95% CI**	**p**	**HR**	**95% CI**	**p**
CNAs	no yes	3 5	95 80	0.86-1.00 0.64-1.00	0.58	11 12	64 63	0.45-0.9 0.45-0.86	0.89			
del(20q), del(17q), del(12p)	no yes	1 6	96 75	0.88-1.00 0.57-1.00	**0.03**	13 9	72 48	0.56-0.9 0.26-1.00	0.72			
Number of mutations	0-1 mutation > 1 mutation	2 5	100 72	0.79-1.00 0.52-1.00	**0.04**	7 16	80 44	0.65-0.75 0.3-0.75	**0.001**			
Epigenetic-associated genes mutated	no yes	2 5	100 74	0.75-1.00 0.55-1.00	0.10	8 13	72 58	0.55-0.97 0.45-0.88	**0.03**			
ASXL1 mutated	no yes	4 3	92 77	0.81-1.00 0.54-1.00	0.21	13 9	71 48	0.57-0.9 0.33-0.96	**0.02**	2.4	0.94-6.27	0.07
EZH2 mutated	no yes	6 1	91 0	0.81-1.00 0.13-1.00	**0.01**	19 2	69 0	0.59-0.88 NA	**0.003**	6.8	1.11-413.0	**0.04**
TET2 mutated	no yes	6 1	92 80	0.83-1.00 0.52-1.00	0.76	18 3	59 86	0.47-0.82 0.63-1.00	0.59			
DNMT3A mutated	no yes	6 1	90 50	0.8-1.00 0.13-1.00	0.21	20 2	66 33	0.52-0.84 0.07-1.00	0.17			
Splicing-associated gene mutated	no yes	4 2	95 0	0.87-1.00 0.43-1.00	0.07	16 5	69 21	0.54-0.87 0.5-1	**0.004**			
SF3B1 mutated	no yes	6 0	87 NA	0.76-1.00 NA	0.65	19 2	66 0	0.56-0.86 NA	0.17			
SRSF2 mutated	no yes	6 2	94 0	0.88-1.00 0.13-1.00	**0.0002**	21 3	65 0	0.50-0.84 NA	**0.01**	6.2	1.66-23.00	**0.007**
Signaling-associated gene mutated	no yes	0 6	NA 84	NA 0.69-1.00	0.20	1 19	NA 58	NA 0.47-0.79	0.08			
JAK2 mutated	no yes	2 6	94 83	0.84-1.00 0.69-1.00	0.54	NA NA	NA NA	NA NA	NA NA			
LNK mutated	no yes	7 0	88 NA	0.77-1.00 NA	0.67	NA NA	NA NA	NA NA	NA NA			
NF1 mutated	no yes	3 1	86 0	0.7-1.00 NA	0.21	NA NA	NA NA	NA NA	NA NA			

Supplemental Figures

Supplemental Figure 1. Examples of aCGH profiles. A: aCGH profile of chromosome 1 in case HD-1427 showing a deletion including *CSMD2*. **B**: aCGH profile of chromosome 8 in case HD-1362 showing a deletion including *TRPS1.* **C**: aCGH profile of chromosome 14 in case HD-0728 showing a deletion including *RCOR1.* **D**: aCGH profile of chromosome 18 in case HD-0777 showing a gain including *SALL3*.

Supplemental Figure 2. Deduced localization of mutations in our myelofibrosis series (n=68). ASXL1, ASXH: additional sex combs homology domain; PHD: plant homeodomain; NR box: nuclear receptor box. TET2, BOX1 and BOX2: conserved regions 1134-1444, 1842-1921. EZH2, SANT: putative DNA binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB; C-rich: cysteine-rich domain; SET domain: protein lysine methyltransferase enzymes. DNMT3A, PHD: plant homeodomain; PRC2: polycomb repressive complex 2; PWWP: proline tryptophan tryptophan proline. CBL, TKB: tyrosine kinase binding; RF: ring finger; PPP: proline rich region; LZ: leucine zipper; UBA: ubiquitin associated. NF1, Sec: sec14-like; PH: pleckstrin homology. LNK, Pro/DD: proline-rich dimerization domain; PH: pleckstrin homology; SH2: src homology 2. PTPN11, N-SH2 and C-SH2: amino SRC homology 2; PTP: protein tyrosine phosphatase. SRSF2, RRM: RNA recognition motif; RS: arginine/serine domain. SF3B1, HD: heat domains. Triangles represent nonsense mutations, circles frameshift mutations and squares missense mutations.

Supplemental Figure 3. Histogram comparing alterations found in patients studied at diagnosis and during disease course using aCGH (n=63) and sequencing (n=68). CNA: copy number aberration.

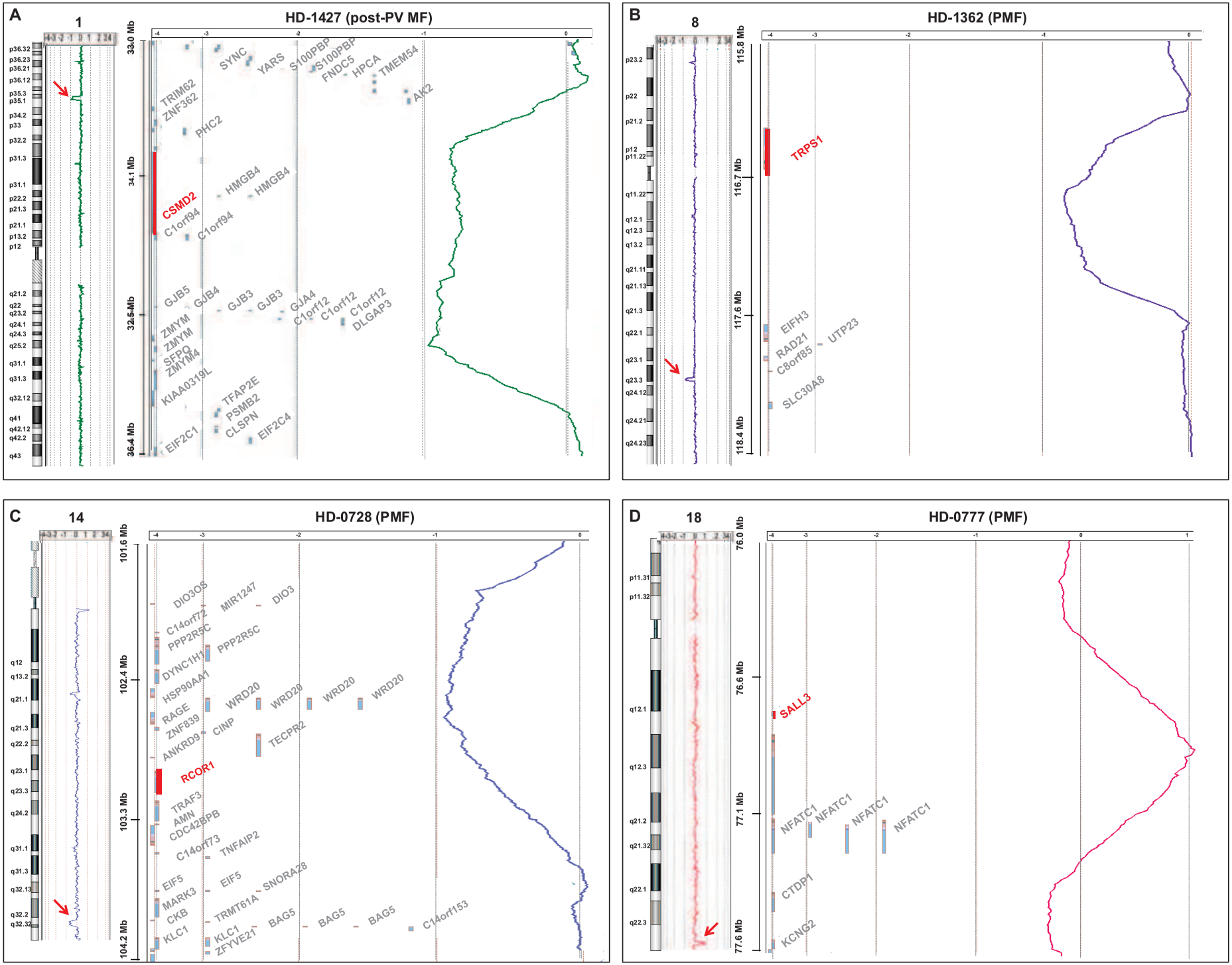
Supplemental Figure 4. Examples of aCGH profiles in blast phase MF. A: aCGH profile of chromosome 12 in 7 samples (HD-1559_1649, HD-1649_1559, HD-1137, HD-1741_1309, HD-1813_1836, HD-1813_1836 and HD-1847) showing recurrent deletions in 12p13 including *ETV6, CDKN1B/p27*. **B**: aCGH profile of chromosome 15 in HD-1167 showing a deletion including *TCF12*. **C**: aCGH profile of chromosome 18 in HD-1167 showing a deletion including *PTPN2*. **D**: aCGH profile of chromosome 21 in HD-1517 showing a deletion including *RUNX1*.

Supplemental Figure 5. Schematic representation of ways to primary or secondary myelofibrosis from a JAK2 mutated or non-JAK2 clone as deduced from the mutation analysis.

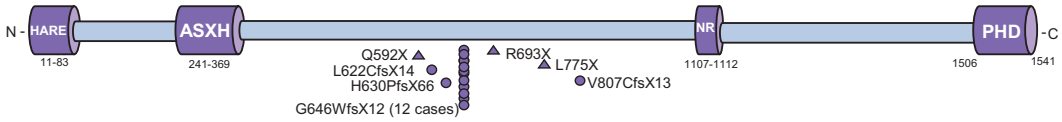
Supplemental Figure 6. Ideogram of the long arm of chromosome 20 comparing deleted regions in myeloid malignancies. Commonly-deleted regions (CDRs) determined in several studies including the present one are shown by

colored rectangles, with position in Mb. The colored lines indicate the deleted region(s) in each patient. aCGH: array comparative genomic hybridization, AML: acute myeloid leukemia, aSNP: array single nucleotide polymorphism, CEL: chronic eosinophilic leukemia, CMML: chronic myelomonocytic leukemia, ET: essential thrombocythemia, FISH: fluorescence in situ hybridization, MDS: myelodysplastic syndrome, MPN: myeloproliferative neoplasm, MF: myelofibrosis, sAML, secondary acute myeloid leukemia, PMF: primary myelofibrosis, PV: polycythemia vera.

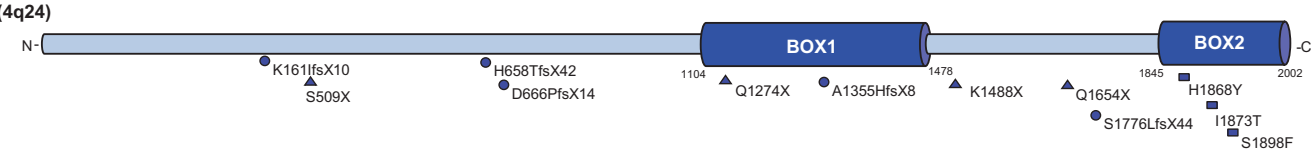
Supplemental Figure 1



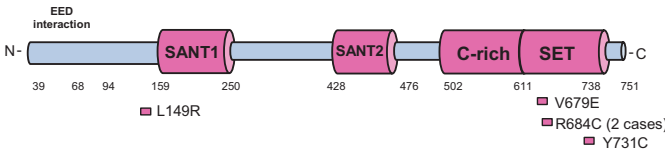
ASXL1 (20q11)



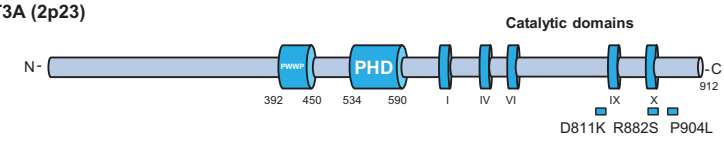
TET2 (4q24)



EZH2 (7q35-q36)

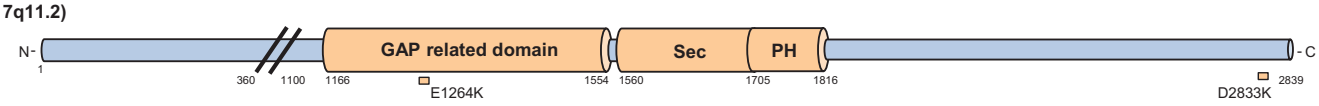


DNMT3A (2p23)

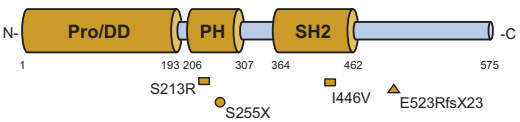


CBL (11q23.3)

NF1 (17q11.2)

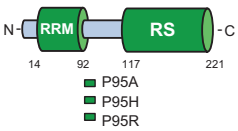


LNK/SH2B3 (12q24)

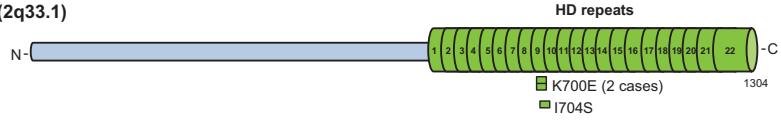


PTPN11 (12q24)

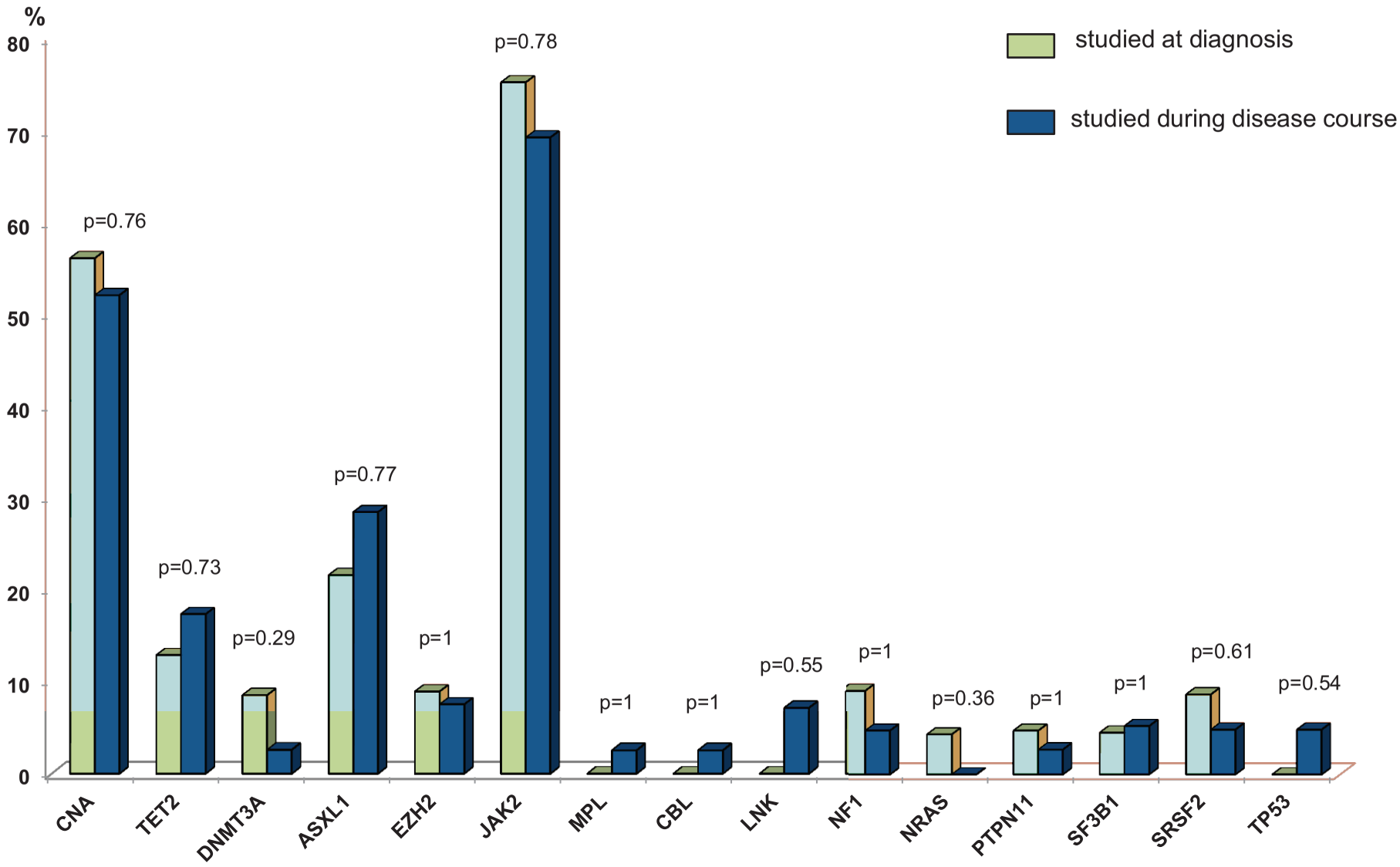
SRSF2 (17q25.1)



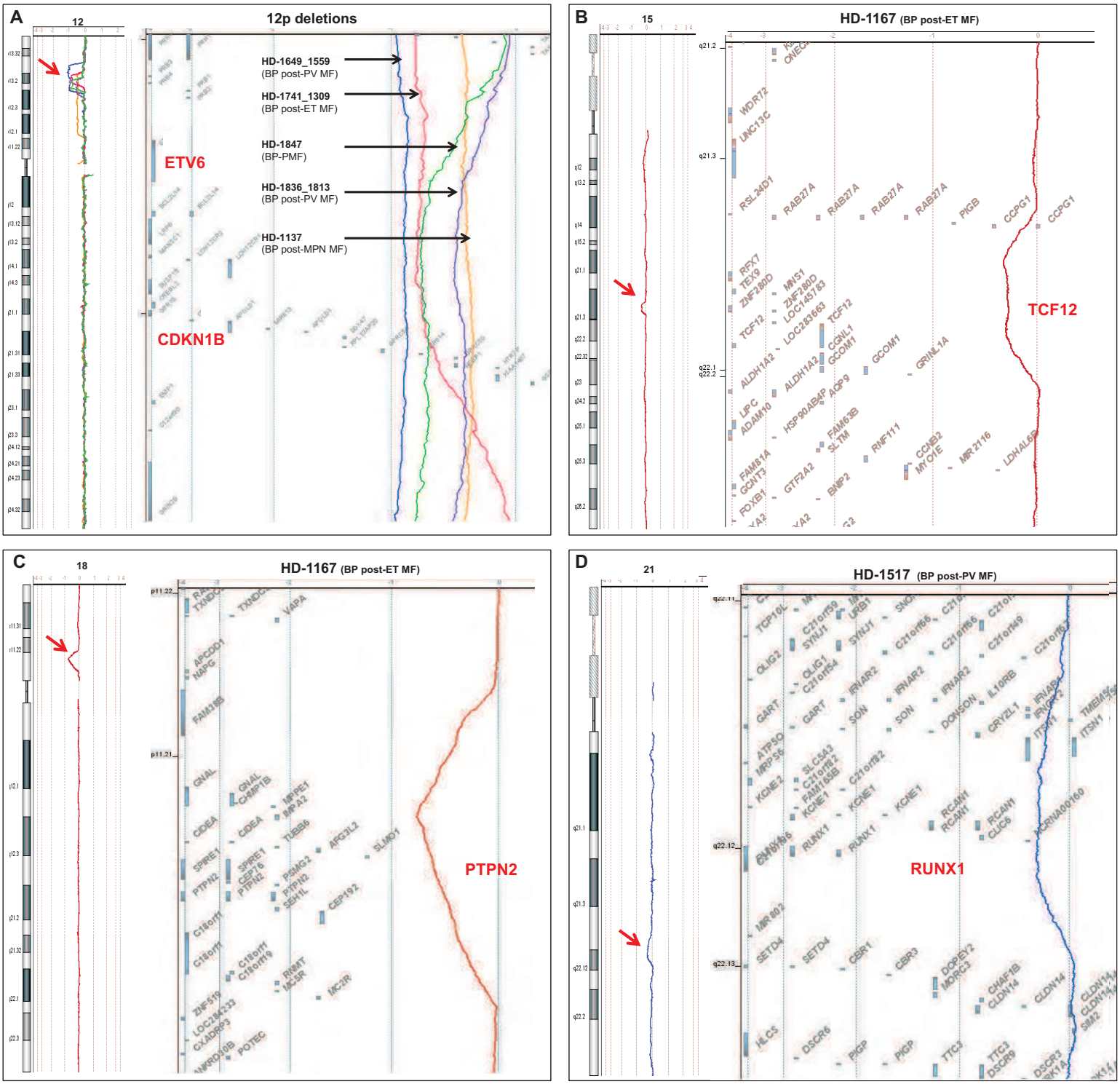
SF3B1 (2q33.1)



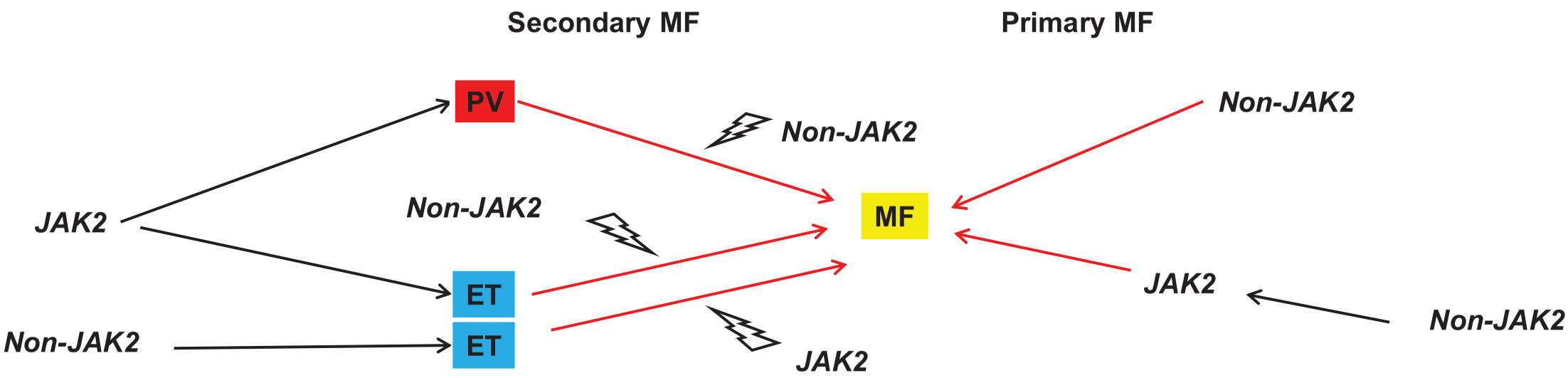
Supplemental Figure 3



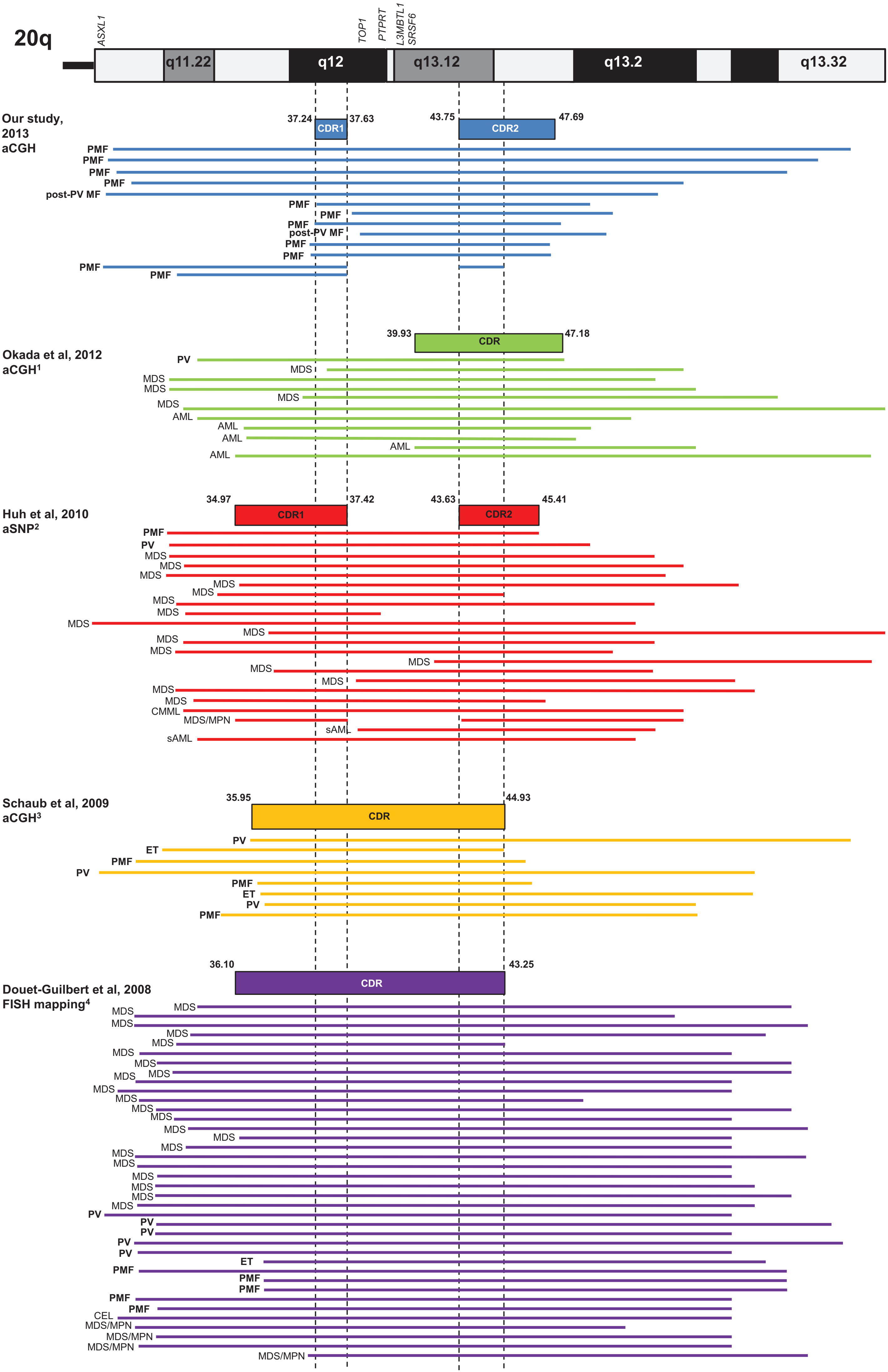
Supplementary Figure 4



Supplemental Figure 5



Supplemental Figure 6



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Résultats non publiés

LAM secondaires aux SMP

Dans le but d'identifier des marqueurs moléculaires impliqués dans la physiopathologie des LAM secondaires aux SMP (post-SMP), nous avons étudié 10 échantillons de LAM post-SMP supplémentaires (8 LAM post-TE, 1 LAM post-PV and 1 LAM post-MFP), en complément des 17 LAM post-MF de l'article 6. Nous avons conjointement mené deux approches : la CGH-array et le séquençage dans le but d'établir un état des lieux des anomalies récurrentes dans les LAM post-SMP.

Ces travaux ont mis en évidence les éléments suivants :
Les résultats de CGH-array ont montré deux anomalies récurrentes : la délétion 12p identifiée dans 7 échantillons et l'altération du chromosome 7 (monosomie 7 ou délétion 7p) identifiée dans 3 LAM post-SMP. La majorité des délétions 12p n'avaient pas été détectées au caryotype ; les gènes *ETV6* et *CDKN1B* y étaient systématiquement inclus.

Compte-tenu des résultats récurrents de CGH-array, nous avons décidé de séquencer les gènes *ETV6* et *CDKN1B* dans une série de 26 LAM post-SMP dont 7 LAM TE, 10 LAM MF post-TE, 5 LAM MF post-PV et 4 LAM post-SMP.

Aucune mutation des gènes *ETV6* et *CDKN1B* n'a été identifiée, suggérant que le mécanisme d'altération n'est pas mutationnel mais seulement la perte d'un allèle, car la délétion n'a été retrouvée qu'à l'état hétérozygote.

Notion d'oligoclonalité

Afin de savoir si les mutations des gènes *ASXL1*, *JAK2* et *SRSF2* du patient HD-1853 (LAM post-MFP avec 40 % de blastes dans la moelle) étaient monoclonales ou oligoclonales, nous avons utilisé les techniques de culture de cellules en milieu liquide (méthylcellulose). Les cellules ont été ensemencées en « duplicate » à 1.10^4 et 5.10^4 cellules pour 1mL de milieu de culture (methocult H4035 optimum without EPO, Stem Cell). Chaque colonie a été dénombrée au 14ème jour et isolée. Puis nous avons séquencé par Sanger les gènes *ASXL1*, *JAK2* et *SRSF2* au sein de 10 colonies formées.

Nous avons identifiées que 5 colonies sur 10 étaient mutées pour les trois gènes *JAK2, ASXL1 et SRSF2*; 3 colonies étaient mutées pour deux gènes : une mutée *JAK2* et *ASXL1*, une autre *JAK2* et *SRSF2* et une mutée *ASXL1* et *SRSF2 ;* une seule colonie était mutée JAK2V617F et une seule colonie était sans mutation (**Table 7**). *A noter qu'au stade chronique (HD-0927, HD-1265, HD-1461) et au stade aigu (HD-1853), les mutations des gènes JAK2, ASXL1 et SRSF2 étaient retrouvées dans les cellules (de sang total) du patient.*

	Diagnostic	Echantillons	Mois (MF)	Caryotype	CGH-array	*JAK2*	*ASXL1*	*SRSF2*
sang total	MFP	HD-0927	<1	nd	No CNA	x	x	x
	MFP	HD-1265	13	nd	délétion 20q11-q13.32	x	x	x
	MFP	HD-1461	8	nd	délétion 20q11-q13.32	x	x	x
	LAM post-MFP	HD-1853	15	46,XY,+8[16]/46,XY[6]	trisomie 8	x	x	x
colonies de moelle totale	LAM post-MFP	HD-1853_B01	15	nd	nd	x	x	x
	LAM post-MFP	HD-1853_C01	15	nd	nd	x	x	x
	LAM post-MFP	HD-1853_A02	15	nd	nd	x	x	x
	LAM post-MFP	HD-1853_A03	15	nd	nd	x	x	x
	LAM post-MFP	HD-1853_D04	15	nd	nd	x	x	x
	LAM post-MFP	HD-1853_B04	15	nd	nd	x	x	wt
	LAM post-MFP	HD-1853_A04	15	nd	nd	x	wt	x
	LAM post-MFP	HD-1853_E02	15	nd	nd	wt	x	x
	LAM post-MFP	HD-1853_B02	15	nd	nd	x	wt	wt
	LAM post-MFP	HD-1853_D01	15	nd	nd	wt	wt	wt

Table 7 : Mutations des gènes ASXL1, JAK2 et SRSF2 au sein de 10 colonies d'un patient atteint de LAM post-MFP

Mois (MF) : correspond au temps (en mois) entre le diagnostic de la MF et l'échantillonnage, nd : not done, wt : wild-type.

Analyse de l'expression des gènes dans 72 SMP non LMC

Afin d'apporter de nouveaux éléments pour la compréhension des liens entre la PV, la TE et la MF, nous avons poursuivi notre analyse génomique intégrée avec l'étude des profils d'expressions génique. Au total, nous avons étudié 93 échantillons, dont 72 SMP (16 PV, 22 TE, 23 MFP, 4 MF post-PV et 7 MF post-TE), 19 formes réactionnelles (FR) (10 polyglobulies réactionnelles (PR) à des insuffisances respiratoires, à des apnées du sommeil, au tabagisme et 9 thrombocytoses réactionnelles (TR) à des syndromes inflammatoires, à des splénectomies, à des carences en fer), ainsi que 2 échantillons de sang normal (NB). Dans un premier temps, nous avons établi la classification des gènes et des échantillons selon leurs similarités d'expression. Dans un second temps, nous avons comparé les formes malignes versus les réactionnelles, et deux à deux les PV, les TE et les MF. Pour finir, nous nous sommes intéressés aux MF primaires versus les MF secondaires. Voici les résultats préliminaires de ces travaux :

A-Analyse de l'expression des gènes au sein des 93 échantillons

Analyse non supervisée: le Clustering Hiérarchique

- **Au niveau de l'axe des abscisses : 93 échantillons**

Selon le spectre de la classification hiérarchique (**Figure 23**), les échantillons se classaient en trois groupes anti-corrélés représentés par les branches I, IIa et IIb. Les échantillons (PV, TE et FR) se regroupaient au sein des clusters I et IIb, suggérant la proximité d'expression génique entre les polyglobulies et thrombocytoses quel que soit le mécanisme de survenue (primitif ou réactionnel).

Par ailleurs, la quasi-totalité des MF (MFP et MF secondaires) se regroupaient dans le cluster IIa, témoignant que les MF sont différentes des PV/TE et relativement homogènes; et aussi que les MF post-PV/TE sont éloignées de leur maladie d'origine mais très proches des MFP.

- **Au niveau de l'axe des ordonnées : les gènes**

Quatre clusters anti-corrélés définissaient majoritairement l'ensemble de la classification des échantillons :

Le **cluster A** qui était sous-exprimé dans les MF, regroupait les gènes impliqués dans les voies de signalisations des chimiokines (*VAV3*, *FOX3*), dans les voies de la machinerie d'épissage (*DHX8, SF3A1, SF3A2, SF3B4*) et dans la voie du TGFβ (*SMAD3, TGFβ1).*

Le **cluster B** le plus grand, était associé aux voies de signalisation FAS (*RB1, PAK1, PAK2*), JAK/STAT (*JAK2, STAT2, STAT5B, FGR, SOS1, SOS2*), NOTCH (*NOTCH1, NOTCH2, PSEN1, PSEN2*), MAPK (*MAPK1, MAPK14, PTPN11, PIK3CA*), et comprenait des gènes impliqués dans les LAM (*TP53, MYC, BCL2L1, TGFBR1, TGFBR2)*.

Le **cluster C**, le plus petit, montrait une surexpression dans les MF des gènes impliqués dans les voies de signalisation du récepteur NOD-like *(IL6, TNF, NFKB1A, NFKB1B, BIRC3, TNFAIP3*), TGFβ (*BMP2, SMAD7, SMURF1, SKP1, CUL1, TFDP1*) et des gènes impliqués dans les LAM (*KRAS, BRAF, PIK3CB, ARAF, PIM1, PIM2, PML, RUNX, BAD, STAT3*)

Le **cluster D** qui était surexprimé dans les MF, contenait les gènes de la prolifération et du cycle cellulaire (*CDK1, CDK2, CCNE1, CCNB1, CHECK1*), ainsi que les gènes de la réplication de l'ADN (*DNA2, MCM2, MCM4, MCM7*).

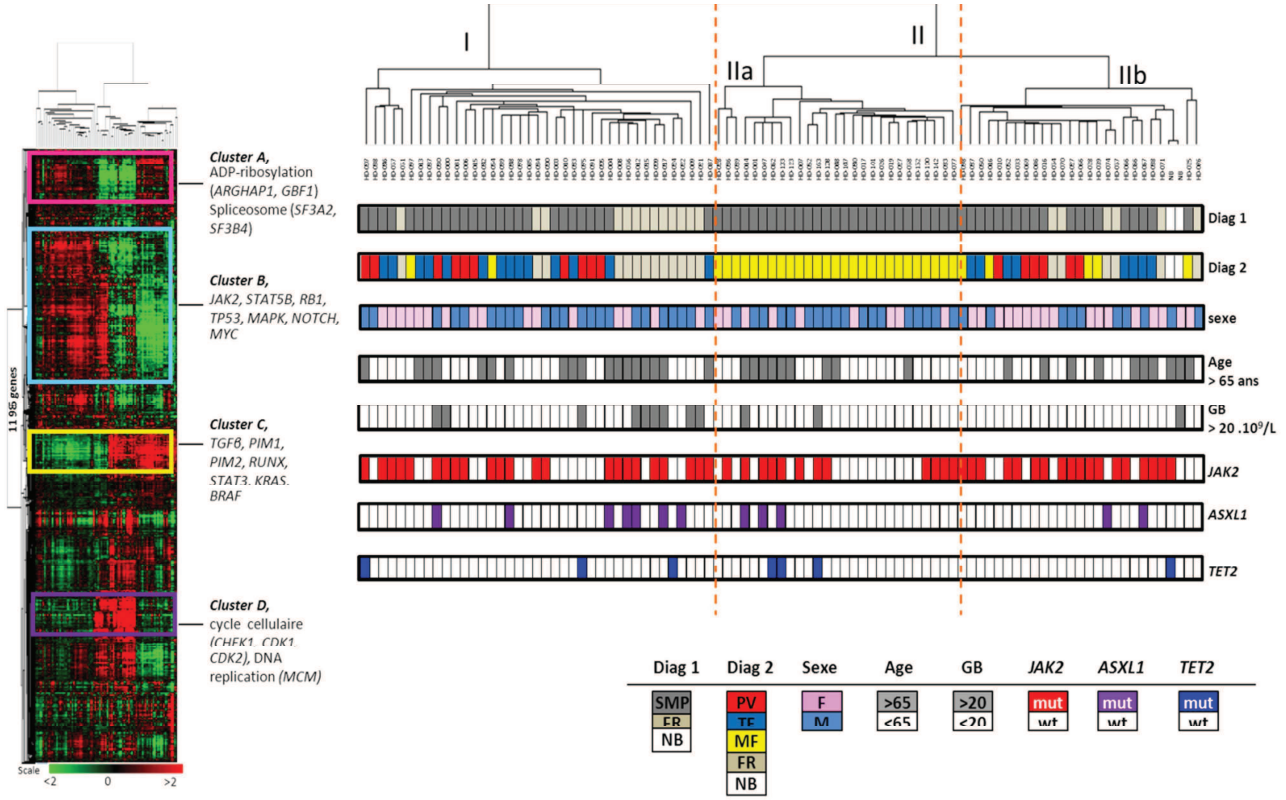

Figure 23 : Classification hiérarchique des 72 SMP non LMC, des 19 formes réactionnelles et des 2 normaux

Le diagnostic 1 correspond à la distinction des SMP (gris foncé) des formes réactionnelles (gris clair) et le diagnostic 2 à celui des sous-types de syndrome myéloprolifératifs (voir code couleur ci-dessus). Diag : diagnostic, F : féminin, FR : forme réactionnelle, M : masculin, MF : myélofibrose, mut : muté, NB : normal blood, PV : polyglobulie de Vaquez, SMP : syndrome myéloprolifératif, TE : thrombocytémie essentielle, wt : wild-type.

Analyse de l'expression des gènes *JAK2*, *STAT3* et *STAT5B*

Tous les échantillons de SMP et les formes réactionnelles surexprimaient les gènes *JAK2*, *STAT5B*, et sous-exprimaient le gène *STAT3* par rapport aux deux échantillons de sang normal (NB), témoignant de l'implication de la voie JAK/STAT quel que soit le type de polyglobulies, de thrombocytoses (primaire ou réactionnelle) et le statut mutationnel *JAK2* (**Figure 24A**). Toutefois, chez les mutés *JAK2*, l'expression des gènes *JAK2* et *STAT5B* était plus importante, l'expression de *STAT3* plus faible comparé aux non-mutés *JAK2*, suggérant l'existence d'un lien entre l'activation constitutive de la voie JAK/STAT induite par la mutation JAK2V617F (**Figure 24B**).

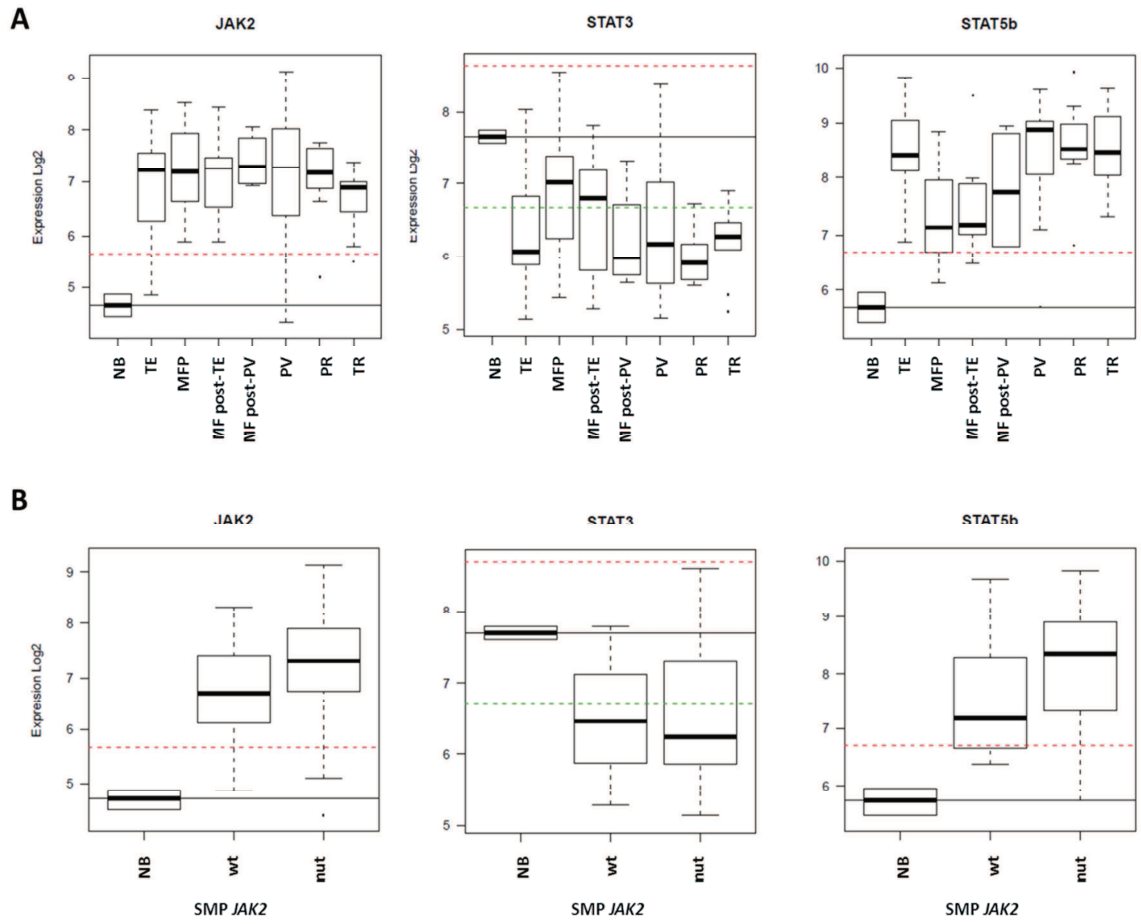

Figure 24 : Box-Plots corrélant l'expression et le statut mutationnel JAK2 dans les 72 SMP et les 19 FR par rapport à la normale.

A : box-plots en fonction des sous-types SMP et des formes réactionnelles. B : box-plots dans les SMP en fonction du statut mutationnel de *JAK2*. MF : myélofibrose, mut : muté, NB : normal, PV : polyglobulie de Vaquez, SMP : syndrome myéloprolifératif, TE : thrombocytémie essentielle, wt : wild-type.

B-Analyse de l'expression des gènes au sein des 72 SMP classiques non-LMC

Analyses supervisée: PV vs TE vs MF

De façon à mieux situer les trois sous-types (PV, TE et MF) entre eux, nous avons réalisé trois analyses supervisées.

Les résultats des analyses supervisées réalisées deux à deux sont i) PV (n=16) vs TE (n=22) : 17 gènes ; ii) PV (n=16) vs MF (n=34) : 1697 gènes ; iii) TE (n=22) vs MF (n=34) : 1360 gènes. Au final, nous avons identifié 52 gènes discriminants entre les PV, les TE et les MF. Parmi ceux-ci se trouvent *PRV1* et *MPL*.

Que ce soit en utilisant la classification hiérarchique ou l'analyse en composante principale (PCA) (**Figure 25**), il existe une signature moléculaire propre aux MF qui se regroupent ensemble.

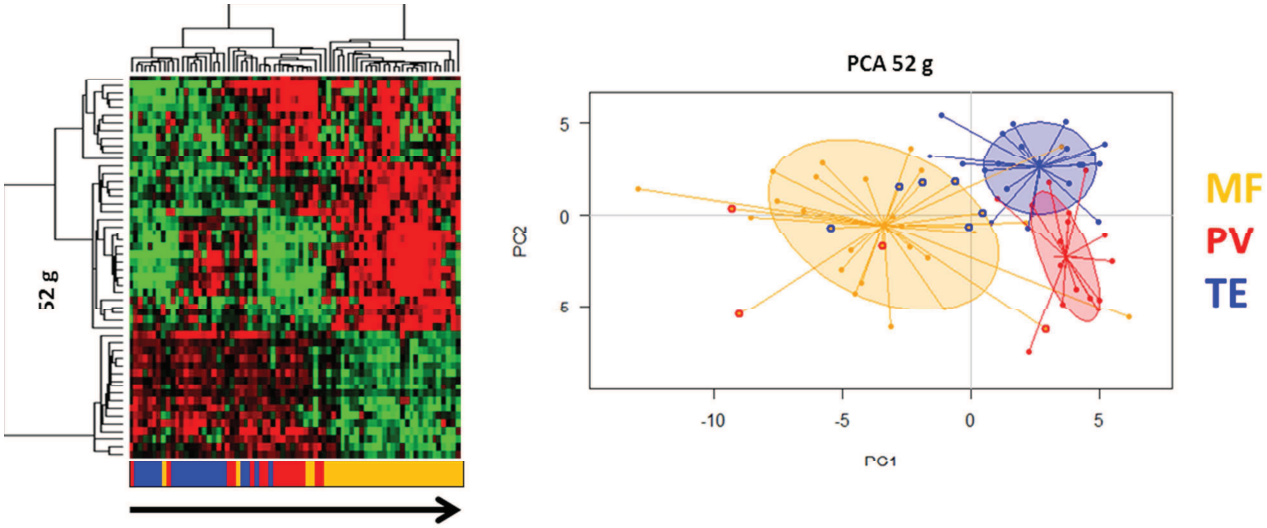

Figure 25 : Analyse de l'expression des 52 gènes selon le type de SMP non LMC et PCA

Les trois analyses supervisées entre les sous-types PV, TE et MF ont permis d'identifier 52 gènes discriminants. Ces derniers ont permis de reclasser les échantillons selon leur sous-type (à gauche, en bleu les TE, en rouge les PV et en jaune les MF) et de construire l'analyse en composante principale (à droite). Ces deux études témoignent de la signature propre des MF qui se regroupent ensemble suggérant un mécanisme de continuum de la TE, à la PV jusqu'à la MF.

C-Analyse de l'expression des gènes au sein des 34 MF

Selon le spectre de la classification hiérarchique (**Figure 26**), les échantillons de MF, qu'ils soient primaire ou secondaires, se classaient de façon homogène. De plus l'analyse supervisée réalisée entre les MF primaires (n=23) et les MF post-PV/TE (n=11) n'a révélé aucun gène différentiellement exprimé entre ces deux groupes. Les profils d'expression génique des MFP et des MF secondaires sont proches et ne permettent pas de distinguer les formes primaires des secondaires.

La surexpression du gène *PIM1*

De façon intéressante, nous avons identifié une surexpression du gène *PIM1* dans les MF primaire et secondaire. *PIM1* appartient à la famille des sérine/thréonine kinase PIM, qui contient deux autres membres. Les PIM jouent un rôle dans la transduction du signal des cellules sanguines via les oncogènes ABL, JAK2 et FLT3. Elles sont de ce fait impliquées dans la prolifération et la survie cellulaire. Des études ont montré que la surexpression des kinases *PIM* conduit à une résistance aux inhibiteurs de mTOR (rapamicine) (Swords *et al.* 2011). *De nouvelles molécules inhibitrices de ces kinases sont en cours d'étude dans les hémopathies myéloïdes et en particulier dans les LAM* (Chen *et al.* 2011; Yuan *et al.* 2013).

Analyses supervisée selon les scores IPSS, DIPSS, DIPSSplus dans les MFP

De façon à mieux situer les MFP de mauvais pronostic, nous avons réalisé trois analyses supervisées dans les MFP selon les scores IPSS, DIPSS et DIPSSplus.

Ces trois analyses supervisées ont conduits à une liste de 479 gènes communs aux trois analyses. Cette liste permet de discriminer le groupe 1 (« high »-« intermediate-2 ») du groupe 2 (« low »-« intermediate 1 »). Selon l'analyse ontogénique faites par le logiciel *DAVID*, les gènes surexprimés dans le groupe 1 étaient associées aux fonctions cellulaires telles que l'oxydoréduction et à la régulation des peroxysomes. Les gènes surexprimés dans le groupe 2 étaient impliqués dans les processus liés à l'inflammation.

Discussion générale

Le travail présenté dans ce manuscrit de ***« caractérisation moléculaire des syndromes myéloprolifératifs non leucémie myéloïde chronique »*** *a permis d'amorcer les réponses à plusieurs questions soulevées dès 2009.*

Existent-ils d'autres acteurs que JAK2 impliqués dans la signalisation des facteurs de croissance hématopoïétiques ?

Au sein de la voie JAK/STAT, des délétions des gènes *SOCS* et de rares mutations des gènes *CBL*, *PTPN11*, et *LNK,* non exclusives de JAK2V617F, ont été retrouvées lors de nos travaux (Brecqueville *et al.* 2013) et complètent ceux de précédentes études publiées (Grand *et al.* 2009; Tartaglia *et al.* 2003; Pardanani, Lasho, Finke, Oh, *et al.* 2010) (**Figure 26**). De plus, ces études ont permis d'identifier, d'autres voies de signalisation cellulaire également affectées dans les SMP, notamment la voie RAS-MAPK (ex : délétions et mutations du gène *NF1,* mutations des gènes *K* et *NRAS*). Toutefois, certaines anomalies (*CBL*, *PTPN11* et *NF1)* ne sont pas spécifiques des SMP puisque celles-ci sont également retrouvées dans d'autres hémopathies myéloïdes chroniques telles que les SMD, LMMC, LMMJ et LAM.

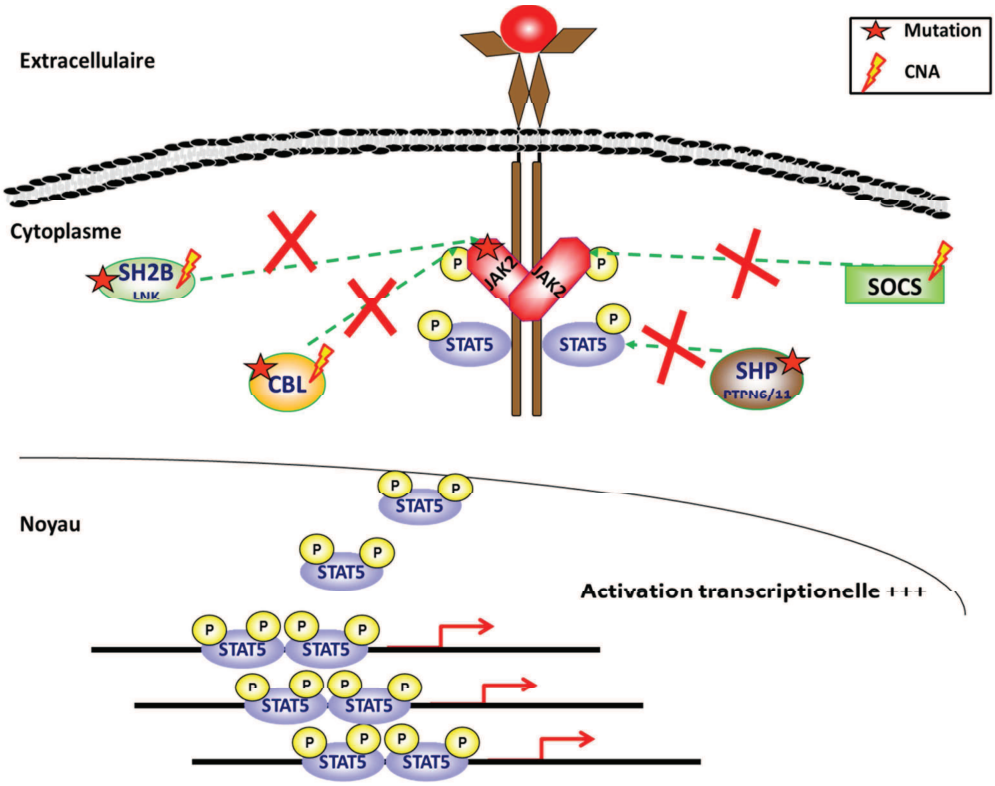

Figure 26 : Schéma des altérations possibles des acteurs de la voie JAK/STAT (en dehors de JAK2)

Quels sont les autres fonctions cellulaires altérées ?

Les voies de signalisation ne sont pas les seules impliquées dans les SMP, d'autres processus tels que la régulation épigénétique (pour revue Shih *et al.* 2012) et l'épissage des ARN (pour revue Damm *et al.* 2012) y participent également. Schématiquement, il semblerait qu'il y ait cinq classes de gènes (regroupés selon leur fonction cellulaire) associés aux SMP. Les cinq classes regroupent les gènes de la signalisation cellulaire (*JAK2, MPL, NF1, PTPN11, NRAS, CBL, LNK,* …), des facteurs de transcription (*ETV6,* ..), des régulateurs épigénétiques (*ASXL1, RCOR1, EZH2, EED, SUZ12, BMI1, DNMT3A, IDH1/2, TET2,* …), des gènes suppresseurs de tumeurs (*CDKN1B, TP53,* …) et des membres de la machinerie d'épissage (*SF3B1, SRSF2,* …)(**Figure 27**).

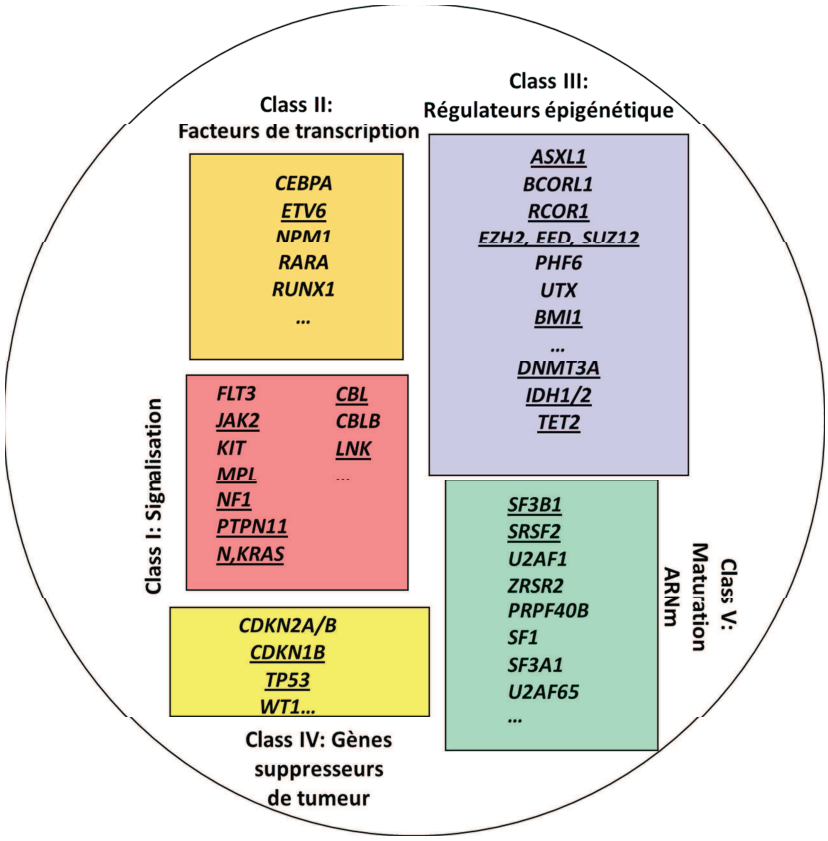

Figure 27: Schéma des cinq classes de gènes impliquées dans la leucémogénèse

(Murati, Brecqueville *et al.* 2012). A l'heure actuelle, ce schéma peut être universel à la leucémogenèse des hémopathies malignes myéloïde. Les gènes soulignés sont spécifiquement altérés dans les SMP. Les régulateurs épigénétiques (classe III) peuvent être classés en deux groupes (associés à la méthylation de l'ADN et associés aux modifications covalentes des histones).

Quelle est la proportion des gènes mutés dans les PV, TE et MF ?

Selon les résultats de nos études, la proportion des gènes mutés diffère au sein des trois sous-types : PV, TE et MF (**Figure 28**). Dans la **PV**, la plupart des patients sont mutés JAK2V617F (97%). Des mutations plus rares sont retrouvées dans les gènes *TET2* (10%), *ASXL1* (7%), *SUZ12* (3%) *et DNMT3A* (3%) et/ou des CNA telles que la délétion (20q) (9%). Dans la **TE**, les patients mutés JAK2V617F (55%) peuvent également présenter des mutations rares de *TET2* (8%), *ASXL1* (4%), *CBL* (2%) et *SF3B1* (2%). Dans la **MF**, les patients portent de nombreuses altérations (mutations et CNA), témoignant de la complexité moléculaire de ce sous-type. Les altérations impliquent plusieurs voies (signalisation, épigénétique et épissage) et plusieurs membres qui peuvent ou non être associés à la mutation JAK2V617F. Les mutations d'*ASXL1* (26%), *TET2* (14%), *EZH2* (8%) sont les plus fréquentes après la mutation JAK2V617F (69%).

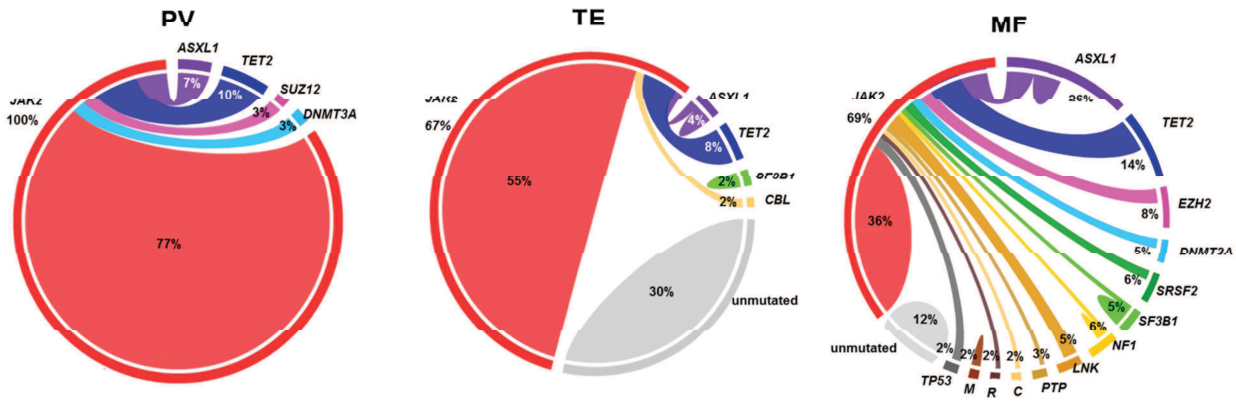

Figure 28 : Représentation sous forme de circos des gènes mutés dans la PV, la TE et la MF. (Brecqueville *et al.* 2013)

Existent-t-ils des anomalies spécifiques d'un sous-type de SMP?

Nous avons identifié que plusieurs mutations sont récurrentes dans les sous-types SMP. Celles des gènes *SUZ12* et *DNMT3A* ne sont identifiées que dans la PV et la MF (**Figure 28**), celles des gènes *SF3B1* et *CBL*, que dans la TE et la MF (**Figure 28**). *A noter que les mutations*

de SF3B1 ont également été décrites dans les anémies réfractaires avec sidéroblastes en couronne (RARS) et thrombocytose marquée (RARS-T) (Papaemmanuil *et al.* 2011). Ces données suggèrent un lien entre les mutations du spliceosome et la prolifération de la lignée des mégacaryocytes. De plus, les mutations du gène *ASXL1* sont significativement plus récurrentes dans la MF (**Figure 29)**, alors que celles du gène *TET2* sont réparties de façon homogène au sein des trois sous-types.

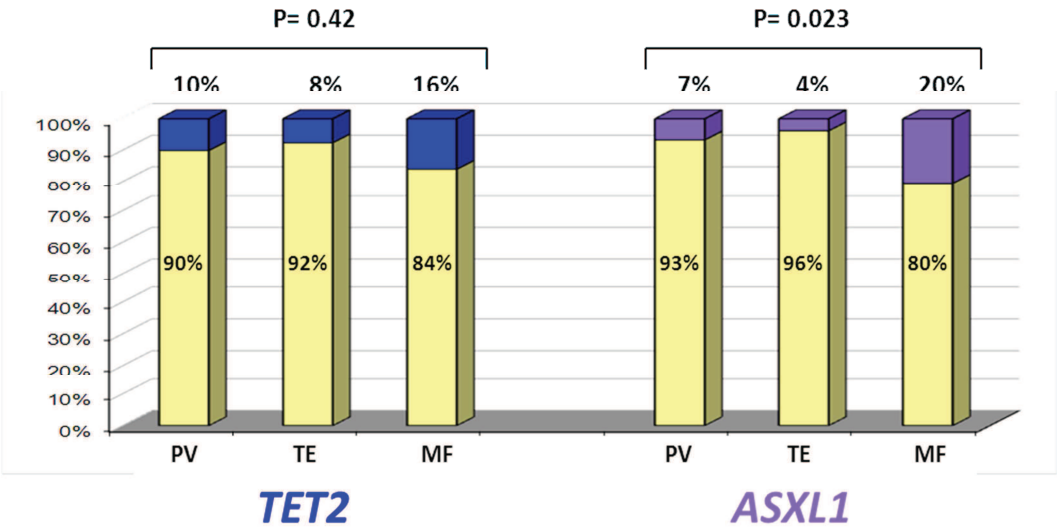

Figure 29 : Répartition des mutations des gènes ASXL1 et TET2 dans les PV, TE et MF

(Brecqueville *et al.* 2012) Parmi les SMP mutés *ASXL1*, les MF sont significativement plus mutées que les PV ou TE (P=0.023), ce qui n'est pas le cas des patients mutés *TET2* (P=0.42).

Quelles sont les particularités moléculaires dans les myélofibroses ?

La complexité moléculaire de la MF a été confirmée, du fait du nombre important de mutations (84%) et CNA (54%) retrouvées. Dans la MF qu'elle soit primaire ou secondaire à une PV/TE, nous avons remarqué plusieurs associations possibles de mutations des gènes impliqués dans la signalisation cellulaire (*CBL, JAK2, MPL, NF1, RAS, PTPN11*), dans les régulations épigénétiques (*ASXL1, SUZ12, DNMT3A, IDH1/2, TET2*) ainsi que dans la machinerie d'épissage (*SRSF2*, *SF3B1*) (**Figure 30**). Par ailleurs, plusieurs mutations au sein d'une même classe de gènes (*ASXL1* et *TET2*) peuvent être associées (Brecqueville *et al.* 2013) , ce qui n'est pas le cas dans les PV et TE.

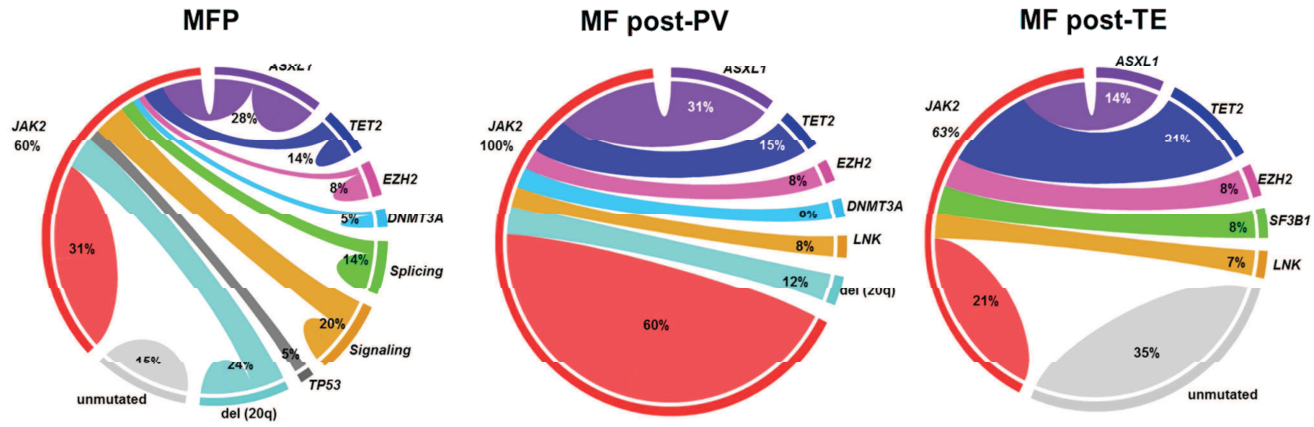

Figure 30 : Représentation sous forme de circos des gènes mutés dans les trois sous-types de MF (MFP, MF post-PV, MF post-TE)

(Brecqueville *et al.* 2013). Le ruban représentant la voie de la maturation des ARN ("splicing") est composé des mutations des gènes *SF3B1* et *SRSF2* ; celui qui représente la signalisation cellulaire ("signaling") est composé des mutations des gènes *CBL, LNK, MPL, NF1, NRAS,* et *PTPN11*.

Comment expliquer l'évolution des PV/TE en MF?

Nos observations suggèrent que différentes voies moléculaires peuvent conduire à la transformation en MF (**Figure 31**) :

1-Il n'y avait aucune différence en termes d'expression des gènes entre MF primaire et secondaire. De même il n'y avait aucune différence dans le nombre total de CNA entre MF primaire et secondaire. Toutefois dans les MF secondaires, les co-mutations étaient toujours associées à la mutation JAK2V617F alors que ce n'était pas le cas pour la MFP.

2-Les patients atteints MF post-TE/PV peuvent avoir acquis une mutation d'un gène impliqué dans la régulation épigénétique ou dans l'épissage associé à la mutation JAK2V617F isolée, alors que les patients atteints de MFP, ne portent pas toujours la mutation JAK2V617F (**Figure 30**).

3-Que ce soit dans les MF primaires ou secondaires, certains patients ne sont mutés que JAK2V617F.

4-Pour 35% des patients atteints MF post-TE et 15% des patients atteints de MFP, nous n'avons identifié aucune mutation. Le pourcentage de MF post-TE sans mutation et CNA

est à peu près similaire à celui trouvé dans TE. Cette observation soulève la problématique non résolue des TE et des MF post-TE non mutées.

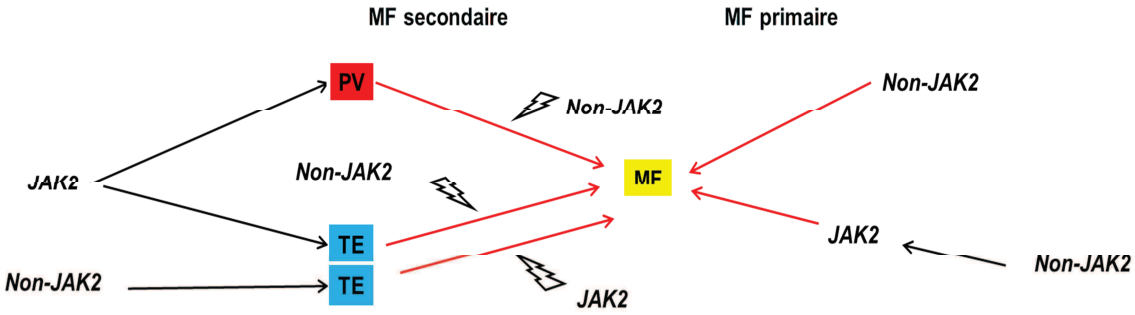

Figure 31 : Schéma des voies de MFP et MF post-PV/TE des clones JAK2 muté ou non muté

(Brecqueville *et al.* 2013). Ce schéma illustre les possibles voies pouvant conduire à l'apparition d'une MF primaire et à la transformation d'une PV ou d'une TE en MF secondaire.

Existent-t-ils des anomalies spécifiques d'une évolution en LAM ?

Chez les patients atteints de LAM, les cellules à l'origine de la tumeur semblent dériver de progéniteurs ou de CSH qui ont conservé leur potentiel d'auto-renouvellement mais ont perdu leur capacité de différenciation. Les SMP se transforment dans près de 5 à 10% des cas en LAM. La transformation leucémique est plus fréquente chez les patients atteints de MFP (8-23%) que chez les patients atteints de TE (0,5-1%) et PV (1-4%) au cours des 10 premières années après le diagnostic (Abdulkarim *et al.* 2009).

Les anomalies moléculaires qui participent à la leucémogenèse peuvent intéresser soit un gène (par exemple les mutations, ou les méthylations de promoteurs) soit des portions entières de chromosome (translocation, inversion, délétion, amplification).

Nos études des LAM post-SMP ont permis d'identifier des mutations prédominantes dans les formes aiguës (*TP53, DNMT3A, IDH1/2*) à l'instar des formes chroniques (*JAK2*) (**Figure 32**).

Nous avons également retrouvé une fréquence élevée de délétion 12p par CGH-array dans les LAM post-MF (25% des cas) alors que sa fréquence habituelle dans les LAM et les SMD

n'est que de 5% (Andreasson *et al.* 1997). De plus, nous avons montré que dans certains cas de SMP, lorsque le caryotype révèle une monosomie 7 en phase chronique, une analyse par CGH-array peut permettre l'identification d'une délétion 12p associée. Cette information pourrait aider à la décision thérapeutique si l'on tient compte du fait que la délétion 12p est de mauvais pronostic dans les MF (Tam *et al.* 2009; Tefferi *et al.* 2001; Caramazza *et al.* 2011) et que nous l'avons retrouvée dans 25% des LAM post-SMP.

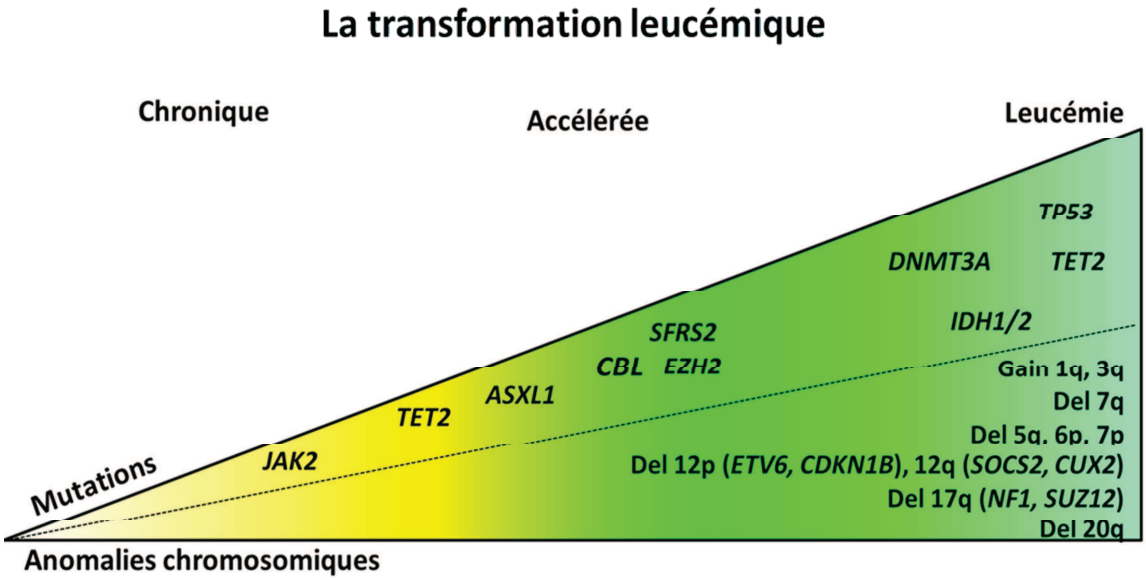

Figure 32 : Schéma des anomalies au cours de la transformation LAM post-SMP

Selon les résultats de nos études, certaines mutations sont spécifiques des phases chroniques, alors que d'autres des phases aigues.

Quelles sont les combinaisons des gènes mutés dans les LAM post-SMP ?

Nos résultats soutiennent le développement d'une LAM post-SMP suivant un modèle de machine à sous ("slot machine") (**Figure 33**). Dans ce modèle, la LAM post-SMP résulterait d'une combinaison d'au moins quatre altérations coopératrices (bobines). Les altérations pourraient affecter des FT, des molécules de signalisation cellulaire, des régulateurs épigénétiques, des gènes suppresseurs de tumeurs (TSG) et des membres de la machinerie d'épissage. A noter qu'au stade chronique, les étapes se combinent de façon variable.

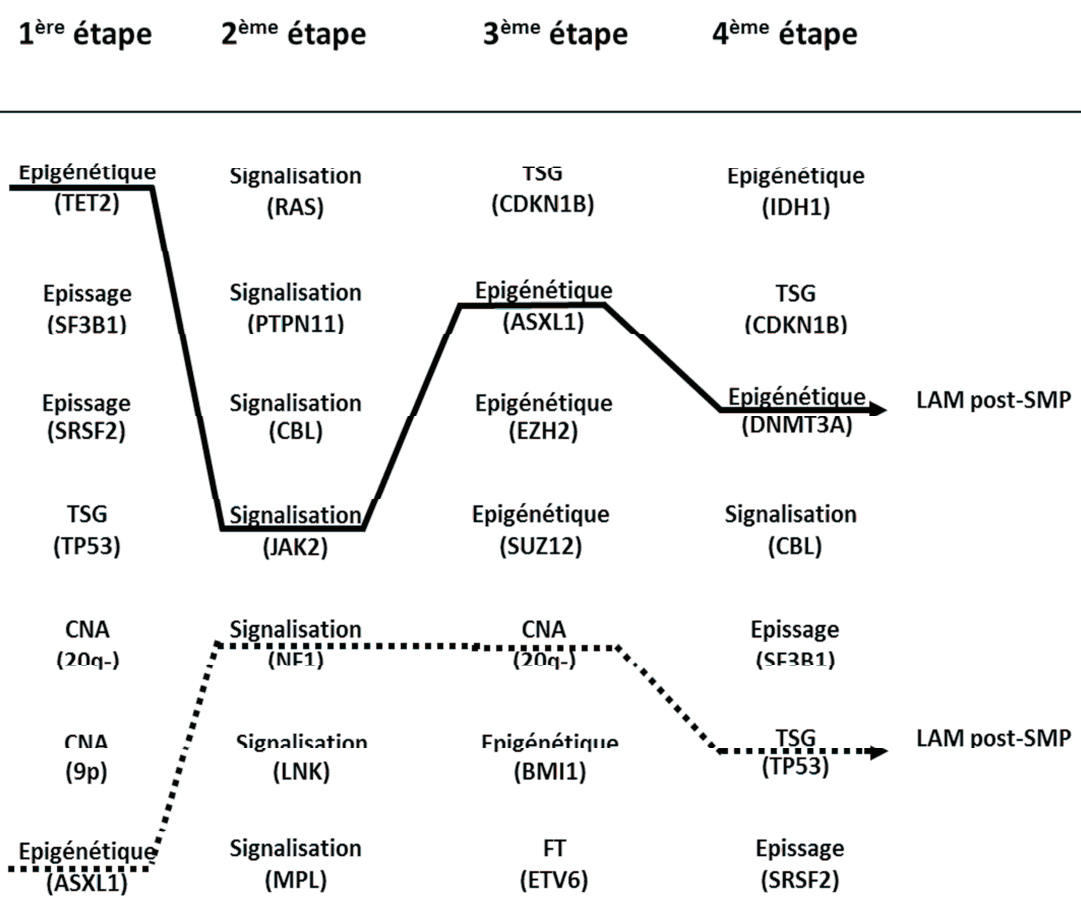

Figure 33 : Modèle de leucémogenèse façon « machine à sous »

D'après (Murati, Brecqueville et al. 2012). Dans ce schéma, la LAM résulte d'une combinaison d'au moins quatre altérations (bobines). *Deux exemples de tirage menant à la LAM post-SMP sont représentés par les traits plein et pointillé.*

Quel modèle proposé pour expliquer la physiopathologie des SMP ?

Les anomalies moléculaires décrites à ce jour témoignent de la complexité moléculaire et du caractère évolutif au cours du temps d'une même maladie. Ceci nous conduit à la notion de « continuum » avec le passage d'une forme à l'autre suivant l'acquisition d'anomalies moléculaires (**Figure 34**).

Dans le schéma ci-dessous, la présence d'haplotype de susceptibilité y est ajoutée. En effet, à ce jour ceux-ci sont décrits comme participant à la physiopathologie des SMP :

i) **L'haplotype de susceptibilité JAK2 46/1** (rs12343867 retrouvé plus fréquemment dans les formes familiales et sporadiques des SMP que dans la population générale (Pardanani, Lasho, Finke, Gangat, *et al.* 2010; Olcaydu *et al.* 2009; Jones *et al.* 2009). Il entrainerait un risque 3 à 4 fois plus élevé de survenue de la mutation JAK2V617F et également une susceptibilité à développer une thrombocytose indépendamment du statut JAK2.

ii) Le **polymorphisme A3669G** (rs6198) du gène *GR* (récepteur glucocorticoïde) décrit comme facteur de prédisposition de l'érythrocytose dans les PV (Varricchio *et al.* 2011).

iii) Le **polymorphisme du gène *XPD*** (Xeroderma Pigmentosum group D) semble être un facteur de risque de transformation en LAM chez les patients atteints de TE et PV (Hernández-Boluda *et al.* 2012) ; par contre, dans les MFP, ce résultat n'est pas retrouvé (Poletto *et al.* 2013).

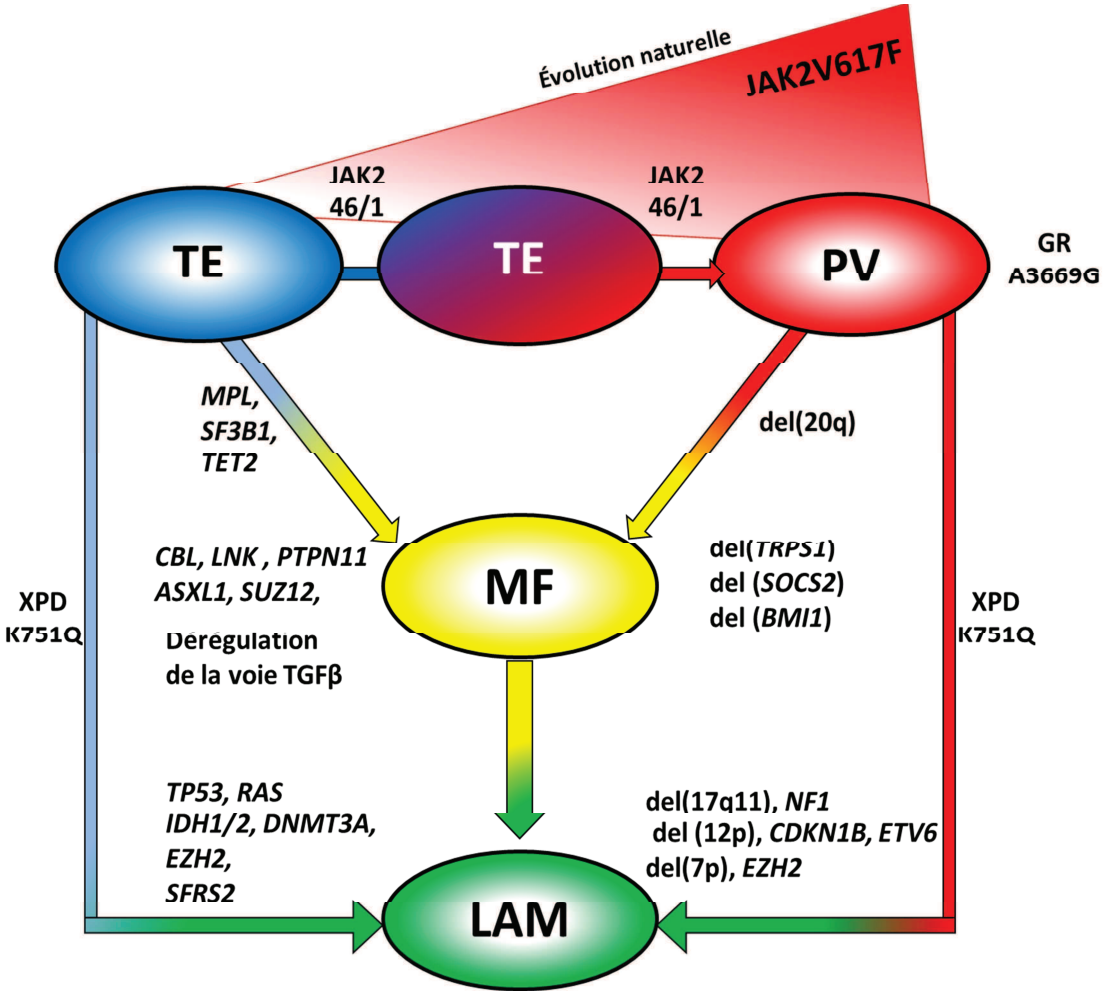

Figure 34 : Modèle proposé afin d'expliquer l'apparition et les évolutions des SMP classiques non LMC

Avons-nous identifié les acteurs principaux des voies affectées dans les SMP ?

Pour plusieurs raisons, nous pensons que les gènes des principales voies affectées dans les SMP ont été identifiés :

1 – Plus d'une centaine de mutations ont été rapportées mais seulement un nombre limité d'entre-elles sont des mutations conductrices (« drivers »).

2 – Quelles que soient les nouvelles altérations identifiées, ce sont les mêmes voies impactées. Par exemple, les délétions des gènes *NF1* et *PTPN11* auraient le même effet que la mutation du gène RAS. De même, les rares délétions d'*EED* et *SUZ12* affecteraient la même voie que les mutations *EZH2*, étant donné que ce sont des gènes qui codent pour des protéines d'un même complexe.

3 – La mutation d'un gène n'est pas le seul mécanisme d'inactivation ; des délétions ou des points de cassure peuvent également l'être (ex : *ETV6*, *CDKN1B*).

4 – La plupart des gènes importants dans la régulation cellulaire sont affectés par des mécanismes autres que mutationnel tels que la méthylation anormale de l'ADN, les modifications covalente d'histones, l'épissage des ARNm, l'interférence des ARN, ou les produits de dégradation.

5 – La leucémogenèse est une combinaison de modifications (mutations et CNA) affectant un répertoire défini de gènes « drivers ».

Existent-t-il des mutations spécifiques d'un pronostic ?

Nos travaux ont souligné l'impact pronostic important de certaines altérations sur la survie et sur le temps de transformation en LAM (TTAT). L'analyse du pronostic dans notre série modeste de 44 MF avait permis de souligner pour la première fois, les mutations d'*ASXL1* associées à un mauvais pronostic (Brecqueville *et al.* 2012) . Nous avons récemment compléter ce résultat dans notre dernière étude (Brecqueville *et al.* 2013) identifiant les co-mutations des gènes *ASXL1* et *JAK2* associées à une plus faible survie. A noter que le

mauvais pronostic des mutations ASXL1 est également retrouvé dans les SMD (Boultwood *et al.* 2010; Gelsi-Boyer *et al.* 2012), LMMC (Gelsi-Boyer *et al.* 2010) et LAM de novo (Chou *et al.* 2010). Nos résultats, associés aux travaux récemment publiés de Vannuchi et al. soulignent le mauvais pronostic des mutations *ASXL1*, *SRSF2*, *EZH2* et *IDH*, (Vannucchi *et al.* 2013) et renforcent l'idée que certaines anomalies moléculaires devraient être recherchées au diagnostic permettant une orientation thérapeutique en fonction du statut mutationnel (**Figure 35**). Ceci pourrait permettre une meilleure gestion thérapeutique des patients, orientés vers les traitements des patients de haut risque comme l'allogreffre ou inclus dans de nouveaux protocoles associant les anti-JAK2 avec des déméthylants, des inhibiteurs d'HDAC ou des inhibiteurs de la voie mTOR (Tefferi 2011).

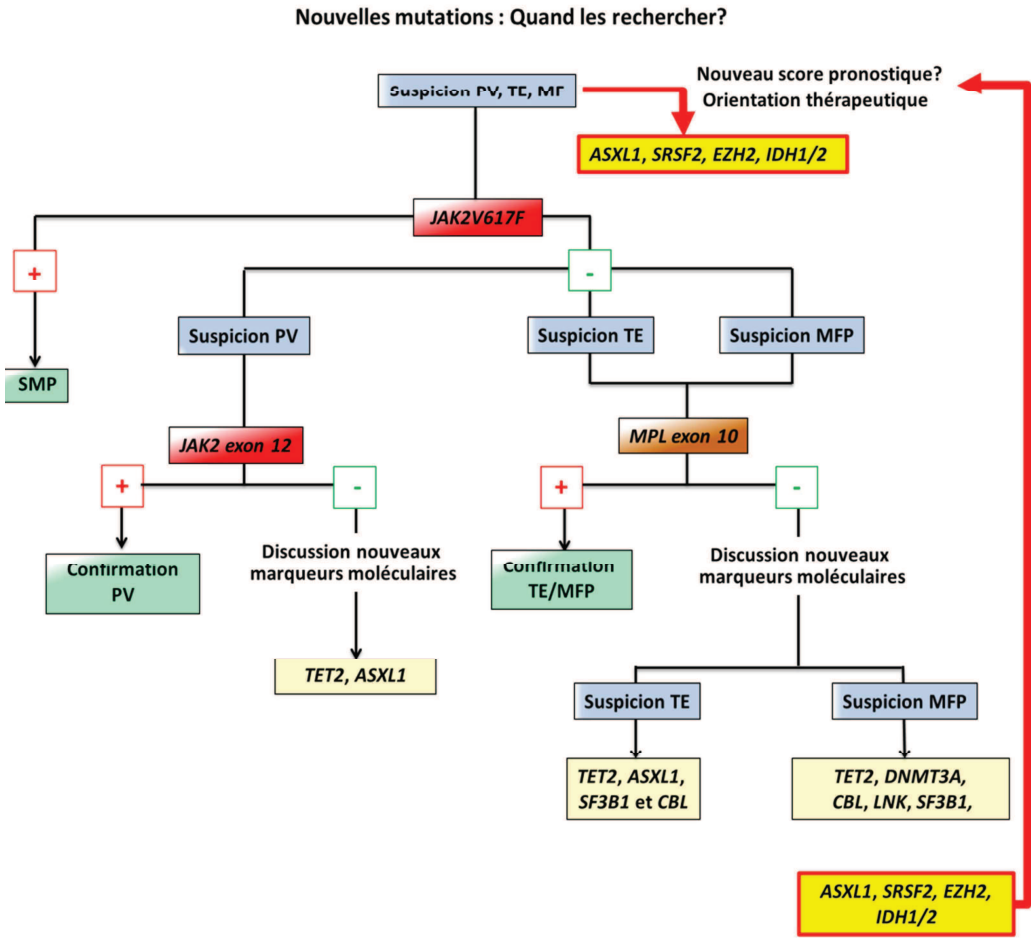

Figure 35 : Arbre décisionnel proposé pour la recherche des marqueurs moléculaires au sein des PV, TE et MF

Conclusion

En conclusion, bien que de nombreuses questions restent non résolues, les travaux présentés dans ce manuscrit décrivent et soulignent la complexité moléculaire des SMP classiques non LMC. Nos résultats soutiennent l'intérêt d'établir une nouvelle classification moléculaire et de nouveaux scores pronostiques prenant en compte les anomalies chromosomiques et mutationnelles. Ceci afin d'améliorer la prise en charge diagnostique, le suivi de la maladie et la thérapeutique. Très prochainement, le répertoire complet des anomalies moléculaire (certainement révélé par le NGS (séquençage de nouvelle génération)) et les progrès dans la compréhension des anomalies, nous permettront une orientation vers une médecine de précision, adaptée à chaque patient.

Perspectives

Quelle est la cellule à l'origine d'un SMP ?

Les évènements initiateurs de la leucémogenèse au sein des SMP classiques non LMC sont à ce jour peu caractérisés. Deux hypothèses non antinomiques existent sur l'origine de la cellule initiatrice (ou cellule souche leucémique) qui serait la CSH ou une cellule somatique. En effet, les SMP sont décrits par définition comme des hémopathies clonales des CSH. Celles-ci persistent tout au long de la vie, ce qui sous-entend qu'elles auraient plus de risque d'accumuler certaines anomalies moléculaires que les cellules plus matures à durée de vie plus courte. De même, des propriétés proches des CSH (capacité auto-renouvellement) pourraient être acquises par la cellule somatique suite à certaines anomalies moléculaires (Misaghian *et al.* 2009). Les travaux sur les cellules iPS (inductible pluripotent stem cell) soutiennent cette hypothèse puisque sous l'action d'un cocktail de 4 gènes (*MYC, KLF4, OCT4, NANOG*), des cellules somatiques sont reprogrammées en cellules pluripotentes.

Afin de mettre en évidence la cellule initiatrice, il faudrait isoler les cellules souches leucémiques des patients en utilisant une combinaison de marqueurs spécifiques. A l'heure actuelle, le phénotype complet de ces marqueurs n'a toujours pas été établi. Toutefois, les principaux marqueurs semblent être CD34+, CD33-, CD38-, CD45RA-, CD49f+, CD90+, +, Thy1+, ALDH+, Lin- (Misaghian *et al.* 2009; Notta *et al.* 2011; Wiseman *et al.* 2013). *A noter que la cellule initiatrice n'est pas toujours celle qui perdure en cellule leucémique, certaines peuvent être conductrices « driver » ou passagères « passenger ».*

Quels sont les évènements oncogéniques fondateurs dans les SMP ?

Afin de répondre à cette question, une des approches serait d'isoler les CSH des patients avec des marqueurs spécifiques décrits précédemment et les mettre en culture en méthylcellulose ou réaliser des xénogreffes dans des souris immunodéprimées. Ceci permettrait avec l'utilisation des techniques de séquençage, de CGH-array et de transcriptome, de détecter les clones majoritaires et les éventuels minoritaires qui au cours de la progression de la maladie pourraient prendre l'avantage, témoignant de l'hétérogénéité des LAM.

Les anomalies sont-elles au sein d'une ou de plusieurs cellules ?

Chez les patients porteurs de plusieurs mutations, la question est de savoir si ces mutations sont portées par la même cellule (notion de monoclonalité) ou s'il existe plusieurs cellules portant des mutations différentes (notion d'oligoclonalité). L'acquisition des lésions moléculaires au cours de l'évolution des SMP pourrait se faire sur un mode linéaire (un clone acquiert successivement plusieurs mutations), sur un mode indépendant (deux ou plusieurs clones acquièrent des mutations indépendamment), ou bien selon les deux modes (Itzykson *et al.* 2013).

Le séquençage par NGS sur des clones isolés issus de culture en méthylcellulose à partir de plusieurs prélèvements de moelle à des temps différents de la phase chronique et lors de l'évolution de la maladie vers une LAM permettrait d'observer l'évolution des clones mutés ou l'apparition de nouvelles mutations. Cette approche pourrait mettre en évidence des clones mineurs potentiellement plus agressifs. La suite de cette démarche serait d'étudier la progression du clone qui devient majoritaire. Les SMP comportent souvent en plus d'un clone majoritaire des clones mineurs qu'il est important de détecter. Au cours de l'expansion clonale, de nouveaux clones peuvent apparaître et acquérir de nouvelles anomalies et de nouveaux avantages prolifératifs. Il y aurait alors compétition entre les clones et peut être une nouvelle sélection. Cette étape est généralement longue au cours du temps, car de multiples mutations sont retrouvées. Le suivi par NGS (plus sensible et rapide que le séquençage Sanger classique) des clones mineurs mutés chez les patients traités ou non permettrait d'évaluer l'impact pronostique et thérapeutique de ces mutations.

Quelles sont les anomalies moléculaires des SMP JAK2V617F négatifs ?

De nombreux gènes ont été retrouvés altérés (CNA et mutations) dans les SMP : *ASXL1, BMI1, CBL, DNMT3A, EZH2, IDH1, IDH2, JAK2, KRAS, LNK, MPL, NF1, NRAS, PPP1R16B, PTPN11, RCOR1, SF3B1, SOCS2, SRSF2, SUZ12, TET2, TP53*. Cependant il reste encore certains échantillons de TE (30%) et MF (12%) non mutés. L'analyse par NGS de l'exome chez ces patients non mutés pourrait permettre l'identification de nouvelles mutations. De plus, l'étude des SMP d'origine familiale est également à exploiter. En effet dans le passé,

les recherches sur les formes familiales ont permis de mettre en évidence des anomalies moléculaires causales (ex : la mutation de TPO-R/MPL au sein de thrombocytoses familiales) (Kondo et al., 1998).

Quel est le rôle du microenvironnement médullaire dans les SMP ?

Les résultats présentés ici ont très peu abordé le rôle du microenvironnement alors qu'il est indispensable à toutes les étapes de l'hématopoïèse : survie, auto-renouvellement, détermination, prolifération et différenciation. Dans certains cas, une altération des cellules mésenchymateuses et une augmentation de la micro-vascularisation médullaire est observée. La recherche des altérations serait certainement contributive (cf modèle Bad « Seed » and Bad « Soil » (Le Bousse-Kerdilès 2012)) à la caractérisation des SMP.

Quels sont les mécanismes de la transformation en LAM ?

Nos résultats ont souligné la fréquence importante de la délétion 12p cryptique détectée par CGH-array et associée à une transformation en LAM. Si l'on se réfère aux recommandations actuelles (Mascarenhas *et al.* 2012), la délétion 12p n'est à ce jour pas recherchée systématiquement dans les MF et LAM post-MF. Une étude comparative entre la phase chronique et la phase aigüe d'une grande série de patients permettrait de confirmer la nécessité de rechercher cette délétion lors de la phase chronique et au diagnostic de la LAM.

Quel est le rôle des anomalies dans les SMP ?

Afin d'étudier les dérégulations épigénétiques, l'analyse des profils de méthylation (ADN et des histones) et d'acétylation est à sérieusement envisager. *A noter, que celles-ci doivent être confrontées aux génotypes des patients puisque certaines dérégulations épigénétiques peuvent être dues à des mutations.* Par ailleurs, il reste beaucoup à faire sur le versant fondamental. En effet de nombreuses questions persistent, comme par exemple : quels

sont les gènes impliqués dans les délétions 20q ? Est-ce que la perte des régions non-codantes pourrait participer à la physiopathologie des SMP ? Quel est le rôle des SNORNA retrouvés dans cette région ?

Quelle est la conséquence de la coopération des mutations *JAK2* et *ASXL1 in vivo*?

Afin de répondre à cette question, l'utilisation de modèle animaux est à envisager. En effet, à partir des modèles de souris JAK2 existants, il faudrait greffer, chez ces souris immunodéprimées, des plasmides contenant un shRNA *ASXL1*. Les souris JAK2 KO se transforment naturellement après environ 9 mois en MF (Marty *et al.* 2010); il serait intéressant de contrôler si l'addition du shRNA d'*ASXL1* n'accélère pas ce phénomène. Ce modèle d'étude peut être également envisagé avec d'autres gènes qu'*ASXL1* tels que *SRSF2* et *EZH2* afin d'étudier les coopérations entre *JAK2* et les autres gènes mutés au sein des SMP.

Quelles sont les perspectives thérapeutiques potentielles dans les SMP ?

L'identification de stratégies thérapeutiques pertinentes dans les SMP constitue l'ultime étape de l'étude de la physiopathologie de la maladie. A l'heure actuelle, les thérapies déjà existantes telles que **les chimiothérapies** ont montré leur limite ; elles n'éliminent pas les CSH leucémiques, puisqu'elles ciblent les cellules en cycle. Toutefois, de nouvelles thérapies telles que **les anti-JAK1/JAK2** ont des résultats prometteurs sur l'amélioration de la qualité de vie (taille de la rate diminuée de façon surprenante) (Ganetsky 2013; Verstovsek *et al.* 2013), mais n'ont pas d'impact sur la charge allélique de *JAK2* (Quintás-Cardama 2013).

De plus, étant donné le grand nombre de gènes mutés et la présence de mutations différentes pour un même gène (la plupart entrainant des pertes de fonctions), on ne peut à l'heure actuelle envisager une thérapeutique ciblée. Toutefois, pour les mutations gain-

de-fonction comme celle d'IDH1/2 (l'accumulation de 2-hydroxyglutarate), des inhibiteurs ciblant les mutations sont en développement. Récemment, l'équipe de K. Yen a développé une molécule (AGI-6780) inhibant de façon sélective et intense la mutation « hotspot » R140Q avec pour conséquence la différenciation des cellules de LAM (Wang *et al.* 2013).

D'autres approches sont également intéressantes, telles que l'établissement d'un **chemogramme** qui est l'étude *in vitro* de la sensibilité aux drogues des cellules de patients atteints de MF ou LAM post-SMP en fonction du statut mutationnel.

En conclusion, en attendant les progrès thérapeutiques innovants (des inhibiteurs des histones déméthylases par exemple), pour les patients qui s'avèrent être de mauvais pronostic ; des traitements combinant une action anti-JAK2 avec des modulateurs de l'épigénétique (agents hypométhylants et HDAC inhibiteurs) pourront être envisagés dans des essais cliniques. En effet, l'efficacité thérapeutique des **agents hypométhylants** (5-azacytidine et la décitabine) supérieures aux traitements classiques a été démontré dans les SMD et les LAM (Estey 2013). De plus, l'impact potentiel thérapeutique des **HDAC inhibiteurs** dans les SMP a récemment été montré chez des lignées cellulaires de LAM post-SMP (HEL et K562). L'utilisation des HDAC inhibiteurs (de sodium butyrate) avait induit une surexpression des protéines inhibitrices SOCS1 et SOCS3, entrainant une inhibition importante de la voie JAK/STAT (Gao *et al.* 2013). Enfin, récemment des essais cliniques sur **les inhibiteurs des protéines kinases PIM**, sont en cours d'étude dans plusieurs hémopathies et serait à envisager chez les patients atteints de SMP.

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Annexes

Article 7 - Myeloid malignancies: mutations, models and management.

ARTICLE n°7

Myeloid malignancies: mutations, models and management.

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REVIEW Open Access

Myeloid malignancies: mutations, models and management

Anne Murati, Mandy Brecqueville, Raynier Devillier, Marie-Joelle Mozziconacci, Véronique Gelsi-Boyer and Daniel Birnbaum*

Abstract

Myeloid malignant diseases comprise chronic (including myelodysplastic syndromes, myeloproliferative neoplasms and chronic myelomonocytic leukemia) and acute (acute myeloid leukemia) stages. They are clonal diseases arising in hematopoietic stem or progenitor cells. Mutations responsible for these diseases occur in several genes whose encoded proteins belong principally to five classes: signaling pathways proteins (e.g. CBL, FLT3, JAK2, RAS), transcription factors (e.g. CEBPA, ETV6, RUNX1), epigenetic regulators (e.g. ASXL1, DNMT3A, EZH2, IDH1, IDH2, SUZ12, TET2, UTX), tumor suppressors (e.g. TP53), and components of the spliceosome (e.g. SF3B1, SRSF2). Large-scale sequencing efforts will soon lead to the establishment of a comprehensive repertoire of these mutations, allowing for a better definition and classification of myeloid malignancies, the identification of new prognostic markers and therapeutic targets, and the development of novel therapies. Given the importance of epigenetic deregulation in myeloid diseases, the use of drugs targeting epigenetic regulators appears as a most promising therapeutic approach.

Introduction

Myeloid malignancies are clonal diseases of hematopoietic stem or progenitor cells. They result from genetic and epigenetic alterations that perturb key processes such as self-renewal, proliferation and differentiation. They comprise chronic stages such as myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML) and acute stages, i.e acute myeloid leukemia (AML). AML can occur *de novo* (~80% of the cases) or follow a chronic stage (secondary AML). According to the karyotype, AMLs can be subdivided into AML with favorable, intermediate or unfavorable cytogenetic risk [1]. MPNs comprise a variety of disorders such as chronic myeloid leukemia (CML) and non-CML MPNs such as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).

Molecular biology has always been important in hematology, especially myeloid malignant diseases. Currently however, except in some specific examples such as the *BCR-ABL1* fusion in CML, and *NPM1* or *FLT3* mutations in *de novo* AML, molecular data are not associated with optimal clinical and therapeutic exploitation in the clinic. This may change with the flurry of new data that are being generated. It all started with the discovery of the JAK2V617F mutation in MPNs [2–5]. Like the characterization of the BCR-ABL1 fusion kinase, which has led to the development of an efficient targeted therapy [6], this breakthrough showed how much progress can be made by the identification of a single molecular event regarding disease definition, understanding and classification, prognosis assessment, clinical monitoring and treatment. Since then, many new mutated genes have been identified. They affect various cell processes such as signaling, regulation of gene transcription and epigenetics, mRNA splicing and others. The aim of this review is not to describe these results in detail; this has been done in several excellent recently-published reviews [7–16]. Without putting emphasis on a particular gene, disease or cell process, it is more to discuss how the new data may improve our global vision of leukemogenesis and may be used for progress in at least three directions.

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Review

Understanding molecular leukemogenesis

Identification of new mutations

The genetic events involved in leukemogenesis have been deciphered by using two approaches. First, genomic alterations have been identified by using karyotype analysis and DNA hybridization onto oligonucleotide arrays (SNP-arrays, array-CGH); several types of genomic profiles have been found: lack of detectable changes, uniparental disomies (UPD), losses of chromosomes or large chromosomal regions, trisomies, losses or gains of small regions or genes. Second, small gene mutations have been detected by classical Sanger sequencing [17–22] or, more recently, by the use of new technologies such as next generation sequencing (NGS) [23–31].

These studies, together with previous ones that had identified *JAK2, NPM1, MPL, RAS* and *RUNX1* mutations, among others, led to the discovery of several major players in leukemogenesis: *ASXL1* [21], *BCORL1* [25], *CBL* [19], *DNMT3A* [24,32], *EZH2* [20,22], *IDH1/IDH2* [26], *TET2* [18] and *UTX* [33]. The mutational frequencies of these genes range from a few percent to more than 50%, or even virtually 100%, depending on the gene, the disease and the series studied. Thus, almost all cases of PV have a mutation of *JAK2* [34,35]. Not counting the latter, mutations in *ASXL1* and *TET2* are frequently observed throughout the whole myeloid spectrum (Figure 1), reaching 40-50% in CMML [33,36]. Mutations in *DNMT3A* and *IDH1/2* are rare in the chronic stages but reach 15-20% in AML and exhibit a strong association with monocytic features [30]. Genes encoding components of the splicing machinery that is involved in the splicing of introns during pre-mRNA maturation (mainly *SF3B1, SRSF2, U2AF35/U2AF1*, and *ZRSR2*) have been found frequently mutated in MDSs and CMML, and more rarely in MPNs and AML (Figure 1) [31,37–42]. Mutations in splicing factors are found in more than 60% of MDS with ring sideroblasts and in more than 50% of CMML [31].

Mutations in leukemogenic genes have been described in detail in recent reviews [7,9,10,12–16,43]; and will not be reviewed here. We will rather delve on the questions aroused by these recent data.

Have we already identified the entire repertoire of mutated genes?

We may have identified (most of) the major culprits [14]. First, there are hundreds of background mutations (i.e. that do not provide selective advantage) but only a limited number of driver mutations (i.e. that cause the disease) in each malignant disease. Second, many of the newly discovered mutated genes may affect the same pathways or networks as the major mutated genes. For example, deletions and mutations of *NF1*, which have been recently identified [17,44,45], or *PTPN11* [46] are thought to have the same effect as a *RAS* mutation; a mutation of the *SHKBP1* gene [47] or a duplication of the *SH3KBP1* gene [48], which both encode cytoplasmic regulators of the CBL pathway, may have the same effect as a *CBL* mutation [49,50]. Because EZH2, EED and

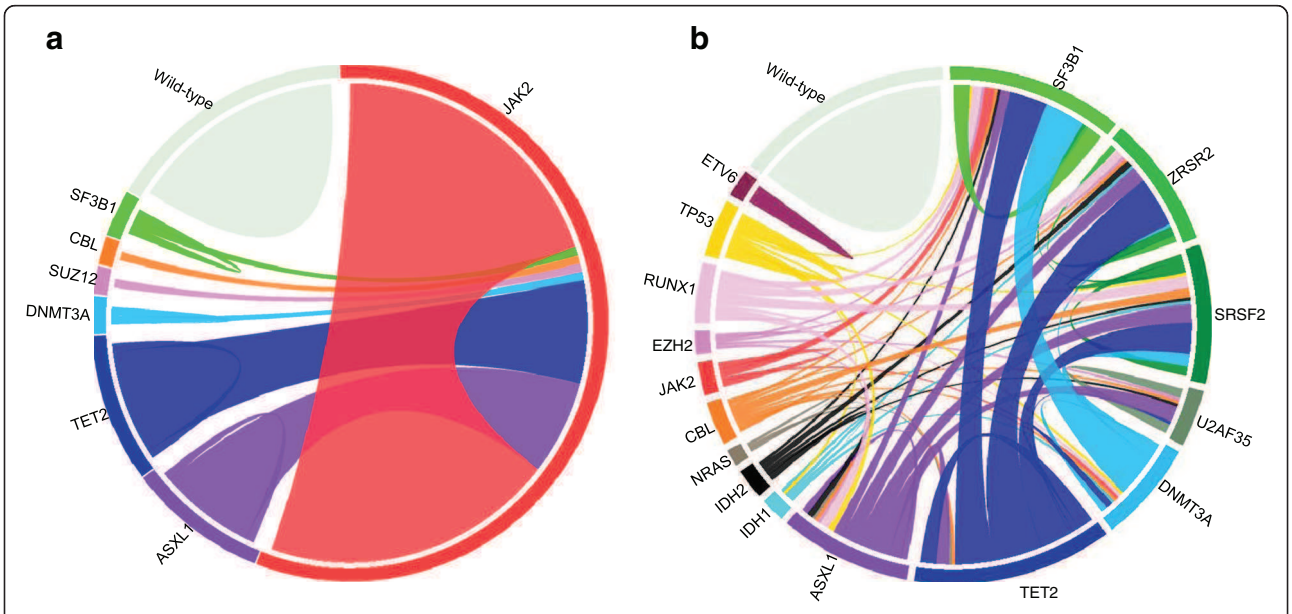

Figure 1 Circos diagrams depict the relative frequency and associations of the major mutations in MPNs (a) and MDSs (b), respectively based on data from our work [37] **on 127 classic MPNs and from Damm's study** [38] **on 221 MDSs.** Wild-type means that no disease allele has been detected in the genes listed.

SUZ12 proteins all belong to the same polycomb complex 2 (PRC2) the rare deletions or mutations of the *EED* [23,51] and *SUZ12* genes [17,51] could have the same effect as *EZH2* mutations. Third, several genes (e.g. *ETV6* [52] or *RUNX1*) can be structurally altered by mechanisms other than mutation, such as deletions and breakages. Fourth, some important regulatory genes could be affected not by structural alteration but through other mechanisms such as abnormal DNA methylation (e.g. *CDKN2A/B* [53], *TRIM33* [54], *CTNNA1* [55], *SOCS1* [56,57]), histone modifications, mRNA splicing, microRNA or long non-coding RNA (lncRNA) modulation, or product degradation. Fifth, when all known mutated genes are analyzed in a series of cases, the percentage of samples with at least one mutated candidate driver gene varies from 50% [58] to over 90% (in CMML; [33]; Gelsi-Boyer et al., submitted). Moreover, most samples studied by NGS were shown to harbor gene mutations [23,26]. Thus, we are soon approaching the days where all cases can be defined by combination of several alterations. The practical definition of leukemogenesis will then be based on a specific and limited repertoire of alterations, including translocations, mutations and copy number changes, affecting a defined set of driver genes.

However, some issues still need be addressed. First, many genes may be mutated or deleted with a very low frequency (i.e. under 1%); their involvement and recurrence may be hard to demonstrate. Second, because NGS studies of several malignancies have shown that hundreds of genes can be mutated in a single tumor, background mutations should be discarded and driver genes validated. Third, we still miss information in some diseases such as essential thrombocythemia (ET), in which *JAK2* mutations are found in only half the cases, and *TET2* mutations in less than 10%. We also lack knowledge about the targeted genes of some frequent genomic alterations such as the 20q11-q13 deletion (*ASXL1* and *DNMT3B*, more centromeric, are not involved). Fortunately, this lack of information is bound to disappear. The example of refractory anemia with ring sideroblasts (RARS) is instructive; in three-quarters of RARS, mutations have been recently found in *SF3B1*, a gene encoding a subunit of a splicing factor (U2 snRNP) and histone acetyltransferase (STAGA) complexes [27,29,31].

Is there some specificity in gene alterations?

Gene fusions (e.g. *BCR-ABL1, PML-RARA, FGFR1*-associated fusions…), 5q deletion and *JAK2* mutations are specific of some forms of myeloid diseases, although *JAK2* mutations occur in three distinct subtypes of MPN. *RUNX1* mutations are frequent in MDSs, CMML and AML but rare in MPNs. Among splicing factor genes, mutations in *SF3B1* are highly specific of MDS with ring sideroblasts and *SRSF2* mutations are most frequent in CMML [31]. In contrast, some mutated genes (e.g. *ASXL1, DNMT3A, EZH2, TET2*) occur in a wide range of myeloid diseases and with various frequencies. Future studies may identify mutations or combinations of mutations that drive a specific phenotype.

What are the functions of the mutated proteins ?

Leukemogenic alterations mainly affect five classes of proteins (Figure 2): signaling pathway components, such as ABL, CBL, CBLB, FGFR1, FLT3, JAK2, KIT, LNK, MPL, PDGFRs, PTPN11, PTPRT [23,59] and RAS, transcription factors (TFs) such as CEBPA, ETV6 [58], GATA2 [30], IKZF1 [60], RARA and RUNX1, epigenetic regulators (ERs), such as ASXL1, BCORL1 [25], DAXX [23], DNMT3A, EZH2 [20,22], MLL, MYST3, NSD1 [30], PHF6 [61], SUZ12 [17,51], TET2 and UTX [28], tumor suppressors (TSG), such as CDKN2A, TP53, and WT1 and components of the spliceosome [27,29,31,38,39,41,42]. However, additional alterations occur in genes encoding proteins that it is too early to classified into these defined categories, such as DIS3, DDX41 [23], mitochondrial NAPDH dehydrogenase ND4 [62], or cohesin complex proteins [23,63].

In chronic stages, alterations in signaling molecules can be grouped in two major categories, a first one that is found in MPNs and affects oncogenic tyrosine kinases (ABL1, JAK2, FGFR1, PDGFRs) and the downstream JAK-STAT and/or PI3-kinase pathways, and a second one that is mutated in CMML and affect the RAS-MAP kinase pathway (RAS, PTPN11, NF1). CBL alterations occur in a wide variety of myeloid diseases [50].

TFs and ERs constitute the largest classes, which involve several categories of proteins (Figure 3); because there are many ways to affect gene expression it is probable that not all of these categories are known yet. The existence of epigenetic alterations in myeloid malignancies has been known for long time [64,65]. For example, alterations of MLL, a histone methyltransferase (HMT), and MYST3, a histone acetyltransferase (HAT), have shown the importance of epigenetic deregulation in AMLs with translocation [45,64,65]. However, in chronic diseases and in AMLs with normal karyotype, the extent, causes, identities, exact roles and consequences of epigenetic alterations have long remained elusive. Molecular studies have recently shown that both DNA methylation and histone regulation are affected, and that epigenetic alterations may be due to genetic alterations, (i.e. mutations in genes encoding epigenetic regulators). The latter phenomenon has been observed in genome-wide analyses of many neoplasias [28,66,67]. However, not all epigenetic alterations may be due to an abnormal genetic background [1,53,54].

The recent reports of the interrelated functions of IDH1/2 and TET2 in DNA methylation represent a major

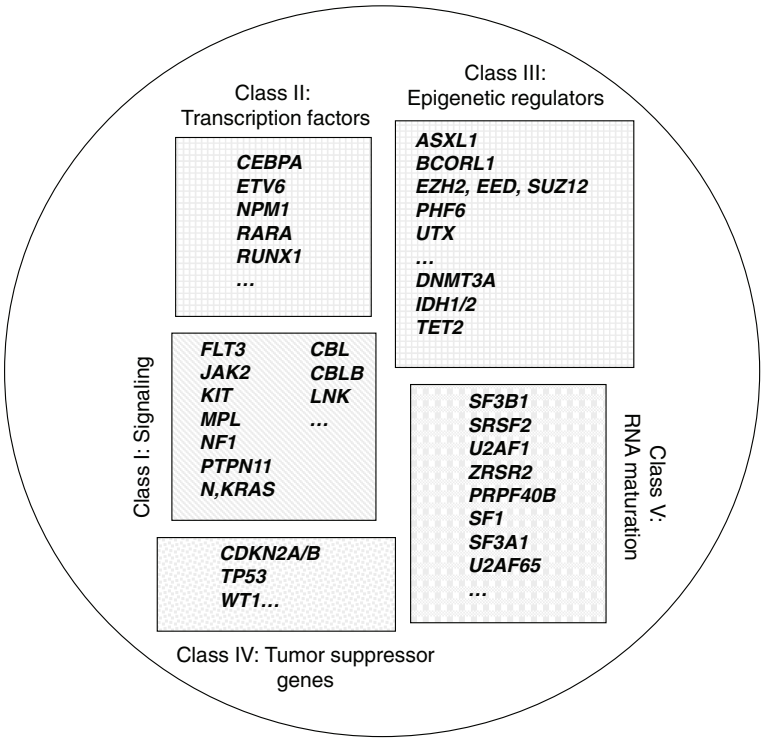

Figure 2 Schematic representation of five classes of leukemogenic genes. ERs (class III) can be subdivided into two subclasses (DNA methylation-associated and histone-associated).

breakthrough in our understanding of leukemogenesis [68]. It was initially hard to associate mutations of IDH1 and IDH2, two metabolic enzymes, with mutations in TET2, an unknown gene product, and as hard to suspect their role on DNA methylation. A very rapid series of elegant studies have shown i) that *IDH1/2* and *TET2* mutations are mutually exclusive in myeloid malignancies [68], ii) that mutated IDH1 and IDH2 produce 2-hydroxyglutarate instead of alpha-ketoglutarate (αKG) [69,70], iii) that *TET2* encodes an αKG–dependent methyl cytosine dioxygenase whose mutation alters the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) [68,71] and iv) that both *IDH1/2* and *TET2* mutations impact on DNA methylation and are involved in the same biochemical pathway [72]. In addition, TET proteins can generate from 5-hmC 5-formylcytosine and 5-carboxylcytosine, but their roles are currently unknown [73]. The recent studies on TET proteins suggest a role in removing aberrant DNA methylation to ensure DNA methylation fidelity [74]. This has opened a new area of research since first, other factors involved in DNA demethylation may exist and second, several αKG–dependent enzymes, such as jumonji histone demethylases [75] are epigenetic regulators; therefore, some of these proteins could also be involved in malignancies. However, *IDH1/2* and *TET2* mutations, while mutually exclusive, are not equivalent because *IDH1/2* mutations are more frequent in acute than in chronic myeloid diseases, whereas it is not the case for *TET2* alterations, which are more evenly distributed between chronic and acute stages. Inactivation of TET2 increases self-renewal in hematopoietic stem cells and induces a disease resembling CMML in mouse models [76,77]. Mutated IDH1/2 enzymes may impact on self-renewal but with a different strength. The likely explanation is that IDH1/2 and TET2 have other, non-overlapping functions on the regulation of DNA methylation and histone marks. Also, an IDH-mutated product may depend on another, rate-limiting factor to exert a leukemogenic effect. DNMT3A is a *de novo* DNA methyltransferase involved in the formation of 5-mC and has complex interactions with polycomb and HMT proteins [78]. How DNMT3A mutations affect DNA methylation remains to be defined [24,30,79]; they probably do so in a different way from *TET2* or *IDH1/2* mutations since they may co-occur with either of them. A recent study showed that DNMT3A loss leads to upregulation of hematopoietic stem cell genes and downregulation of differentiation genes but is alone insufficient to induce a malignant disease in a mouse model [79].

Mutations in regulators of histone marks have become a major subject of research and the relationships between them are quickly unveiled. Central regulators of

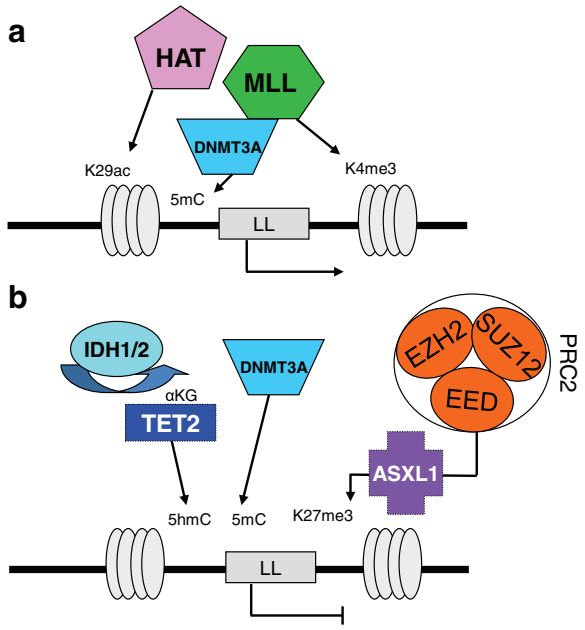

Figure 3 Schematic representation of epigenetic regulation of a leukemogenic locus (LL) framed by histone H3. (**a**) Histone acetyltransferases (HAT; e.g. MYST3) and histone methyltransferases (HMT; e.g. MLL) can activate the locus. (**b**) Reciprocally, the locus is repressed by polycomb complex PRC2 (which comprised EED, EZH2 and SUZ12 proteins). ASXL1 would direct PRC2 to the locus. Loss-of-function mutations in PRC2 components or in ASXL1 remove PRC2 repression. DNMT3A is involved in the formation of 5-methylcytosines (5mC) from cytosines and interacts with HMTs as well as with PRC2 components. TET2 mediates hydroxylation of 5mC to 5hmC. To function, TET2 requires α-ketoglutarate (αKG), which is provided by IDH1/2 proteins. Aberrant methylation patterns are caused by mutation in TET2 or in IDH1/2, which produces 2-hydroxyglutarate instead of αKG.

myelopoiesis and key players in leukemogenesis seem to be the polycomb regulatory complexes, especially PRC2, which, in addition to direct defects of its components (EED, EZH2, SUZ12), could be affected in its concerted action with several ERs, such as ASXL1, cohesins, DNMT3A, IDH1/2, MLL, TET2 and UTX. TET proteins could regulate pluripotency and self-renewal through interaction with PRC2 [74,80,81]. The cohesin complex is encoded by four genes (*SMC1, SMC3, RAD21* and *STAG2*), which have been found mutated [23] and deleted [63]. A major interactor of cohesin complex is CTCF. PRC2 is recruited to specific loci through interaction of SUZ12 with CTCF [82]. Another main leukemogenic interactor of PRC2 components is ASXL1. A recent study showed that ASXL1 loss affects PRC2 complexes and H3K27me3 histone marks, and induces a strong hematopoietic phenotype consistent with an MDS in a conditional knock-out mouse model [83]. ASXL1 would direct PRC2 to leukemogenic loci such as *HOXA* genes. Thus, through direct alterations of its components or of proteins or lncRNAs [84] that recruit the complex, PRC2 has emerged as a key node in a network regulating hematopoietic stem cell self-renewal and proliferation and as a major factor in myeloid leukemogenesis. This is also true for T-cell leukemogenesis [85]. Correct functioning of polycomb repressive complex 1 (PRC1) seems also to be important for myeloid cells since the loss of BMI1 (a component of PRC1) in the mouse leads to a disease similar to PMF [86]. Structural alterations of the *BMI1* gene occur but are rare in human myeloid diseases [87].

Whether other chromatin-associated complexes play a role in leukemogenesis should soon be revealed. ASXL1 could play a role in a cross-talk between major chromatin silencing systems, PRC1/PRC2, HP1α/CBX5 heterochromatin repressive complex and polycomb repressive deubiquitinase (PR-DUB) complex. Mutations in *BCOR* and *BCORL1* suggest that the RAF/BCOR complex [84,88] might be involved in AML. The recent identification of a mutation in the *DAXX* gene in an AML case [23] further supports a wide participation of chromatin-regulatory complexes in leukemogenesis and cancer in general. DAXX and ATRX (which is mutated in X-linked α-thalassemia) are subunits of a chromatin remodeling complex and are both mutated in solid tumors [89,90].

The importance of the fifth class of mutated genes was more unexpected. Mutations in components of the spliceosome, which are mutually exclusive, lead to splicing defects including exon skipping, intron retention and use of incorrect splice site [31]. A recent study showed that a consequence of splicing gene mutations is accumulation of unspliced transcripts affecting a specific subset of mRNAs [41].

What are the effects of the gene mutations?

The dominant-positive effects of oncogenes such as *BCR-ABL1*, mutated *FLT3, JAK2* or *RAS,* have been easy to apprehend. *CBL* and *LNK* mutations inactivate brakes on signaling pathways and may have a dominant-negative effect. *TET2* is inactivated in the manner of a tumor suppressor. *EZH2* is frequently associated with UPD and acts as a TSG. A frequent form of defect seems to be haplo-insufficiency [91], which could be associated with the (generally) heterozygous loss or mutation of *ASXL1, NF1, NPM1, TP53, RUNX1* or *TET2*. Neo-functionalization results from *IDH1/2* mutations, which are always mono-allelic. For genes altered through different mechanisms (mutations, deletions or translocations) such as *RUNX1* or with different types of mutations (hotspot or dispersed) such as *DNMT3A*, the function might be variably affected and some mutants may have a dominant-negative effect. Mutations in spliceosome genes are mostly missense and could result in proteins with a modified but not inactivated function.

Mutations in signaling pathways, transcription networks and splicing machinery have many downstream consequences. Modifications in epigenetic regulation of DNA and histones may have a strong amplifying effect since they impact on the transcription of thousands of genes. This in turn impacts on the properties of hematopoietic stem cells, favoring self-renewal and proliferation over differentiation, thus promoting leukemogenesis [92]. However, chimeric proteins involving TFs and ERs (e.g. MLL, MYST3, NSD1 ...) may induce a stronger effect than mutations in other TFs and ERs (such as ASXL1, EZH2 or TET2), which may need to co-occur with several other alterations to trigger AML, often after a chronic phase. Perhaps like the difference between a water jet and a sprinkling rain, this difference may have to do with the specific functions of TFs and ERs [64]. TF and ER fusion proteins assemble in complexes that are directly recruited to their target genes where they modify the local histone marks, drastically altering transcription. In contrast, mutated ERs may moderately perturb the epigenetic network, resulting in global gene deregulation.

Mutations in spliceosome components may lead to several types of deregulation, including alterations of the epigenetic control of differentiation and self-renewal; they may thus result in the same defects as TF and ER mutations. This may derive from splicing aberrations of leukemogenic genes (e.g. *RUNX1*) [41] or from other specific but indirect defects. SF3B1 for example interacts with components of the polycomb repressor complex 1 (PRC1) and *SF3B1* mutations may compromise PRC1 regulation of leukemogenic loci [93]. Reciprocally, the function of the pre-mRNA splicing machinery involves the reading of histone marks, and defective chromatin regulators may affect splicing [94]. Directly or indirectly, *SF3B1* mutations, which are associated with the presence of ring sideroblasts, are likely to affect genes involved in red cell biology and mitochondria function. Because mutations in splicing genes, in TFs and in ERs are not mutually exclusive it is probable that the three types of alterations have additive rather than interchangeable effects.

Modeling molecular leukemogenesis

Are there preferential combinations and mutual exclusions?

Two driver mutations may never occur together (mutual exclusion) in the same cell because of epistasis (two hits in the same pathway are not selected because they do not provide a growth advantage) or synthetic lethality (two hits are counter-selected because they compromise the life of the leukemic cell). Associations and cooperation can occur in all other cases.

Some chronic myeloid malignancies, such as CMML (myeloproliferative form, MP-CMML) and MPNs, have a proliferative component. This component is driven by alterations in signaling molecules, such as *CBL, CBLB, FLT3, JAK2, LNK, MPL, NF1, PTPN11* or *RAS*. These mutations are generally mutually exclusive. However, *JAK2* mutations can be found in patients with mutations of *CBL*, *LNK* or *MPL* [95–97]. In most cases when two signaling mutations are found in the same patient they are not in the same cellular clone. Signaling mutations associate with mutations in genes from the other classes (TSGs, TFs, ERs). *CBL* and *KIT* mutations are more frequent in AML with t(8;21) and inv(16), i.e. with alterations of the core binding factor (CBF), a dimeric transcriptional factor containing the RUNX1 protein [98].

With rare exceptions, mutations in genes encoding splicing factors do not synergize and are mutually exclusive [31,38,41,42].

As already mentioned, *IDH1* or *IDH2* mutations are mutually exclusive with *TET2* mutations. Except for this, *TET2* mutations seem to be able to cooperate with either of the other recurrent alterations. *ASXL1* mutations, which occur preferentially in secondary AML, are mutually exclusive with *NPM1* mutations, which occur in *de novo* AML [99]. Although ASXL1 interacts with PRC2 proteins [83] *ASXL1* and *EZH2* mutations are not mutually exclusive [58]. Mutations in *EED* and *SUZ12* may even be found in the same AML case [23]; however, they may affect different clones. *RUNX1* mutations are frequently associated with *ASXL1* defects in MDSs [100]. Mutations in *ASXL1* and *TET2* can be concomitant (Figure 1), and each can co-occur with mutations in signaling molecules [58,100]. In MDSs, *U2AF1* mutations are more frequent in *ASXL1*-mutated than in *ASXL1*-wildtype cases [38,42]. *TP53* mutations and losses, likely associated with genetic instability, are found in MDSs with karyotypic alterations but not in cases with normal karyotype [58]. *DNMT3A* mutations are more frequent in AML with *NPM1* and *FLT3* mutations, infrequently found in *ASXL1*-mutated cases, and very rare in cases with translocations [24,101]. Overall, while *IDH1/2* and *TET2* mutations are equally distributed, there seem to be two major associations in AMLs with intermediate cytogenetic risk, *ASXL1/RUNX1* on the one hand (secondary, dysplastic AMLs), *NPM1/FLT3/DNMT3A* on the other hand (primary, non-dysplastic AMLs) [99]. These and other associations and exclusions not described here or yet to be discovered will help understand the major leukemogenic pathways. An important issue is to demonstrate that mutations found in the same case are actually cooperating mutations that co-occur in the same cell progeny and not in different clones.

How many hits are necessary to trigger a malignant myeloid disease?

Early studies of chronic and acute hematopoietic malignant diseases have shown that some cases may display a

single mutational event whereas others harbor several hits [100]. This difference may just be due to the low mutational frequency of many driver genes (e.g. *NF1A, EED*) [102] and to our current ignorance of other targets. Actually, NGS studies have shown that the general rule is to find several altered genes in each case [23,26,59,103] and murine models have shown that single alterations are, except in rare cases, not sufficient to cause AML [104,105]. In the years to come mouse models will have to challenge many combinations of mutations.

The study of matched chronic and acute stages has shown that progression is associated with additional alterations. However, the chronic stages are already characterized by the presence of several mutations. We found that many cases of CMML have already four mutations [36], and this was without counting mutations in splicing factors. *JAK2* and *TET2* concomitant mutations are frequent in MPNs [16,37]. Whether they are both necessary for the various phases of the disease and their order of appearance are a matter of debate [106]. An NGS study indeed showed that the ten mutations identified in an MDS patient can be detected together in most studied single cells, suggesting a linear evolution of the disease and the existence of a dominant clone [103]. Regarding evolution of AML after therapy, a recent NGS study has revealed two major patterns at relapse [23]; the first pattern is the persistence of a dominant clone and the second pattern is the selection and expansion of a minor clone; in both cases the relapse clone had gained additional mutations. Another recent NGS study showed that genetic evolution of secondary AML is a dynamic process shaped by multiple cycles of mutation acquisition and clonal selection. MDS are oligoclonal with founding clones; these clones persist in secondary AML, which shows at least an additional subclone with new "progression" mutations [107]. Founding mutations may occur in various genes, such as *U2AF1* [39] or *TET2*. Many different genes may be involved in progression. Thus, several steps are necessary to trigger a myeloid disease, even a so-called chronic one, and progression involves additional hits.

How many of these steps are there?

A first step in the leukemogenic process is likely to be a mere clonal expansion. Several gene mutations may play a role at this stage. Their identity may depend on whether they target a hematopoietic stem cell or a progenitor. In the first case the initial hit should provide a proliferation boost, in the second the hit should bestow self-renewal on the proliferating progenitor [108]. Mutations in a TSG, splicing gene, or in some ERs such as *TET2,* could occur at this initial step. It is also possible that, in a susceptible background, several clones emerge independently early on [12,109].

Then, because of increasing proliferation and genetic instability, a cell from the affected clone (or clones) undergoes various additional mutations (including many background mutations), leading to an oligoclonal malignant tumor. Some of the early mutations may not be present in the clone that eventually becomes leukemic. Thus, for each case, only the determination of all potential mutations and the reconstitution of the mutation profile and clonal evolution will help understand the pathophysiology of the disease. This is now achievable by using NGS. How many steps can eventually be individualized may depend on how many clones are initially expanded, on the level of genetic instability that results from the initial hits, and on the impact of the mutations on self-renewal, differentiation and proliferation. Some mutations in epigenetic regulators may have a milder effect on genetic reprogramming than a gene fusion involving a master transcription factor, which will induce a strong block of differentiation in a hematopoietic precursor [92]. The latter event is prominent in *de novo* AMLs, which accordingly display only few or none of the other recently-discovered mutated genes.

A previous scheme of leukemogenesis [110] was based on the minimal cooperation of two oncogene classes, proliferation-drivers (kinases, RAS) and differentiation-blockers (mostly transcription factors), to trigger AML. The ever-increasing molecular complexity of myeloid malignancies is now obvious and calls for an update of this model. First, it is now routinely possible to observe the cooperation, already at the chronic stage, of three, four or more mutated genes (to speak only of known or suspected drivers), whose products belong to at least five classes, class I signaling molecules class II TFs, class III ERs, class IV TSGs and class V splicing factors [100]. Second, not all mutations of a class are equivalent; mutations in *ASXL1, RUNX1* or *TET2* occur almost as frequently at the chronic stages as in AML whereas mutations of *IDH1/2* or *DNMT3A* are preferentially found at the acute stage. The reason for this remains obscure but may have to do with the different intensities in the differentiation block induced by the mutations. Third, the classes are not well individualized. For example, *EZH2, RUNX1* and *TET2* are both TSGs and TF/ERs. *NF1* is both a TSG and a regulator of signaling pathways. Because it induces phosphorylation of histone H3 and PRMT5 arginine methyltransferase, JAK2V617F may also be an ER [111]. Fourth, all classes may not be systematically affected in each case. Fifth, if classes I and V are relatively well individualized, with genes whose mutations are generally mutually exclusive, the definition of the other classes may evolve. However, despite all this, the initial schematic model might not be so far off. The two key processes of differentiation and self-renewal seem to be always altered and proliferation is frequently affected. It may just be that

the oncogenic hits required to achieve each step might be more numerous than initially expected. This model will apply to cases with intermediate or normal cytogenetic risk; a different leukemogenesis pathway linked to genetic instability may be involved in cases with *TP53* mutation and complex karyotype [112].

Considering all this, several pathways to leukemia can be envisaged (Figure 4). The first pathway could be direct and trigger *de novo* AML with a gene fusion as the major event and few other alterations. The second pathway is characterized by *NPM1* mutations, which are rarely associated with mutations in other known TFs or ERs except in *DNMT3A* and *IDH1/2* [23,101]. AML with complex karyotype can derive from genetic instability, with or without *TP53* mutations. A fourth pathway would be the accumulation of several hits in signaling molecules, TFs, ERs and splicing factors, which induce either secondary AML after a chronic phase (Figure 5) or *de novo* AML; however, some so-called *de novo* AMLs with several ER mutations could actually be secondary to a non-detected chronic phase. Mutations in TFs and ERs are not major events in chronic myeloid leukemia (CML), which is triggered by the *BCR-ABL1* fusion; however, mutations in ERs such as *ASXL1, IDH1/2* and *TET2* may participate to CML progression to AML [113,114].

Overall, the development of an AML may follow a "slot machine" model (Figure 6), in which the late steps would be, to some point, constrained by the initial ones (clonal dominance, cooperations/exclusions). Oligoclonality would be due to several possible draws at each step. It is important that we determine the exact number of "reels" (hits) and "symbols" (genes) and the possible combinations.

Utilizing molecular leukemogenesis

Understanding and modeling leukemogenesis will have a major impact on the management and treatment of

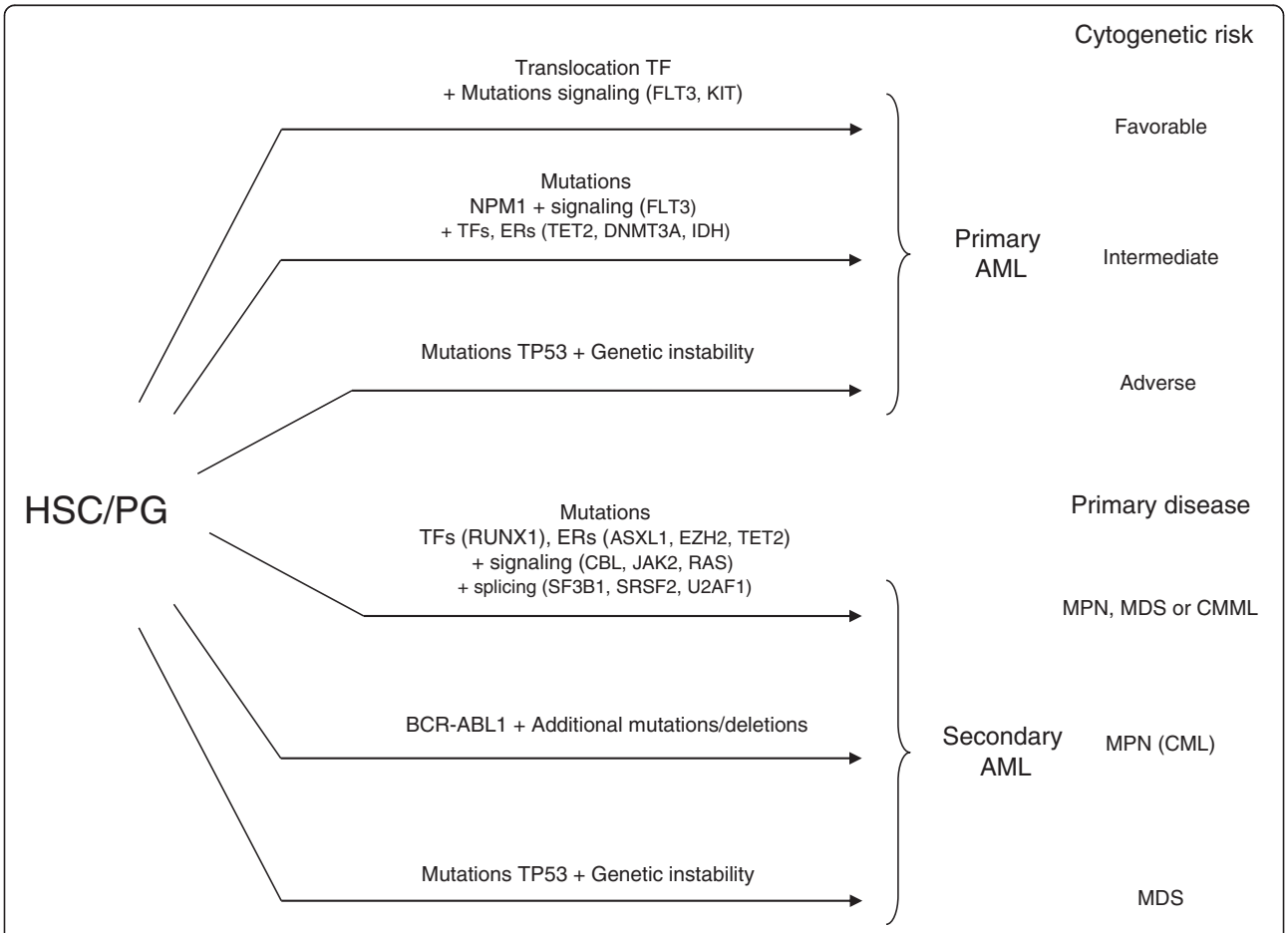

Figure 4 Schematic representation of pathways leading to acute myeloid leukemia (AML) from hematopoietic stem cell (HSC) or progenitors (PG). Gene fusions and *NPM1* mutation are major events in the induction of primary AMLs with favorable and intermediate cytogenetic risk (they correspond respectively to mutation groups A and B of Ley et al [24], and to mutation groups 2 + 3 and 1 of Shen et al. [101]. Secondary AML following MPN or MDS (see Figure 1) could occur after a series of gene mutations in transcription factors and epigenetic regulators combined with a mutation in a signaling pathway (see Figure 3), after TP53 mutation and a series of mutations and karyotype alterations due to genetic instability, or after additional mutations in BCR-ABL chronic myeloid leukemia.

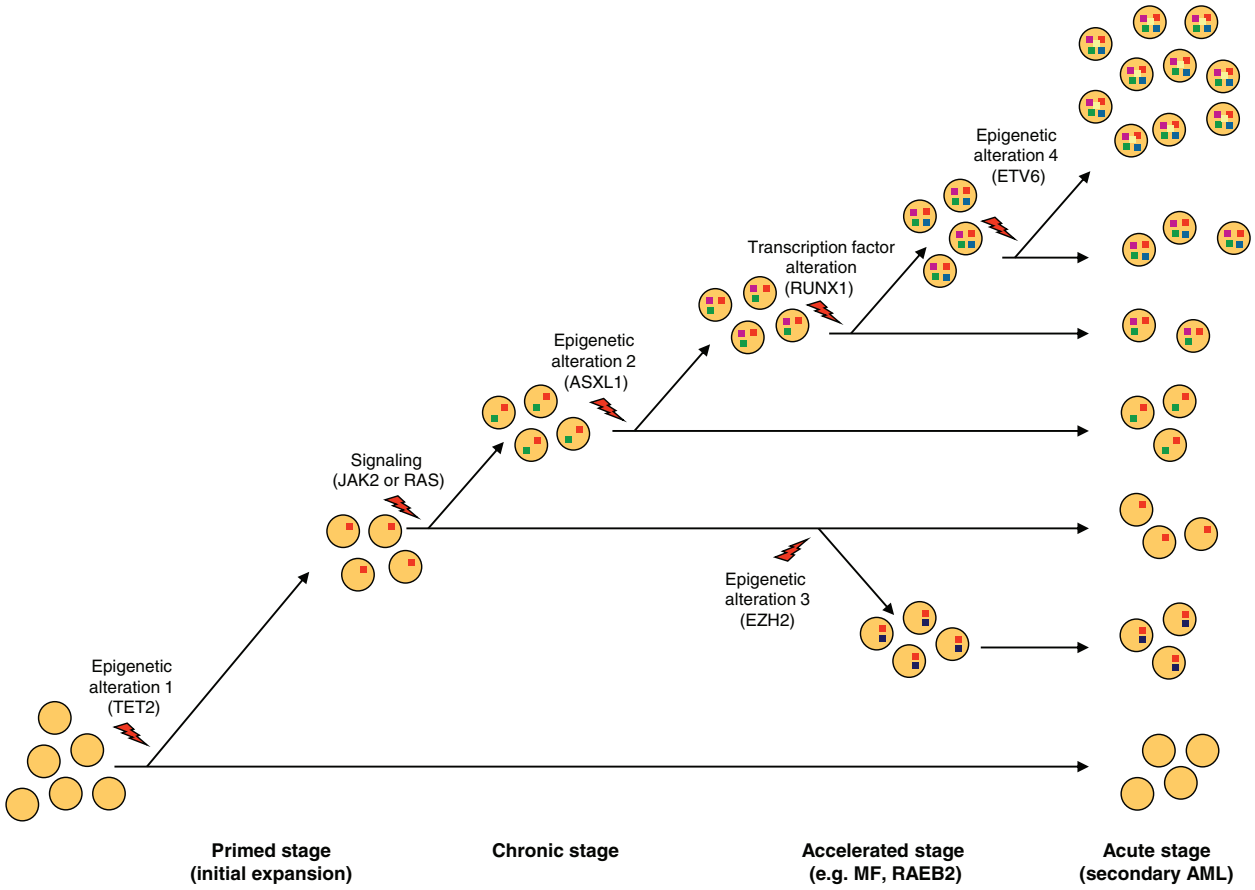

Figure 5 Schematic representation of a case of malignant myeloid disease evolving in four stages along one pathway. Clones with different gene mutations (color squares in cells) represent various ratios of the oligoclonal leukemia. The order and nature of the mutations (or genome alterations) is given as an example and may differ from one case to another. However, in contrast to JAK2V617F, which has a mild effect on hematopoietic stem cell (HSC) [16], *TET2* mutation has the property to initiate the amplification of HSC and to pave the way to secondary mutations [77]. Mutations in signaling molecules, which have a major impact on the disease phenotype, will vary with the type of chronic stage, for example it could affect *JAK2* in case of MPN, *RAS* in case of MP-CMML and be absent in case of MDS. MF: myelofibrosis, RAEB: refractory anemia with excess of blasts, AML: acute myeloid leukemia.

hematopoietic malignancies. Molecular biology already helps establish the diagnosis (*JAK2*), classification (*BCR-ABL1, FGFR1, PDGFRs*), prognosis (*FLT3, NPM1, CEBPA*) and treatment (BCR-ABL, 5q-, JAK2) of myeloid diseases. Due to the increasing simplification and accessibility to clinical laboratories of NGS equipment, the repertoire of all genetic alterations will soon be determined for any new case as a routine practice.

The establishment of a precise taxonomy comprising homogeneous pathophysiological entities is a major goal in hematology. It relies heavily on molecular data. It started with the karyotype and has continued with gene expression profiles [115,116]. Gene mutations will nicely complete the picture. Other factors such as microRNAs and long non-coding RNAs status [1], methylation profiles [117] and histone marks may have to be integrated too.

Several studies have shown that gene mutations have indeed a major impact on prognosis of myeloid diseases. This is the case in MDSs for mutations in five genes, *ASXL1, ETV6, EZH2, TP53* and *RUNX1* [58]. Mutations in *ASXL1* seem to be associated with an aggressive phenotype in all myeloid malignancies [8]: they are frequent in high-risk MDSs and correlate with poor prognosis in MDSs [118–120] and with acute progression in CMML [36], they are more frequent in myelofibrosis than in other MPNs [37,121], and characterize secondary AML. *DNMT3A* mutations are frequent in younger patients with AML and are associated with an unfavorable prognosis in MDS and AML [15,24,30,101]. Among splicing gene mutations, those in *U2AF1* and *SRSF2* seem to be associated with aggressive forms of myeloid diseases and those in *SF3B1* with good prognosis [38,39,41,42]. Molecular data will allow the establishment of an upgraded index of prognosis. For example, in MDSs, it is highly conceivable that the current prognostic index used for the evaluation of the disease (IPSS), which already

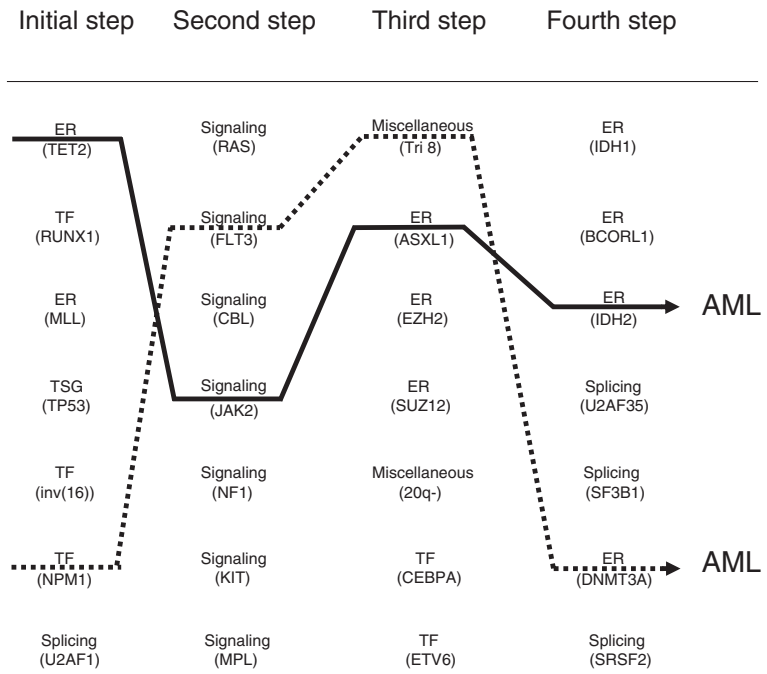

Figure 6 "Slot machine" model of leukemogenesis. Alterations in signaling molecules, transcription factors (TFs), epigenetic regulators (ERs), tumor suppressors (TSG), spliceosome components and various genome abnormalities (examples are given) fall into (at least) four "reels" (steps) that combine to induce a malignant myeloid disease. Acute myeloid leukemia (AML) results from one of the allowed combinations of four (at least) cooperating alterations. At chronic stages, the steps are variably combined, some may be absent (e.g. signaling), some may be specific (e.g. *SF3B1* splicing mutations in RARS). Each step can be achieved by alterations in one of several genes. The initial step leads to expansion of a founding clone. Two examples of draw (plain and dotted lines) leading to AML are shown.

includes karyotypic data, can be improved by a molecular index regrouping the mutations that impact on the patient's outcome [112]. Whether *TET2* mutations are to be included is a matter of debate [122–124]. In AML, a thorough study of 18 genes, including *ASXL1* and *DNMT3A*, proposed an updated and precise risk stratification based on gene mutations [125].

New therapeutic targets can be found in two of the five major classes of leukemogenic genes. Following the successful use of imatinib in CML, abnormal signaling pathways associated with myeloproliferation, be it the JAK-STAT pathway [126–128] or another pathway, represent appealing targets. Drugs targeting epigenetic modifications, i.e. epidrugs, such as histone deacetylase inhibitors and hypomethylating agents (DNMT inhibitors), are currently developed or used in clinics, and many new ones are studied in preclinical assays and clinical trials [1]. Targeting histone methyltransferases (e.g. MLL) or lysine acetyltransferases (e.g. P300) [129] is also a promising area of development. The determination of gene mutations and their consequence on gene regulation and cell programming will help treat myeloid malignancies in providing a rationale for the use and development of new epidrugs, in directing the choice of the drug cocktails, and in allowing the design of drug delivery and the monitoring of drug response and disease progression. For example, agents directed against TET2-, IDH- and DNMT3A-associated methylation defects may represent a new area of development. To date, the use of *TET2* mutations status to evaluate the response to DNMT inhibitors is still debated [130,131]. Because many mutations compromise PRC2 function drugs antagonizing this defect hold great promise.

Proteins of two other leukemogenic classes may also serve as therapeutic targets. For example, the antitumor macrolide pladienolide targets SF3B1 [132] opening new opportunities to develop treatments against RARS. Compounds aiming at restoring a normal P53 pathway are in development [133,134].

The existence of concomitant mutations is an incentive for combinatorial therapies; for example, therapeutic synergy may be obtained by the combined use of signaling inhibitors and epidrugs.

Finally, the complete determination of the mutation repertoire will provide novel therapeutic targets. For some diseases, such as CMML, it is already possible to identify at least one target for nearly nine cases out of ten [33,36]. However, the development of resistance, as observed with imatinib [135], is a critical issue.

Hopefully, target identification will allow for the development of new combinatorial strategies, such as the one based on synthetic lethality [136,137]. If two mutations never occur together it may mean that their combined effect is deleterious. Thus, opportunities for deriving synthetic lethality drugs could stem from the observation of exclusions in mutations patterns.

Conclusions

Thus, mutations and models ("M and Ms") will help manage myeloid malignancies. The eventual comprehensive determination in any given case and at diagnosis, of the set of altered genes, underlying affected pathways and disease stage, will guide towards an optimal treatment based on an appropriate combination of drugs targeting the various affected processes of the disease. Clinically-oriented laboratories should already be preparing for that challenge. Meanwhile, there is much to mull over the "M and Ms" of myeloid malignancies.

Competing interests

The authors have no competing interests.

Author's contributions

All authors have contributed ideas, discussions, and have participated in the writing of the manuscript. All authors read and approved the final manuscript.

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Article 8 - Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases.

ARTICLE n°8

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REVIEW Open Access

Mutations in *ASXL1* are associated with poor prognosis across the spectrum of malignant myeloid diseases

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Abstract

The *ASXL1* gene is one of the most frequently mutated genes in malignant myeloid diseases. The ASXL1 protein belongs to protein complexes involved in the epigenetic regulation of gene expression. *ASXL1* mutations are found in myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML). They are generally associated with signs of aggressiveness and poor clinical outcome. Because of this, a systematic determination of *ASXL1* mutational status in myeloid malignancies should help in prognosis assessment.

Keywords: *ASXL1*, Gene mutations, Myeloid diseases

Mutations in the *ASXL1* (additional sex combs like 1) gene were first reported in 2009 in myelodysplastic syndromes [1]. *ASXL1* maps to chromosome region 20q11, close to the *DNMT3B* gene, and belongs to a family of three paralogs. *ASXL1* comprises 12 exons and is expressed in most hematopoietic cell types.

Function of the ASXL1 protein

ASXL1 codes for a nuclear protein of 1084 residues characterized by an N-terminal helix-turn-helix domain, HARE-HTH [2], and an unusual C-terminal plant homeodomain (PHD), which may bind methylated lysines (Figure 1). The central part of ASXL1 contains an ASXH globular domain that may interact with a polycomb-associated deubiquitinase (DUB) [2,3]. ASXL1 regulates epigenetic marks and transcription through interaction with polycomb complex proteins and various transcription activators and repressors [3-5]. In *Drosophila*, ASX forms a complex with the ubiquitin carboxy-terminal hydrolase calypso to constitute the recently identified polycomb repressive deubiquitinase (PR-DUB) complex [3,6]. Human wild-type ASXL1 associates with the calypso ortholog BAP1 [7]. The calypso/BAP1 DUB deubiquitylates K119ub on histone H2A, leading to gene repression. However, the role of ASXL1 in leukemogenesis does not seem to be mediated by the DUB complex [7]. Recent data have shown that ASXL1 interacts with components of the polycomb complex PRC2, namely EZH2 and SUZ12, two proteins involved in the deposition of H3K27me3 histone repressive marks. These two PRC2 components are also mutated in myeloid malignant diseases [8-11]. Inhibition of ASXL1 function leads to loss of H3K27me3 histone marks. ASXL1 role could be to recruit the PRC2 complex to known leukemogenic loci such as *HOXA* genes [7]. ASXL1 also associates with HP1α/CBX5, a component of the heterochromatin repressive complex [6,12]. HP1α binds to histone H3. JAK2 phosphorylates histone H3 and excludes HP1α from chromatin [13]. Thus, a potential functional link may exist between *ASXL1* and *JAK2* mutations but this remains to be demonstrated.

The functions of the other ASXL proteins are poorly defined. ASXL2 has been shown to regulate heart [14] and bone development, as well as adipogenesis. Mouse ASXL2 has been identified as a regulator of bone mineral density and osteoclastogenesis [15] and whereas ASXL1 represses, ASXL2 increases the expression of

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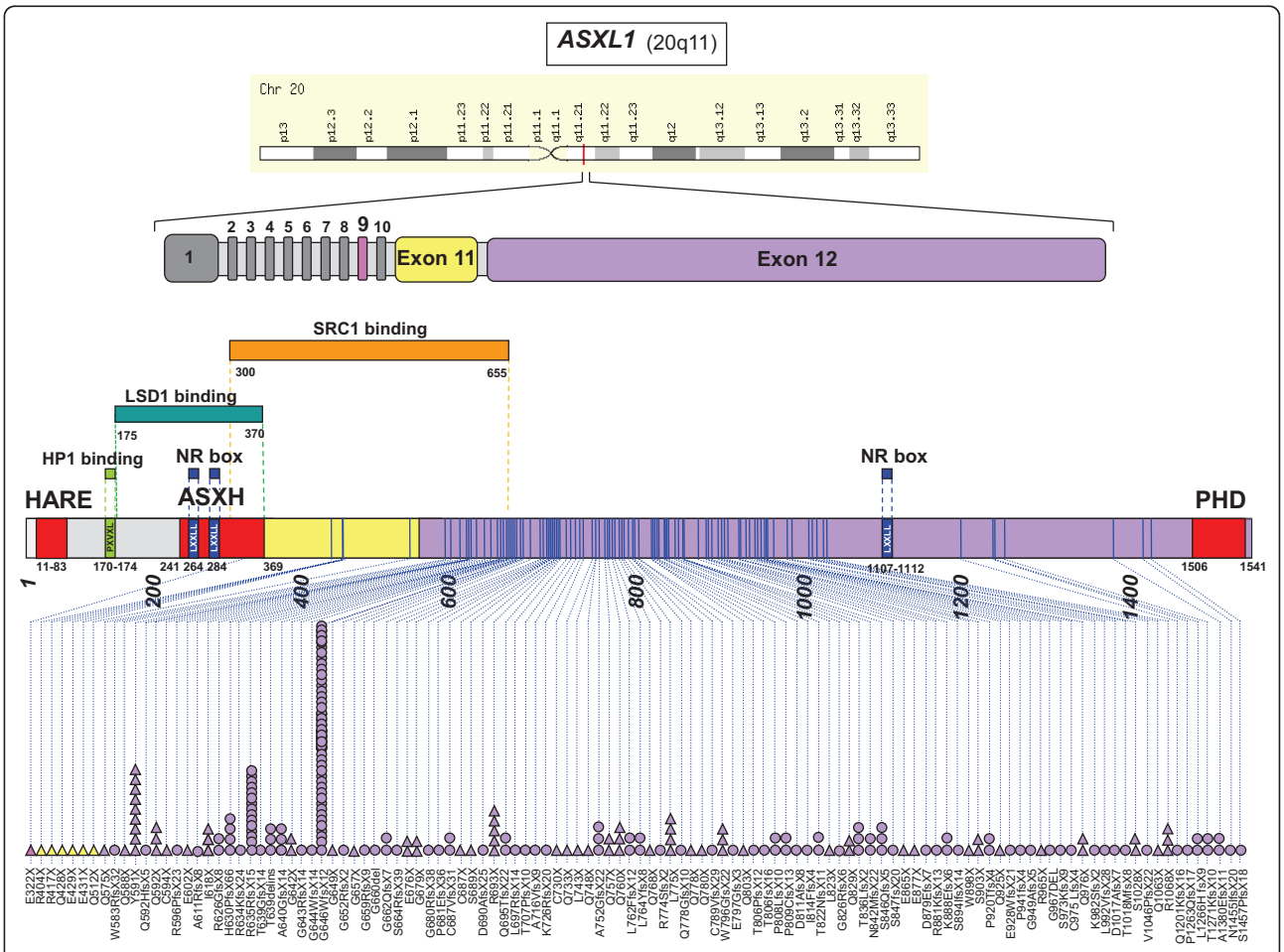

Figure 1 Distribution of ASXL1 mutations along the protein. From top to bottom are shown the localization of the *ASXL1* gene on chromosome region 20q11, the exon structure of *ASXL1*, and the ASXL1 protein with its conserved motifs and binding regions: HARE helix-turn-helix at the N-terminus, HP1/CBX5 binding region, ASXH, an α-helical domain that contains LXXLL (nuclear receptor boxes), and the C-terminal plant homeodomain (PHD) finger. Below reported mutations (see Table 1) are shown along the protein: circles and triangles indicate frameshift and nonsense mutations, respectively, and the colors correspond to the exon location.

adipogenic genes [16]. ASXL3 expression and functions remain to be determined [17].

ASXL1 and concomitant mutations in myeloid malignancies

The vast majority of the *ASXL1* mutations found in myeloid malignancies affect the twelfth exon of the gene although rare mutations in other exons have been detected [18]. *ASXL1* mutations are frameshift and nonsense mutations that are supposed to result in C-terminal truncation of the protein upstream of the PHD finger (Figure 1). The functional relevance of some reported missense mutations is not clear. The most frequent mutation, which accounts for more than 50% of all *ASXL1* mutations, is a duplication of a guanine nucleotide (c.1934dupG); it leads to a frameshift (p.Gly646TrpfsX12). One study has described this mutation as a PCR artefact [19], but because it is not found in germ-line DNAs, control DNAs or other studied types of cancers such as breast cancer, it is now generally considered to be a *bona fide* mutation.

ASXL1 mutations are usually heterozygous, suggesting that haplo-insufficiency is the key pathological factor; however, the truncated ASXL1 protein could also have a dominant negative role in titrating out an interacting protein. Actually, recent data have demonstrated a loss of ASXL1 protein in leukemia samples with ASXL1 mutation, indicating that these mutations are loss-of-function disease alleles [7].

ASXL1 is mutated in all types of malignant myeloid diseases, including myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML). According to the series studied, *ASXL1* mutation frequency varies from a few percent to more than 50% of cases (Table 1). *ASXL1* mutations are most

Table 1 Mutations in *ASXL1* gene in published studies

Selected Ref.	MDS n (%)	CMML n (%)	MPN n (%)	Secondary AML n (%)	*De novo* AML n (%)
*Abdel-Wahab et al., [25]				12/63 (19.3)	
*Abdel-Wahab et al., [18]		3/24 (12.5)	3/46 PMF (6.5)		
Béjar et al., [21]	63/439 (14.4)				
Brecqueville et al., [26]			17/149 (11.4): 6/30 PMF (20), 2/30 PV (7), 2/53 ET (4),		
Boultwood et al., [5]	28/182 (15.4)	17/51 (33.3)		9/40 (22.5)	8/27 (29.6)
Boultwood et al., [27]			+6/41 (CML) (14.6)		
Carbuccia et al., [28]			5/64 (7.8)		
Carbuccia et al., [20]				9/17 (58)	3/46 (6)
Chou et al., [29]					54/501 (10.8)
Gelsi-Boyer et al., [1]	4/35 (11.4)	17/39 (43.6)			
Gelsi-Boyer et al., [30]		25/53 (47.2)			
Grossmann et al., [31]		41/79 (52)			
Jankowsa et al.,[32]		24/52 (46)			
Pratcorona et al., [33]				3/24 (12.5)	35/775 (4.5)
Ricci et al.,[34]			23/42 PMF (54.8)		
Rocquain et al., [22]	13/65 (20)			9/18 (50)	3/46 (6.5)
Shen et al., [35]					27/605 (4.5)
Stein et al., [36]			12/47 PMF (25.5) 1/42 PV (2)		
Thol et al., [37]	40/193 (20.7)				
Total*	**148/914 (16.2)**	**124/274 (45)**	**41/119 PMF (34.5)**	**30/99 (30.3)**	**130/2000 (6.5)**

* not included in final count because p.Gly646TrpfsX12 had not been taken into account; + including CML cases

frequent in CMML (~ 45%). In MPNs, they are frequent in primary myelofibrosis (PMF)(34.5%) and rare in polycythemia vera (PV) or essential thrombocythemia (ET). In AML, they are found in secondary (30%) rather than in *de novo* cases (6.5%), and in AML with normal karyotype *ASXL1* mutations are mutually exclusive with *NPM1* mutations [20]. *ASXL1* is the second most frequently mutated gene in MDSs after *TET2* [21]. In MDSs, *ASXL1* mutations are more frequent in refractory anemia with excess of blasts (RAEB) than in the other forms such as refractory anemia with ring sideroblasts (RARS) [1,5,22]. *ASXL1* mutations are further detected in rare cases of juvenile myelomonocytic leukemia (JMML) [23] and in RARS-T [24].

With the exception of *NPM1* and *FLT3*, it seems that *ASXL1* mutations coincide with mutations in many known genes including *EZH2* [18], *IDH1/2, RUNX1* and *TET2* [21,22]. Although ASXL1 functions are related to the PRC2 complex, which includes EZH2, *ASXL1* and *EZH2* mutations are not mutually exclusive [18,38]. *ASXL1* mutations can also cooperate with mutations in genes encoding signaling (*CBL, JAK2, NF1, RAS*) and splicing proteins (*SF3B1, SRSF2, U2AF35*). For example, in MDSs, *ASXL1* mutations are more frequent in *U2AF35*-mutated patients than in *U2AF35* wild-type patients [39]. In MPNs, *ASXL1* mutations are found with the same frequency in JAK2V617F and JAK2 wild-type cases [26,36]. In MDSs, *ASXL1* mutations are often associated with *RUNX1* mutations, and, in AMLs, with *RUNX1* and *CEBPA*. [29,33,40].

Other alterations in *ASXL1, ASXL2* and *ASXL3*

Few deletions of the gene have been reported and *ASXL1* is generally not included in the more telomeric 20q13 deletion that is often observed in myeloid diseases. The *ASXL1* gene can be translocated and fused to the *PAX5* gene in acute lymphoblastoid leukemia [41] and altered by germ-line mutations in the Bohring-Opitz syndrome; this severe syndrome leads to death at an early age preventing to know whether susceptibility to hematopoietic diseases might result from *ASXL1* germ-line mutations [42]. In recent genome sequencing studies rare mutations in *ASXL1* and *ASXL3* have also been found in chronic lymphocytic leukemia [43] but not in T-cell acute leukemia [44]. Mutations in *ASXL2* and *ASXL3* have not been found in myeloid diseases so far, but *ASXL2-MYST3* and *EPC1-ASXL2* fusions have been identified in myelodysplastic syndrome and T-cell acute leukemia, respectively [45,46]. Both MYST3 and EPC1 are epigenetic regulators and these fusion proteins probably disrupt epigenetic protein complexes.

Animal models of ASXL1 loss

In a first model of *Asxl1* gene knock-out in the mouse ASXL1 loss mildly perturbed myelopoiesis but did not trigger an actual hematological malignancy [47].

However, the effect of the absence of ASXL1 protein may have been masked by partially penetrant perinatal lethality. In another, more recent model of conditional *Asxl1* gene knock-out, the animals developed a strong hematopoietic phenotype consistent with an MDS with myeloproliferative features. In cooperation with NRAS oncogenic mutation the absence of ASXL1 triggered an MDS/MPN. These observations were confirmed by experiments in hematopoietic cells using shRNA directed against *ASXL1*, which were highly coherent with the expected role of ASXL1 in leukemogenesis [7].

ASXL1 mutations in disease evolution

Like *TET2* mutations, *ASXL1* mutations are found in chronic and acute stages of myeloid malignancies. In a study of MPNs, with the exception of a single patient who acquired both *ASXL1* and *TET2* mutations, all patients with *ASXL1* mutation at leukemic transformation already had *ASXL1* mutation at the chronic stage [25]. In a series of secondary AML with multilineage dysplasia we found that in cases resulting from a transformation of a known MDS the same *ASXL1* mutation was present at both the chronic and acute stages (Devillier et al., submitted). These observations suggest that *ASXL1* mutations may constitute early hits in leukemogenesis and precede other alterations such as *JAK2* and *TET2* mutations [24,25,28]. However, there is also evidence to suggest that the opposite is true in some cases. In MPNs, for example, the proportion of *ASXL1* mutations is higher in post-PV myelofibrosis (MF) and post-ET MF than in PV and ET. This suggests that the *ASXL1* mutation may follow a JAK2 mutation and could therefore help predict the risk of evolution from PV and ET to MF [26,36,48]. As such, *ASXL1* mutations may play a crucial role in the pathogenesis of PMF, as well as in the molecular progression from the chronic phase of a previous PV or ET to MF. Finally, in MDSs and CMML, *ASXL1* mutations seem to be present in chronic phases and precede transformation and in rare cases, *ASXL1* mutations can be lost or acquired during relapse of *de novo* AML [29].

ASXL1 mutations in disease outcome

A number of studies have linked *ASXL1* mutations to the outcome of malignant myeloid diseases. In a study of MPNs based on the DIPSS-plus score [49] (Dynamic International Prognostic Scoring System for primary myelofibrosis), *ASXL1* mutation tended to be associated with an aggressive disease and a poor overall survival [26]. In a large study of PMF patients *ASXL1* mutations were associated with shorter overall survival [50]. In CMML, the presence of an *ASXL1* mutation could help predict transformation to AML [30]. In MDSs, *ASXL1* mutations are associated with a reduced time to progression in AML and constitute an independent prognostic marker [37]. Finally, a study of 18 genes in a large cohort of MDSs showed that mutations in 5 genes had prognostic impact: *TP53, EZH2, ETV6, RUNX1* and *ASXL1* [21]. Coupled with the standardized international prognostic scoring system (IPSS), mutations in these five genes could help refine the prognosis evaluation of MDSs.

By contrast, a study of a large cohort of 605 AML cases without cytogenetic prognostic markers other than 11q23 abnormalities, reported that *ASXL1* mutations were not associated with outcome [35]. However, they were associated with shorter overall survival in patients with intermediate-risk AML [29,33]. A recent study of 476 cases with intermediate-risk *de novo* AML showed that *ASXL1* mutations have a major impact on outcome [51]. According to the current European LeukemiaNet (ELN) guidelines for the diagnosis and management of AML, AMLs with normal karyotype are classified into two genetic categories based on their *NPM1, FLT3*-ITD and *CEBPA* mutation status: the ELN Favorable category is defined as mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-IT; all remaining cases (ie, those with wild-type *CEBPA*, and wild-type *NPM1* with or without *FLT3*-ITD or mutated *NPM1* with *FLT3*-ITD) form the ELN Intermediate-I category [52,53]. *ASXL1* mutations have been associated with inferior survival among ELN Favorable, but not among ELN Intermediate-I patients [40]. Taken together, these data show that *ASXL1* mutations have prognostic value in certain subgroups of AML patients.

Conclusion

In almost all studies, and whatever the type of myeloid malignancy, *ASXL1* mutations are associated with adverse features including, but not limited to myelodysplasia, myelofibrosis or progression to AML. Systematic detection of *ASXL1* mutations could thus help in the assessment of disease and should perhaps be implemented in routine practice, whether associated with already systematically-surveyed mutations (*CEBPA, JAK2, FLT3, NPM1*) or in upcoming systematic genome analyses.

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Authors' contributions

All authors have contributed ideas, discussions, and have participated in the writing of the manuscript. All authors read and approved the final manuscript

Competing interests
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Article 9 - Molecular similarity between myelodysplastic form of chronic myelomonocytic leukemia and refractory anemia with ring sideroblasts.

ARTICLE n°9

Molecular similarity between myelodysplastic form of chronic myelomonocytic leukemia and refractory anemia with ring sideroblasts.

Gelsi-Boyer V, Cervera N, Bertucci F, Brecqueville M, Finetti P, Murati A, Arnoulet C, Mozziconacci MJ , Mills K, Vey N & Birnbaum D.

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Molecular similarity between myelodysplastic form of chronic myelomonocytic leukemia and refractory anemia with ring sideroblasts

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ABSTRACT

Chronic myelomonocytic leukemia is similar to but a separate entity from both myeloproliferative neoplasms and myelodysplastic syndromes, and shows either myeloproliferative or myelodysplastic features. We ask whether this distinction may have a molecular basis. We established the gene expression profiles of 39 samples of chronic myelomonocytic leukemia (including 12 CD34-positive) and 32 CD34-positive samples of myelodysplastic syndromes by using Affymetrix microarrays, and studied the status of 18 genes by Sanger sequencing and array-comparative genomic hybridization in 53 samples. Analysis of 12 mRNAS from chronic myelomonocytic leukemia established a gene expression signature of 122 probe sets differentially expressed between proliferative and dysplastic cases of chronic myelomonocytic leukemia. As compared to proliferative cases, dysplastic cases over-expressed genes involved in red blood cell biology. When applied to 32 myelodysplastic syndromes, this gene expression signature was able to discriminate refractory anemias with ring sideroblasts from refractory anemias with excess of blasts. By comparing mRNAS from these two forms of myelodysplastic syndromes we derived a second gene expression signature. This signature separated the myelodysplastic and myeloproliferative forms of chronic myelomonocytic leukemias. These results were validated using two independent gene expression data sets. We found that myelodysplastic chronic myelomonocytic leukemias are characterized by mutations in transcription/epigenetic regulators (*ASXL1, RUNX1, TET2*) and splicing genes (*SRSF2*) and the absence of mutations in signaling genes. Myelodysplastic chronic myelomonocytic leukemias and refractory anemias with ring sideroblasts share a common expression program suggesting they are part of a continuum, which is not totally explained by their similar but not, however, identical mutation spectrum.

Introduction

Chronic myelomonocytic leukemia (CMML) is a malignant hematologic disease of the elderly characterized by peripheral blood monocytosis, overproduction of bone marrow monocytes with dysplasia of one or more lineages, and less than 20% of blasts in the bone marrow. Its prognosis is poor with a median survival of 12-18 months and a 15-20% risk of transformation into acute myeloid leukemia (AML).[1,2] CMML is classified by the World Health Organization (WHO) into the myelodysplastic/myeloproliferative neoplasms and, based on the number of blasts, subclassified into CMML1 and CMML2 (5-9% and 10-19%, respectively).[3] Like myelodysplastic syndromes (MDS), CMML shows dysplastic features that reflect ineffective hematopoiesis; however, dysplasia is associated with bone marrow proliferation.[4,5] Because of this duality, CMML had been separated into a myeloproliferative form (MP-CMML) and a myelodysplastic form (MD-CMML) based on a semi-arbitrary threshold of 13 x 10^9/L for peripheral white blood cell (WBC).[6] However, due to its lack of impact on outcome, this separation is not included in the WHO classification.[3] Yet, the prognosis of MD-CMML but not MP-CMML may be evaluated by the international prognostic scoring system, underlining a similarity of MD-CMML with MDS. Moreover, even if, given the limited treatments currently available, MD and MP-CMMLs have similar outcome, this situation may change with the advent of new therapies, in which case they would each need to be recognized separately.

Because CMML has both dysplastic and proliferative features it is likely that the disease is heterogeneous. We wanted to determine whether these MD and MP features may have any relevant molecular basis that may help classify and understand CMML. To this aim, we established the gene expression profiles and the mutational status of CMML and compared them to those of MDS.

Design and Methods

Patients and samples

We selected 53 CMML and 32 MDS bone marrow (BM) samples previously studied by array-comparative genome hybridization

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(aCGH) and sequencing of candidate genes.[7,8] According to the French-American-British (FAB)[6] and WHO[3] classifications, the CMML series was made up of 31 MP and 22 MD cases (*Online Supplementary Table S1*) and the MDS panel 8 refractory anemia (RA) with ring sideroblasts (RARS), 13 RA with excess of blasts type 1 (RAEB1) and 11 RAEB type 2 (RAEB2). CMML and MDS cases selected for gene expression profiling were collected at the time of diagnosis or in therapeutic abstention; none had been treated. All patients signed an informed consent for research and the study was approved by our institutional review board ("Comité d'Orientation Scientifique" of the Institut Paoli-Calmettes).

CD34 enrichment

Samples were enriched in CD34-positive ($CD34^+$) cells for 12 CMML and 32 MDS cases. Leukocytes were obtained after bone marrow red cell lysis and washing with PBS, and labeled with magnetic bead-conjugated anti-CD34 monoclonal antibody (AC34 MicroBead; Miltenyi Biotec, Auburn, CA, USA). $CD34^+$ hematopoietic stem cell populations were then purified through a miniMACS magnetic cell separation column (Miltenyi Biotec).

RNA/DNA extraction

RNAs and DNAs were extracted from whole BM CMML samples. After BM aspiration, a red cell lysis was carried out, followed by rinses with PBS. Leukocytes were processed immediately or cryopreserved at –80°C at the sample bank of the Institute and processed later. DNA and RNA were extracted using Nucleobond RNA/DNA kit from Macherey-Nagel (Düren, Germany) as recommended by the supplier. RNA from $CD34^+$ cells were similarly extracted using Nucleobond RNA/DNA kit from Macherey-Nagel.

Sequencing of 18 candidate genes

Mutations in *ASXL1* (exon 12), *CBL* (exons 8, 9), *DNMT3A* (exons 15-23), *EZH2* (all exons), *FLT3* (exons 14, 15, 20), *IDH1/2* (exons 4), *JAK2* (exon 14), *NF1* (exons 1-50), *N/KRAS* (exons 1, 2), *PTPN11* (exons 3, 11)), *RUNX1* (exons 1-8), *SF3B1* (exons 12-16), *SUZ12* (exons 14-16), *SRSF2* (exon 2), *TET2* (exons 3-11), *U2AF35/U2AF1* (exons 2, 6), and *ZRSR2* (exons 1-11) were analyzed using BM DNA as previously described.[7-11]

Gene expression profiling

Gene expression profiles of 39 CMML (out of the 53) and 32 MDS (all from $CD34^+$ cells) mRNAs were established. Among the 39 CMML cases, 37 were studied as BM (10 of these were also studied as $CD34^+$) and 2 as $CD34^+$ RNAs. In other words, 10 CMML samples were studied as both $CD34^+$ and whole BM RNAs, and 2 as $CD34^+$ only (12 $CD34^+$ in total).

RNA quality and purity were assessed with Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Preparation of cRNA, hybridizations onto Affymetrix U133 Plus 2.0 human oligonucleotide microarrays, washes and detection were carried out as recommended by the supplier and as previously described.[12] Data were analyzed by the Robust Multichip Average (RMA) method in R using Bioconductor and associated package, as previously described.[12] Before analysis, a first filtering process removed from the data set the probe sets with low and poorly measured expression as defined by an expression value inferior to 100 units, thus retaining 19,730 probe sets in the 12 CMML $CD34^+$ data set and 23,515 probe sets in the 32 MDS $CD34^+$ data set.

Before hierarchical clustering, a second filter, based on the intensity of standard deviation (SD >0.5), was applied and retained 9179 probe sets in the 37 CMML from the whole BM data set, 12,660 probe sets in the 12 CMML $CD34^+$ data set, and 11,623 probe sets in the 32 MDS $CD34^+$ data set. Filtered data were then log2-transformed and submitted to the Cluster program using data median-centered on genes, Pearson's correlation as similarity metrics and centroid linkage clustering. Results were shown using the TreeView program.

Supervised analyses identified and ranked genes that discriminate two groups of samples. A discriminating score (DS) was calculated for each of the 19,730 probe sets for the 12 CMML and of the 23,515 probe sets for the 32 MDS.[13] A 'leave-one-out' (LOO) cross-validation procedure was applied to estimate the accuracy of prediction of the signature and the validity of the supervised analysis. Functional processes and pathways were identified using Ingenuity software (Ingenuity Systems, Redwood City, CA, USA).

To test the performance of our signature on independent panels, we analyzed publicly-available external data sets[14,15] collected from NCBI/Genbank GEO database (series entry GES4619 and entry GES15061). Gene set enrichment analysis (GSEA) was carried out as reported.[16] Fisher's exact test was used when appropriate. All statistical tests were two-sided at the 5% level of significance. All statistical analyses were carried out in R (2.8.0) and its associated packages.

Results

Gene expression analysis separates MD- from MP-CMML cases

We first determined the gene expression profiles of 37 BM CMML samples. Unsupervised analysis identified two clusters (S1 and S2) including 17 and 20 cases, respectively (*Online Supplementary Figure S1*). S1 and S2 cases did not correlate with clinical or hematologic data and were not separated according to MD/MP features. We next determined the gene expression profiles of 12 available RNAs from $CD34^+$ CMML samples (5 MD and 7 MP). Hierarchical clustering separated the 12 samples into two clusters (*Online Supplementary Figure S2*). The two clusters differed (Fisher's exact test, P=0.04) in terms of leukocytosis and overlapped the MP/MD definition: the left cluster contained 4 of 5 MD-CMML cases whereas the right cluster comprised 6 of 7 MP-CMML cases (black boxes). This showed that the MD/MP distinction has a molecular basis at the transcriptional level on a whole-genome scale.

MD-CMML over-expresses genes involved in red blood cell biology as compared to MP-CMML

To understand this MD/MP difference, we compared the gene expression profiles from the 5 MD-CMML samples to those of the 7 MP-CMML samples in a supervised analysis. A total of 122 probe sets (corresponding to 96 unique genes and 6 ESTs; *Online Supplementary Table S2*) were differentially expressed between the two forms. The accuracy of prediction and validity of our procedure was cross validated by LOO with overall accuracy of 92% (Fisher's exact test, P=0.015) with high sensitivity and specificity (86% and 100%; only one MP-CMML was misplaced) and with a theoretical number of false positive of 30.

Among the 122 probe sets, 61 were up-regulated and 61 were down-regulated in the MP samples (the top 20 up-regulated genes are listed in the *Online Supplementary Table S3*). Inspection of the list (hereafter called MD/MP CMML gene expression signature or CMML GES) showed that up-regulated genes in MD-CMML belonged to pathways and cell processes found in red blood cells: they encoded

enzymes involved in heme synthesis (ALAS2, HMBS, FECH), glycophorins (GYPA, GYPB), globins (HBA1, HBB, HBM), and proteins associated with blood groups (RHD, RHCE) and erythrocyte differentiation (TRIM10). Ingenuity analysis of this GES confirmed that the most relevant over-expressed genes in MD-CMML cases were involved in erythropoiesis (*data not shown*). Down-regulated genes in MD-CMML included *ZCCHC11/TUT4, PHC1* and *BMI1*.

We applied this CMML GES to our 12 CD34$^+$ CMML RNAs. As expected, the MD and MP samples were separated (Fisher's exact test, P=1x10^{-3}) (Figure 1A). We applied this GES to the 37 BM CMML RNAs (including 10 of the 10 CD34$^+$ samples and 27 additional samples). Two clusters were observed: in the left cluster, 16 of the 19 samples were MP-CMML, whereas in the right cluster, 13 of the 18 samples were MD-CMML (Figure 1B), supporting the validity of our MD/MP CMML GES (Fisher's exact test;

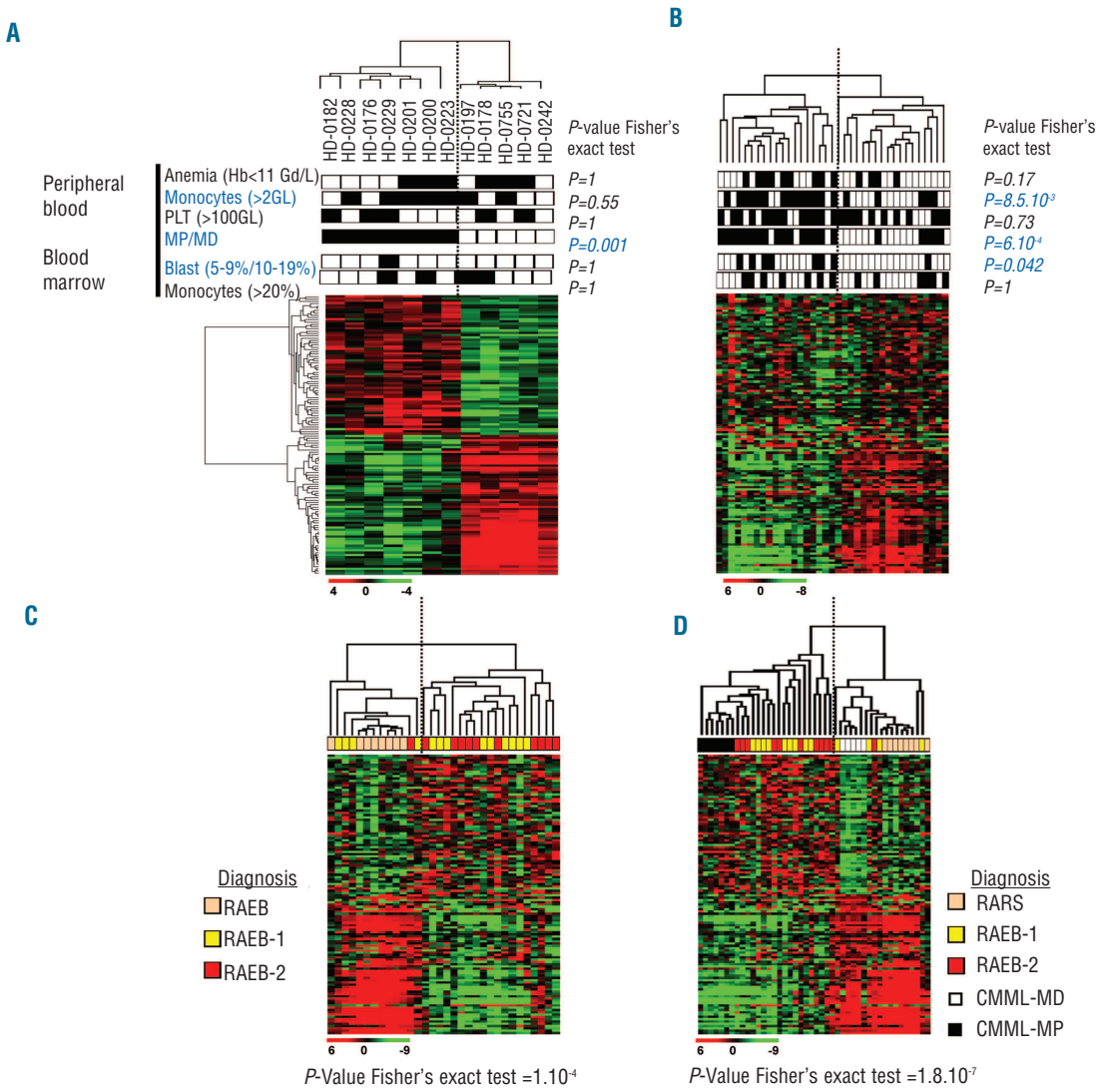

Figure 1. Classification of CMML (A, B) and MDS samples (D, E) using a CMML gene expression signature. (A) Hierarchical clustering of 12 CD34$^+$ CMML RNAs with the 122-gene MD/MP CMML gene expression signature (GES). Each row of the data matrix represents a gene and each column represents a sample. Expression levels are depicted according to the color scale shown at the bottom. Red and green indicate expression levels respectively above and below the median. The magnitude of deviation from the median is represented by the color saturation. Below the horizontal sample dendrogram, are some histoclinical and molecular features of the samples (PLT: platelets), including the MD or MP phenotype (white for MP and black for MD). The GES (vertical dendrogram) classifies the samples into two clusters (black vertical line) associated with the MD/MP definition. The correlation of the two clusters with the histoclinical data is indicated using the *P* value of Fisher's exact test to the right of the stripes (significant *P*-values are in blue). (B) Similar to (A), but applied to 37 bone marrow CMML samples. The genes are not clustered and are ordered as in (A). (C) Hierarchical clustering of 32 CD34$^+$ MDS samples using the 122-gene MD/MP CMML gene expression signature (GES). Legend as in (A). RARS and RAEB are indicated by colored boxes under the dendrogram. The GES significantly distinguishes the RARS from the RAEB samples. (D) As in (C), but applied to the 32 mRNAs from CD34-positive MDS samples and 12 mRNAs from CD34-positive CMML samples. The genes are not clustered and are ordered as in (A). The CMML GES separates the RARS and MD-CMML samples from the MP-CMML and RAEB samples. MD/MP forms are indicated by black and white boxes as indicated.

P=6x10^{-4}). Using GSEA, we confronted the gene expression profiles of our 37 BM CMML mRNAs to the 122 gene signature. We found a significant enrichment in the red cell genes of this signature in the MD-CMML samples (Enrichment Score=0.76; Normalized Enrichment Score=2.08; FDR q-value<0.01).

CMML gene expression signature classifies MDS samples

Overexpression of genes involved in red cell biology has been observed in previous gene expression analyses of RARS samples.[14,17] When applied to our 32 CD34$^+$ MDS samples, the CMML GES perfectly separated RARS from RAEB samples (Fisher's exact test, P=1x10^{-4}, Figure 1C). When the CMML GES was applied to the pool of 12 CMML and 32 MDS CD34$^+$ samples the MD-CMMLs clustered with the RARS samples and the MP-CMMLs with the RAEB samples (Figure 1D) (Fisher's exact test, P=1.8x10^{-7}). These results showed that MD-CMML and RARS share gene similar expression programs.

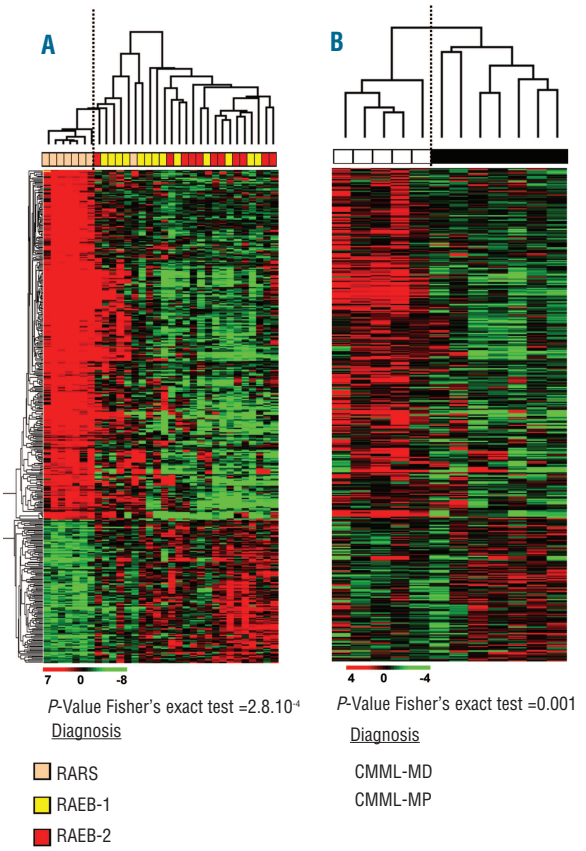

Figure 2. Classification of MDS samples using an MDS gene expression signature. (A) Hierarchical clustering of 32 CD34$^+$ MDS samples using the 428-gene MDS gene expression signature. Legend as in Figure 1A. MDS classes are indicated by colored boxes in the stripe under the dendrogram. The GES significantly distinguishes the RARS from the RAEB samples. (B) As in (A), but applied to the 12 CD34-positive CMML samples. The genes are not clustered and are ordered as in (A). The GES significantly distinguishes the MD from the M$^-$-CMML samples. MD/MP forms are indicated by white and black boxes in the stripe under the dendrogram.

MDS gene expression signature classifies CMML samples

Then we derived an MDS GES by comparing the gene expression profiles of our 8 RARS to those of our 24 RAEB samples. A total of 428 probe sets (295 unique genes and 25 ESTs; *Online Supplementary Table S4*) were differentially expressed between RARS and RAEB (hereafter called MDS GES). The accuracy of prediction and validity of our procedure was cross-validated by LOO with overall accuracy of 78% (Fisher's exact test, P=6x10^{-4}) with high sensitivity and specificity (72% and 100%, respectively) and with a theoretical number of false positive of 5.

A total of 304 probe sets were up-regulated and 124 were down-regulated in the RARS cases (the top 20 up-regulated genes are listed in *Online Supplementary Table S3*). Up-regulated genes in RARS belonged to the pathway and cell processes found in red blood cells (*Online Supplementary Figure S3*): they encoded proteins involved in heme synthesis (ALAS2, ALAD, HMBS, UROD, CPOX, PPOX, FECH), iron-sulfur cluster biogenesis (SLC25A37, GLRX5), mitochondrial biology (TRAK2), antioxidant defense (HAGH), glycophorins (GYPA, GYPB, GYPE), globins (HBA1, HBA2, HBB, HBD, HBG1, HBM, HBQ1), proteins associated with blood groups (RHCE, RHD, DARC, KEL), red cell structure (ANK1, EPB42, EPB49, ERMAP, SPTA1, SLC4A1), differentiation (TRIM10) and regulation (GATA1, KLF1, TAL1, EPOR). Ingenuity analysis of this GES confirmed that the most relevant over-expressed genes in RARS were involved in erythropoiesis (*data not shown*). When applied to our 32 CD34$^+$ MDS RNAs, the MDS GES perfectly separated the RARS from the RAEB samples (Figure 2A), as expected. More surprisingly, when applied to the 12 CD34$^+$ (Figure 2B) or BM CMML mRNAs (Fisher's exact test, P=8.4.10^{-4}; *data not shown*) it perfectly separated the MD-CMML from the MP-CMML samples.

Thus, MD-CMML and RARS share expression of genes involved in erythropoiesis and red blood cell biology, with a total of 38 probe sets corresponding to 25 unique genes common to the MD/MP CMML and MDS GESs (*Online Supplementary Table S3*).

External validation on two independent data sets

To validate these unexpected results, we applied our two GESs (CMML GES and MDS GES) to two external published data sets (a CMML and an MDS data set)[14,15] and a GES obtained from one of these external data sets to our CMML and MDS samples.

First, we studied the external data sets with our two GESs. The first data set corresponded to the expression profiles of 25 CMML BM samples profiled using Affymetrix microarrays.[15] Unfortunately, information on MD/MP forms was not associated with the data. Our CMML GES separated these external CMML cases into two groups, one of which over-expressed red blood cell genes (*Online Supplementary Figure S4A*, stripe 1). All but one of the 25 CMML samples was similarly sorted with our MDS GES (*Online Supplementary Figure S4B*, stripe 2). We studied a second external data set, consisting in the expression profiles of 66 CD34$^+$ MDS samples studied by Affymetrix microarrays.[14] Our MDS GES separated the RARS from the other MDS classes (Figure 3A), as did our CMML GES (Figure 3B).

Second, we derived a third GES (hereafter called MDS-ext GES) by comparing RARS and RAEB samples from the MDS external data set.[14] This MDS-ext GES contained 738

probe sets (597 unique genes and 28 ESTs). The genes found over-expressed in RARS are involved in red blood cell biology. This GES was able to separate our RARS from RAEB samples (*Online Supplementary Figure S4C*) and our MD-CMML from MP-CMML CD34-positive samples (Figure 3C). When applied to the CMML external data set,[15] it separated the 25 samples (stripe 3) in the same way as did our CMML and MDS GESs (*Online Supplementary Figure S4D*).

Thus, GESs obtained from comparison from either CMML or MDS were similarly able to distinguish CMML and MDS classes, showing that MD-CMML and RARS share common molecular features. The CMML GES, MDS GES and MDS-ext GES had 26 probe sets in common (corresponding to 16 genes and 1 EST)(Figure 3D), all overexpressed in MD-CMML and RARS and involved in red cell biology (*Online Supplementary Table S3*). This small core GES separated MD-CMML from MP-CMML (Figure 3E) and RARS from RAEB (*Online Supplementary Figure S5*) as efficiently as the three larger GESs.

Analysis of mutated genes in CMML and MDS

These results showed a molecular similarity between MD-CMML and RARS. Could this similarity be the result of gene mutations common and specific to the two diseases? We[7,8,18] and others[19-36] have previously studied several leukemogenic genes in CMML and RARS. However, several of those (e.g. *ASXL1, RUNX1, TET2*) are neither specific of MD-CMML nor of RARS and, therefore, can account neither for the similarity between the two diseases nor for the differences from the other myeloid malignancies.

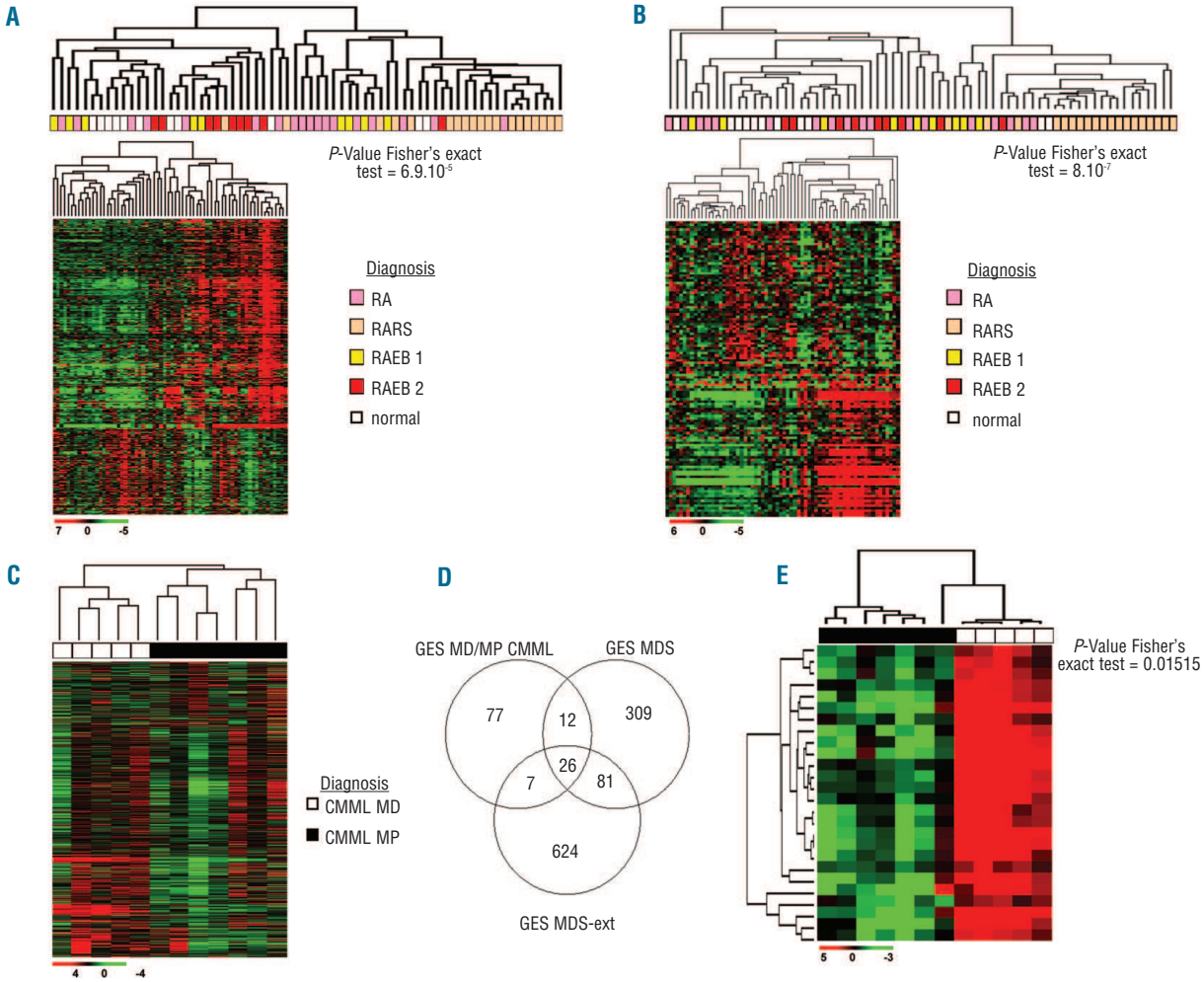

Figure 3. Independent validation of the gene expression signatures. (A) Hierarchical clustering of 66 Pellagatti's CD34+ MDS external samples[14] using our 428-gene MDS GES. Legend similar to Figure 1A. MDS classes are indicated by colored boxes in the stripe under the dendrogram. The GES significantly separates the RARS from the other MDS classes. (B) Similar to (A), but using our 122-gene MD/MP CMML. The GES significantly separates the RARS from the other MDS classes. (C) Hierarchical clustering of our 12 CD34-positive CMML samples using the MDS-ext GES. MD/MP forms are indicated by black and white boxes in the stripe under the dendrogram. The GES significantly separates the MD-CMML samples from the MP-CMML samples. (D) Venn diagram showing the overlap between the three GES: 26 genes overlap the three GES. (E) Hierarchical clustering of the 12 mRNAs from CD34+ CMML samples using these 26 overlapping genes. This 26-gene GES separates the MD-CMML from the MP-CMML samples.

We studied the sequence of 18 genes involved in the regulation of transcription (*ASXL1, DNMT3A, EZH2, IDH1, IDH2, RUNX1, SUZ12, TET2*), splicing (*SF3B1, SRSF2, U2AF1, ZRSR2*) and signaling (*CBL, FLT3, JAK2, KRAS, NRAS, PTPN11*) in our series of 53 CMMLs. Results are shown in Figure 4, *Online Supplementary Table S5* (see also *Online Supplementary Figure S1*). The samples had been studied by aCGH7,[18] and deletions of these genes were taken into account when appropriate. In CMML, splicing mutations have been recently described,[31] but how mutations in 4 splicing genes combine with alterations in the other 14 genes has not yet been reported. Only seven samples (3 MD and 4 MP) did not show any mutation in the genes studied (13%). Among transcription/epigenetic regulators, *ASXL1, RUNX1* and *TET2* were the most frequently mutated genes.

Mutations in these genes were found in both MP and MD cases. *DNMT3A* mutations were only found in MD-CMML and *EZH2* mutations in MP-CMML; however, the number of these events was low, confirming findings of previous studies.[27-30] MD-CMML showed only one CBL mutation and one NF1 deletion, whereas MP-CMML showed 14 mutations in signaling genes and one NF1 deletion. Thus, mutations in these selected signaling genes account for a first difference between MD-CMML and MP-CMML.

Except in one case, mutations in splicing genes were mutually exclusive, as previously described[31] and recently reviewed.[37] We found one mutation in *SF3B1*, 3 in *U2AF1*, 2 in *ZRSF2* and 24 in *SRSF2*. In total, 15 of 22 (68%) MD and 15 of 31 (45%) MP cases were mutated in the splicing genes studied. They were not differentially distributed between the two forms (P=0.17): the 22 MD cases showed no *SF3B1* and 13 *SRSF2* mutations (59%) whereas the 31 MP cases showed one *SF3B1* and 11 *SRSF2* mutations (38.7%). In addition, *U2AF1* and *ZRSR2* were mutated in one and one MD-CMML cases and in 2 and one MP-CMML cases, respectively.

To evaluate the possible relationships between gene mutations and gene expression we looked at the expression of the mutated genes (*Online Supplementary Figure S2B*). As expected, since they were not found in the GES, there was no difference in expression of the 18 genes between MD and MP-CMML. Gene mutations in our series of MDS cases have been described in previous studies[7,8,11] and will not be detailed here again except for the sake of comparison with CMML. We found 6 mutations in *SF3B1* and 3 in *SRSF2* in our series of 32 MDSs (*Online Supplementary Table S6*). They were differentially distributed between RARS and RAEB cases: the 8 RARS showed 5 *SF3B1* and one *SRSF2* mutations whereas the 24 RAEB cases showed one *SF3B1* and 2 *SRSF2* mutations. Three RAEB cases were also mutated in *U2AF1* and *ZRSR2*.

Thus, both MD-CMML and RARS display frequent mutations in genes encoding components of the RNA splicing machinery. However, MP-CMML cases also show alterations of this process. RARS and MD-CMML also show more mutations in *DNMT3A*.[38]

Discussion

We studied CMML by gene expression profiling and by sequencing analyses of 18 candidate genes. Unsupervised analysis of mRNAs from CD34-positive cells separated CMML into two molecular subtypes that overlapped with the MD and MP forms initially distinguished by the FAB classification. Supervised analysis established an MD/MP CMML GES characterized by the overexpression in MD-CMML of genes involved in red blood cells. The comparison of RARS and RAEB samples allowed the establishment of an MDS GES that was also characterized by the overexpression in RARS of the same genes and functions, as observed in previous studies.[14] The CMML GES recognized the RARS and also the MDS GES recognized the MD-CMML samples. Thus, MD-CMML and RARS share

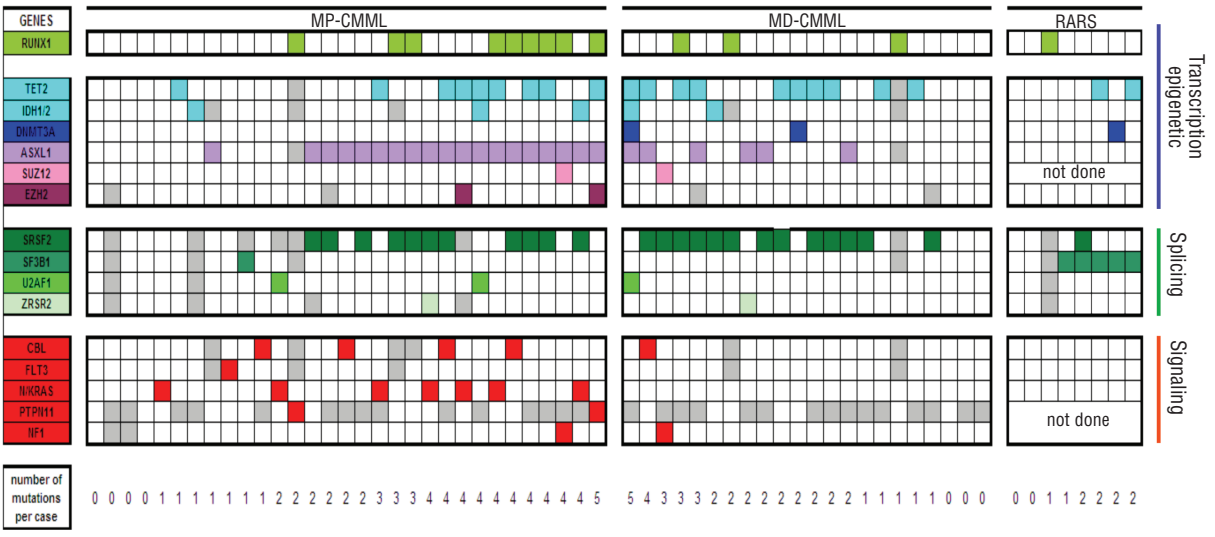

Figure 4. Gene mutations in CMML. Profiles of concomitant gene mutations of 18 genes in 53 CMML cases. Mutations in RARS (*Online Supplementary Table S6*) are shown for comparison. The number of gene mutations per case is presented below. Missing data are indicated by gray squares.

a similar transcriptional program involving red blood cell homeostasis These results were validated by using two external published data sets and a third GES (MDS-ext GES) suggesting the robustness of the results. In MD-CMML and RARS, several red blood cell functions were affected, including heme biosynthesis, iron-sulfur cluster biogenesis, calcium uptake, antioxidant defense, and transcription regulation, suggesting that MD-CMML and RARS share a common differentiation pathway characterized by an erythrocytic program.

In MD-CMML and RARS these results could reflect a mere abundance of erythroid progenitors, an overexpression of the program to compensate for abnormal red blood cell maturation, or a true molecular defect with pathological consequences. This defect in MD-CMML and RARS may be different or similar. In the latter case at least two possibilities should be considered. First, genes present in the signatures and/or involved in the identified functions could be directly affected by mutations. Many of these functions take place in the mitochondrion and it is possible that the defect affects primarily this organelle; the role of the iron transporter gene *ABCB7* had been suspected, but the gene is not mutated.[39] Actually, mutations in mitochondrion genes are associated with congenital rather than acquired anemias. A second hypothesis is perhaps the most likely. Mutations in splicing factors may indirectly affect red cell processes by leading to abnormal processing of mRNAs, including RNAs involved in red cell biology.[40] These frequently show alternative splicing[41] which may make them hypersensitive to splicing mutations. *SF3B1* mutations are prominent in MDS with ring sideroblasts (RS) (a hallmark of iron overload and dyserythropoiesis) such as RARS, and *SF3B1* haploinsufficiency or inhibition leads to the formation of RS.[42] In contrast, *SRSF2* mutations are frequent in CMML.[31] Splicing defects in *SRSF2* in CMML and in *SF3B1* in RARS may affect genes involved in erythropoiesis such as *ABCB7, FTL, GATA1*, or *HAMP* for example, or a master transcription factor of hematopoietic cell lineages such as TIF1γ, which controls erythroid cell fate and acts as a tumor suppressor in CMML.[43-45] However, *SRSF2* mutations are also found in MP-CMML which does not show a red cell program. MP-CMML is characterized by mutations in signaling genes, and this could modify the effect of *SRSF2*. Overexpression of components of the pathway may also compensate for the defect (e.g. *DICER1, CUGBP2/CELF2, ZCCHC11/TUT4* and *SYNCRIP* which are involved in miRNA and mRNA editing).

Current molecular findings are shedding new light on myeloid diseases.[46] Our study establishes a molecular bridge between CMML and MDS, suggesting that these diseases are part of a continuum of pathologies. Interestingly, we (*Online Supplementary Table S5*, HD-0376) and others[47] have observed rare cases of passage from MDS to CMML. This continuum is likely to include RARS associated with marked thrombocytosis (RARS-T, a disease characterized by mutations in *SF3B1* and *JAK2* or *MPL*), since RARS and RARS-T are characterized by common gene expression features[48] and the presence of RS. No such marker has been regularly described in CMML; nor has it been systematically investigated either. Perhaps this is due to a stronger defect in RARS than in MD-CMML because mutations in *SF3B1* have a stronger impact on erythropoiesis than *SRSF2*.

We did not find a difference in overall survival between our 37 MD and MP-CMML cases separated by the CMML GES (P=0.18, Fisher's exact test). Neither did *SRSF2* mutation have any impact on prognosis. If, given the current possibilities of treatment, the prognosis of MD and MP-CMML is similar, in the future these diseases may be treated differently and we will need to be able to identify the difference between them. Our discovery of a molecular similarity between MD-CMML and RARS could be useful by improving the classification of these diseases, providing pathophysiological clues and suggesting the possibility of using treatment approaches common to the two diseases but different from those for MP-CMML.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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Article 10 - Acute myeloid leukemia with myelodysplasia-related changes are characterized by a specific molecular pattern with high frequency of ASXL1 mutations.

ARTICLE n°10

Acute myeloid leukemia with myelodysplasia-related changes are characterized by a specific molecular pattern with high frequency of ASXL1 mutations.

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Acute myeloid leukemia with myelodysplasia-related changes are characterized by a specific molecular pattern with high frequency of *ASXL1* mutations

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To determine whether the distinct and heterogeneous WHO category called "AML with myelodysplasia-related changes" (MRC-AML), presents specific molecular alterations we searched for mutations in genes known to be mutated in malignant myeloid diseases. In 48 MRC-AML patients analyzed, we found 17 mutations in *ASXL1* (35%), eight in *RUNX1* (17%), seven in *TET2* (15%), 12 in *IDH* ($n = 2$) or *IDH2* ($n = 10$) (25%), four in *DNMT3A* (8%), four in *NPM1* (8%), and one in *FLT3* (2%). Mutations were more frequent in the intermediate cytogenetic (IC) subgroup of 36 patients than in the unfavorable karyotype subgroup, with an average ratio mutations/patients of 1.36 [0–3] vs. 0.33 [0–2] ($P < 0.001$). Then, we compared these 36 patients with IC MRC-AML with a control panel of 37 no-MRC-AML patients, who had both IC and no dysplasia. IC MRC-AMLs were associated with higher incidence of *ASXL1* mutations (47% vs. 0%, $P < 0.001$) and lower incidence of *DNMT3A* (6% vs. 38%, $P = 0.001$), *NPM1* (11% vs. 62%, $P < 0.001$) and *FLT3* (3% vs. 49%, $P < 0.001$) mutations. No difference was found in the incidence of *IDH1/2* or *TET2* mutations according to the presence of dysplasia. Complete remission rate after intensive treatment was lower in the MRC-AML group than in the no-MRC-AML group (48% vs. 78%, $P = 0.023$) and in wild type *NPM1* patients (50% vs. 84%, $P = 0.009$). Our study showed that MRC-AML as defined in the WHO 2008 classification presents a specific mutation pattern characterized by a high frequency of *ASXL1* mutations and a low rate of *NPM1*, *FLT3*, and *DNMT3A* mutations. Am. J. Hematol. 87:659–662, 2012. © 2012 Wiley Periodicals, Inc.

Introduction

The 2008 revised WHO classification[1] recognizes a specific category of acute myeloid leukemia (AML) called AML with myelodysplasia-related changes (MRC-AML). MRC-AMLs present myelodysplasia-related phenotype and cytogenetic abnormalities and/or exhibit dysplasia in 50% or more of the cells in two or more myeloid lineages and/or history of myelodysplastic syndrome (MDS). This category includes heterogeneous diseases with distinct clinical and cytogenetic entities. MRC-AMLs are classically considered as high-risk diseases because they are frequently secondary and/or unfavorable cytogenetic cases associated with older age, unfit for intensive care.[2] To our knowledge, no previous study has ever reported the predictive value of dysplasia independently of age, cytogenetic, or previously diagnosed MDS. To date, cytogenetic and molecular alterations but not dysplasia are considered as independent factors influencing both prognosis and therapeutic choices.[3–5] To determine whether this WHO category, currently defined solely upon morphological and cytogenetic criteria, presents specific molecular alterations, we searched for mutations in genes known to be mutated in malignant myeloid diseases. We report clinical, biological, and molecular characteristics of AML according to the presence of criteria for MRC-AML as described in the WHO classification.

Methods

Selection criteria. Patients with the following criteria were included (1) Marrow blast >20% at the time of diagnosis except for erythroleukemia according to the FAB classification (AML6); (2) No favorable cytogenetic abnormalities, i.e., t(8;21), inv(16)/t(16;16), t(15;17); (3) Total bone marrow sample available at diagnosis; and (4) Signed informed consent for somatic genetic analyses.

Cases were separated in two groups according to the presence or not of dysplastic features at diagnosis. Patients with at least one criteria for AML with MRC according to the WHO classification constituted the MRC-AML group: presence of multilineage dysplasia with (secondary MRC-AML subgroup) or without (primary MRC-AML subgroup) history of MDS or myelodysplastic/myeloproliferative syndrome (MDS/MPN); myelodysplasia-related cytogenetic abnormalities (complex karyotype, -5 or del(5q), -7, 11q23 abnormalities except t(9;11), t(6;9), 3q26 abnormalities and 17p abnormalities). Cases without any of these criteria formed the no-MRC-AML group.

Treatment modality and response. Patients received intensive induction therapy (anthracycline and cytarabine), non-intensive treatment (azacytidine, low-dose cytarabine), or supportive care only associated or not with oral chemotherapy such as 6-mercaptopurine and/or methotrexate and/or hydroxyurea. Induction treatment modalities were chosen according to patient's age, performance status, and cytogenetic abnormalities. After induction therapy, response was defined according to Cheson criteria:[6] Bone marrow blast <5%; no Auer rods; platelet count > 100 G/L; absolute neutrophil count > 1 G/L and absence of extramedullary disease defined complete remission (CR). Patients reached CR with incomplete recovery when they presented with all criteria for CR except for residual neutropenia <1 G/L or thrombopenia <100 G/L.

Direct gene sequencing. We used direct Sanger gene sequencing to search for somatic mutations in *ASXL1* (Exon 12), *RUNX1* (Exons 1–8), *TET2* (Exons 3–11), *IDH1* (Exon 4) and *IDH2* (Exon 4), *DNMT3A* (Exons 15–23), *FLT3* (internal tandem duplications—ITD—Exons 14 and 15 and point mutation in Exon 20-encoded kinase domain—TKD) and *NPM1* (Exon 12). Methods have been described previously.[7]

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TABLE I. Patient Characteristics

	MRC-AML (n = 48)		No-MRC-AML (n = 37)		P
Gender					
Men	33	69%	19	51%	0.08
Age					
Median (year) [range]	72	[29–86]	63	[21–83]	<0.001
Occurrence of MRC-AML					
Primary MRC-AML	25	52%			
Secondary MRC-AML	23	48%			
MDS	*18*	*38%*			
CMML	*5*	*10%*			
Cytogenetic risk group					
Intermediate	36	75%	37	100%	0.001
Normal karyotype	18	38%	31	84%	
Other	18	38%	6	16%	
Unfavorable	12	25%	0	0%	
Complex karyotype	8	17%			
Monosomal karyotype	3	6%			
17p abnormality	1	2%			
Induction therapy					
Intensive	26	55%	32	91%	<0.001

TABLE II. Mutations of *ASXL1, RUNX1, TET2, IDH1, IDH2, DNMT3A, NPM1*, and *FLT3* in 48 Cases of MRC-AML According to Cytogenetic Risk Group

	MRC-AML (n = 48)		Intermediate group (n = 36)		Unfavorable group (n = 12)		
	Mutated cases	%	Mutated cases	%	Mutated cases	%	P
ASXL1	17	35	17	47	0	0	0.002
RUNX1	8	17	8	22	0	0	0.080
TET2	7	15	6	17	1	8	0.431
IDH1	2	4	1	3	1	8	0.441
IDH2	10	21	10	28	0	0	0.039
DNMT3A	4	8	2	6	2	17	0.257
NPM1	4	8	4	11	0	0	0.303
FLT3	1	2	1	3	0	0	0.750

Statistical analyses. We used a Chi-square test to compare patient characteristics and mutation frequencies in different groups, and to find predictive factors associated with CR achievement. Overall survival (OS) was calculated from date of AML diagnosis to death or last contact using Kaplan Meier[8] estimates and Log Rank test.

Results

Patient characteristics. Eighty-five patients were studied. Forty-eight and 37 patients formed the MRC-AML and the no-MRC-AML groups, respectively. Patient characteristics are summarized in Table I. MRC-AML patients were older than no-MRC-AML patients (median age of 72 vs. 63, $P < 0.001$). Twenty-three (48%) patients of the MRC-AML group presented with a previously diagnosed MDS ($n = 18$) or CMML ($n = 5$) and were considered as secondary MRC-AML cases. The 25 remaining MRC-AML patients presented with multilineage dysplasia. Twelve (25%) MRC-AML cases had unfavorable cytogenetic abnormalities while all the no-MRC-AML cases presented with intermediate risk karyotype. No-MRC-AML patients were more frequently treated by intensive induction herapy (91% vs. 55%, $P < 0.001$). In the MRC-AML patients, no difference was found in the characteristics of primary and secondary MRC-AML in terms of age ($P = 0.605$), cytogenetic ($P = 0.308$), and intensive induction ($P = 0.06$).

Mutation frequencies in 48 MRC-AML patients. We found 17 mutations in *ASXL1* (35%), eight in *RUNX1* (17%), seven in *TET2* (15%), 12 in *IDH1* ($n = 2$) or *IDH2* ($n = 10$) (25%), four in *DNMT3A* (8%), four in *NPM1* (8%), and one in *FLT3* (2%) (Table II). The profiles of concomitant mutations are shown in Fig. 1. Mutations were more frequent in the intermediate cytogenetic (IC) subgroup than in the unfavorable karyotype subgroup, with an average ratio

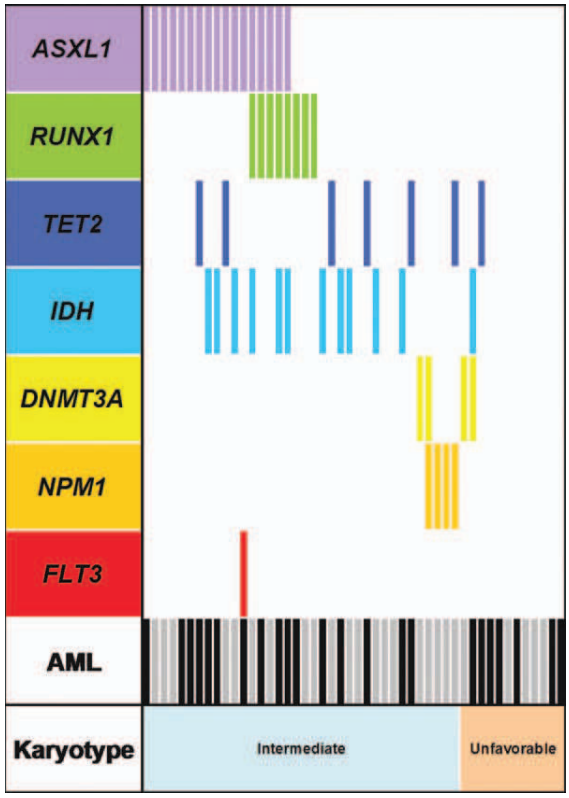

Figure 1. Profiles of concomitant mutations in 48 cases of MRC-AML according to the cytogenetic risk group. Primary and secondary MRC-AMLs are represented with grey and black bars respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III. Mutations of *ASXL1, RUNX1, TET2, IDH1, IDH2, DNMT3A, NPM1*, and *FLT3* in 36 Cases of Intermediate Cytogenetic MRC-AML Cases Compared With 37 Cases of No-MRC-AML

	MRC-AML (n = 36)		No-MRC-AML (n = 37)		
	Mutated cases	%	Mutated cases	%	P
ASXL1	17	47	0	0	<0.001
RUNX1	8	22	3	8	0.087
TET2	6	17	5	14	0.480
IDH1	1	3	3	8	0.318
IDH2	10	28	6	16	0.181
DNMT3A	2	6	14	38	0.001
NPM1	4	11	23	62	<0.001
FLT3	1	3	18	49	<0.001

mutations/patients of 1.36 [0–3] versus 0.33 [0–2] ($P < 0.001$). Mutated *ASXL1* ($P = 0.002$) and *IDH2* ($P = 0.039$) cases were associated with the IC subgroup. Interestingly, no significant difference in the mutation rates was found between primary and secondary MRC-AMLs.

Mutation frequencies in IC AML. We restricted further analysis to AML with IC because most of the mutated cases were found in this entity. The 36 patients with IC MRC-AML were compared with the control panel of 37 no-MRC-AMLs, who had both IC and no dysplasia. We found that IC MRC-AML mutation profile was different from IC no-MRC-AML (Table III; Fig. 2). MRC-AMLs were characterized by the presence of *ASXL1* mutations (47% vs. 0%, $P < 0.001$). There was also a trend toward a higher incidence of *RUNX1* mutations in MRC-AML cases (22% vs. 8%, $P = 0.087$). In contrast, in IC no-MRC-AMLs, we found more frequent mutations of *DNMT3A* (38% vs. 6%, $P = 0.001$),

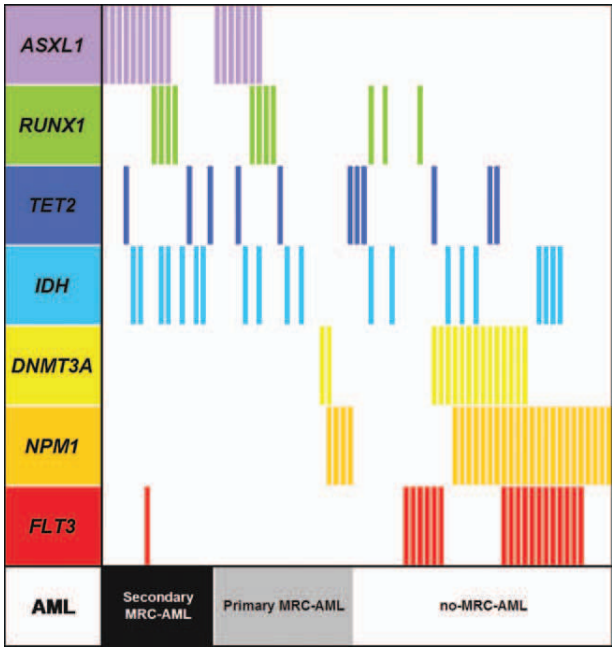

Figure 2. Profiles of concomitant mutations in 36 cytogenetic risk MRC-AML patients compared with the 37 cases of no-MRC-AML. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

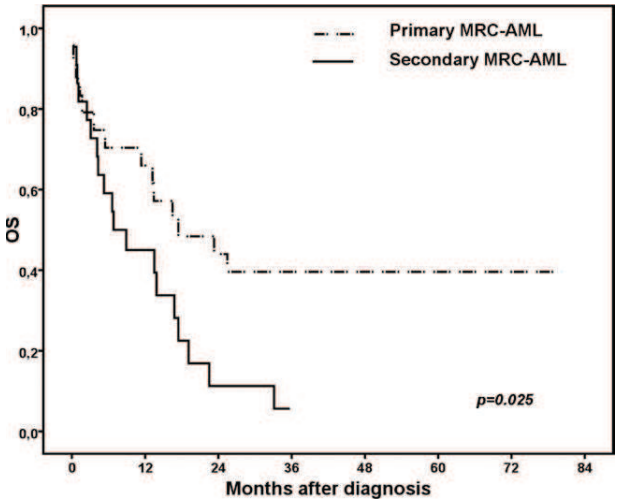

Figure 3. Overall survival of primary versus secondary MRC-AML in 48 MRC-AML patients.

NPM1 (62% vs. 11%, $P < 0.001$), and *FLT3* (49% vs. 3%, $P < 0.001$). *ASXL1* mutations were associated with *RUNX1* mutations ($P = 0.039$) and mutually exclusive with *DNMT3A* ($P = 0.011$) and *NPM1* ($P = 0.001$) mutations in our series. Only one case of MRC-AML carried a FLT3-ITD; it was associated with an *ASXL1* mutation, whereas the overall trend was a mutual exclusivity of *ASXL1* and FLT3-ITD mutations. *NPM1* mutations were associated with mutated *DNMT3A* ($P = 0.001$) and FLT3-ITD ($P = 0.001$) and wild type *RUNX1* ($P = 0.011$). No difference was found between MRC-AMLs and no-MRC-AMLs in the mutation frequencies of *IDH1/2* (31% vs. 24% respectively, $P = 0.369$) and *TET2* (17% and 14% respectively, $P = 0.480$).

Response after induction therapy and survival. Among the 58 patients treated by intensive induction chemotherapy, 35 achieved CR (60%), 10 achieved CRi (9%), and 18 failed (31%). Low CR rates were associated with unfavorable karyotype (0% vs. 66%, $P = 0.007$). After excluding unfavorable karyotype patients, CR rate after intensive treatment remained lower in the MRC-AML group than in the no-MRC-AML group (48% vs. 78%, $P = 0.023$) and in wild type *NPM1* patients (50% vs. 84%, $P = 0.009$). We did not found predictive value of other mutated genes on CR achievement after induction therapy in IC patients. In the MRC-AML patients, secondary MRC-AML was associated with lower CR rate than primary MRC-AML (14% vs. 64%, $P = 0.043$).

No difference was found in 2-year OS between MRC-AML and no-MRC-AML groups after induction therapy (48% vs. 44% respectively, $P = 0.345$). In MRC-AML patients, 2-year OS was 40% and 11% for the primary and secondary MRC-AML subgroups, respectively ($P = 0.025$) (Fig. 3). No mutation was found to significantly influence OS.

Discussion

We have compared here clinical, biological, and molecular features of MRC-AML vs no-MRC-AML. The WHO classification[1] pooled in MRC-AML both secondary and/or unfavorable karyotype AML and primary AML presenting with multilineage dysplasia. We wondered whether the mutation status of genes involved in myeloid malignancies could help better distinguish MRC-AML from no-MRC-AML and/or primary and secondary MRC-AML. Our results showed that mutations of *ASXL1*, *RUNX1*, *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *NPM1*, and *FLT3* mainly occurred in the IC group. This suggests different molecular pathways for monosomal and/or complex karyotype MRC-AMLs. Then we focused our analyses on IC MRC-AMLs and found different mutation profiles according to the presence of dysplasia. We found that MRC-AMLs have a mutation pattern characterized by a high frequency of *ASXL1* mutations, which reached 47% among patients with IC risk. *ASXL1* mutations have been found in only 10% of *de novo* AML cases reported in the literature.[9] *ASXL1* mutations are more frequently found in older patients and in MDS and/or CMML.[9–12] The median age of 72 years in the MRC-AML group and the high proportion of secondary MRC-AML (48%) could explain in part the very high frequency of *ASXL1* mutations in our population. It also seemed that *RUNX1* mutations are more frequently found in MRC-AML (17% in our series and 22% in IC risk patients) than in no-MRC-AML (8%). *RUNX1* mutation frequency was previously described in *de novo* AML and reached 7%.[13] Moreover, we found a lower rate of *DNMT3A* (6%), *NPM1* (11%), and *FLT3* (3%) mutations in our MRC-AML than in our no-MRC-AML patients; in the literature *DNMT3A*, *NPM1* and *FLT3* mutations were found in 34%,[14,15] 60%,[16] and 30%[17] of IC AML, respectively. Conversely, *TET2* and *IDH* mutations were similarly distributed in MRC-AML and no-MRC-AML in our series, as well as in previous reports.[18–20]

We showed that MRC-AML as defined in the WHO 2008 classification presents a specific mutation pattern characterized by a high frequency of *ASXL1* and *RUNX1* mutations and a low rate of *NPM1*, *FLT3*, and *DNMT3A* mutations. Importantly, no difference was found in mutation profile between primary and secondary MRC-AML. This suggests that leukemogenesis of MRC-AML could have a specific molecular pathway contributing to the dysplastic phenotype whatever the presence or not of a previously diagnosed MDS or MDS/MPN. Alternatively, MRC-AML may systematically derive from a previous chronic stage that sometimes goes unnoticed.

It is well documented that MRC-AMLs are associated with unfavorable cytogenetic and older age, leading to a poor prognosis.[2] In our series, we found that the presence

of dysplasia was significantly associated with less CR after induction therapy (48% vs. 78%, $P = 0.023$). However, these results were not translated into an OS benefit. The low numbers and the bias related to patient selection for intensive induction could explain in part this result. It seems that secondary MRC-AML reached lower CR rate (14% vs. 64%, $P = 0.043$) and had unfavorable outcome compared with primary MRC-AML (2-year OS: 11% vs. 25%, $P = 0.025$).

Beyond the specific morphologic and cytogenetic features that define MRC-AML, molecular abnormalities such as *ASXL1* and *RUNX1* mutations, although not specific, could help delineate this recent distinct entity. The high frequency of *ASXL1* mutations in MRC-AML suggests epigenetic deregulation in this kind of myeloid malignancies. This could open perspectives through molecular characterization of MRC-AML and adapted treatment strategies with new drugs such as demethylating agents or HDAC inhibitors.

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