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

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ABSTRACT

Myelofibrosis is a myeloproliferative neoplasm that occurs *de novo* (primary myelofibrosis) or results from the progression of polycythemia vera or essential thrombocytemia (hereafter designated as secondary myelofibrosis or post-polycythemia vera/ essential thrombocythemia 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/NRAS, 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.

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 thrombocythemia (ET) (hereafter called post-PV/ET MF) with no differences in clinical or histological characteristics.[2] MF has a poor prognosis; the median overall survival (OS) is five years.[3] Evolution to acute myeloid leukemia (AML) occurs in approximately 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 decision-making. 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 approximately 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 50-60% and 5-10% 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] There is no difference in the prevalence of an abnormal karyotype 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 studied 80 MF cases by using array-comparative genomic hybridization (aCGH) and Sanger sequencing of 23 genes on 104 MF samples. We 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) (*Online Supplementary Table S1A*) or during disease course (n=44) (*Online Supplementary Table S1B*) and 12 blast phases (BP)-MF (*Online Supplementary Table S1C*; in this table, 5 other BP cases are evolution of 5 of the 68 chronic phases). Based on the World Health

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Table 1. Features of MF cases with CNAs and 20q deletion.

		Total number	CNA	No CNA	*P*	20q deleted	no 20q deleted	*P*
Myelofibrosis		63	34 (54%)	29 (46%)		14 (22%)	49 (78%)	
Gender	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) (range) 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
	inter-2/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 (> 25x10^9/L), on circulating blasts (> or = 1%), on platelet count (< 100x10^9/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 scores were calculated at sampling.

Organization 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 (*Online Supplementary Table S1B*). 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 give their written consent, 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 previously 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 the *Online Supplementary 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 *Online Supplementary Table S2A*. PCR amplifications of DNA were performed as previously described.[23] The PCR program for *TET2* is described in *Online Supplementary Table S2B*. All mutations were confirmed on an independent PCR product.

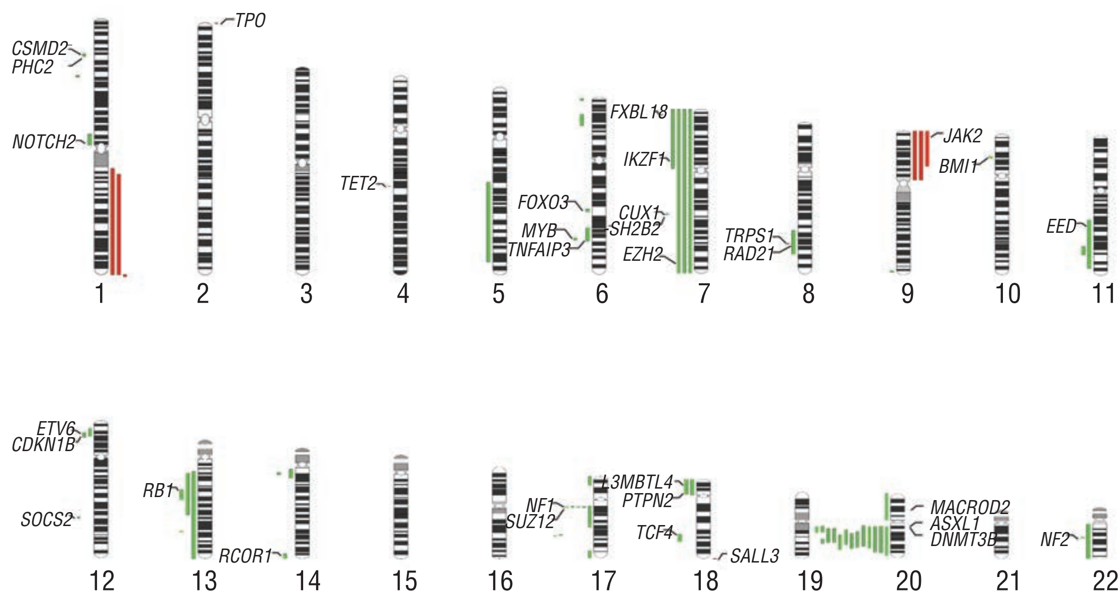

Figure 1. Karyoview of copy number aberrations (CNAs) detected in 63 patients with myelofibrosis by using array-comparative genomic hybridization. The bars depict the physical positions and the size of aberration: green bars indicate deletions and red bars indicate gains. Some genes known or suspected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

Statistical analysis

Analyses were carried out on patients at diagnosis and/or during follow up. Correlations between sample groups and clinico-biological data were calculated with the χ^2 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 as a relevant event for OS while the occurrence of acute transformation was considered as a relevant event for TTAT. Patients without any 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<5% was considered statistically significant.

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 (*Online Supplementary Table S1A*). 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 (*Online Supplementary Table S3*). We identified 72 CNAs including 9 gains and 63 deletions (del) (Figure 1). We classified aCGH profiles into three types. Type 1 (45 of 72) comprised large CNAs (>4Mb) affecting chromosome arms: +1q, -5q, -7, +9p, -8q, -11q, -12p, -13q, -17q, -18p, -20q. Type 2 (27 of 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 (*Online Supplementary Table S3* and *Online Supplementary Figure S1*). In type 3 profiles, observed in 46% (n=29) of patients, no CNA were detected.

We detected no difference in CNAs according to gender (P=0.50), or to the character of primary or secondary MF (P=0.44). Nevertheless, we identified CNAs in 58% of PMF (22 of 38), 71% of post-PV MF (10 of 14), and 18% of post-ET MF cases (2 of 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 1).

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 of 63) of the cases, was the most recurrent alteration (41%, 14 of 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 and 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) (*Online Supplementary Table S3*). 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 and *Online Supplementary Table S4*). Cases with del(20q) were associated with lower leukocyte count, hemoglobin, and hematocrit (P=0.01, P=0.03 and P=0.02, respectively) and the need for red cell transfusion

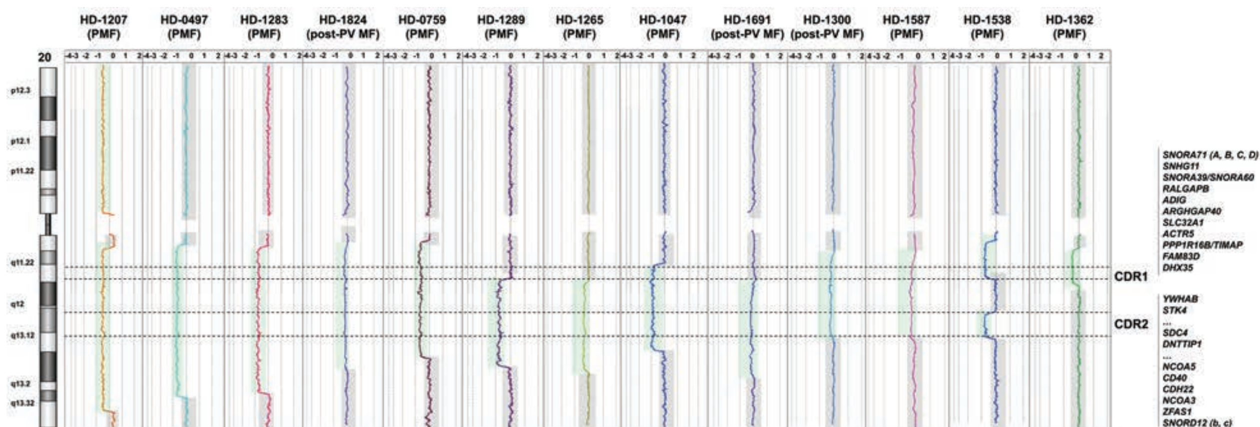

Figure 2. **aCGH profiles of recurrent del(20q). Del(20q) is a recurrent CNA detected in 13 cases by an aCGH. Two separate commonly-deleted regions (CDR), CDR1 and CDR2, were identified, with a respective size of 0.3 Mb (HD-1047;HD-1538) and 3.9 Mb (HD-1538;HD-1587). Genes lost in CDR1 and CRD2 are shown in *Online Supplementary Table S4*.**

(*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 1).

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 and *Online Supplementary Table S3*). *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 2 post-PV MF cases that both evolved in AML, and associated with del(20q) in a patient with post-PV MF who died (Figure 1 and *Online Supplementary Table S3*).

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 (*Online Supplementary Table S5*). 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 and *Online Supplementary Figure S2*). We identified one new gene mutated in MF, *PTPN11*, which was also affected by deletions. In 2 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 of 12, 8%) and PMF (2 of 36, 6%) and *SRSF2* mutations (4 of 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 and 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 and 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) (*Online Supplementary Table S6*). Platelet count was high in *SRSF2*-mutated cases (*P*=0.04) (*Online Supplementary Table S6*). 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 (*Online Supplementary Table S1B*).

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

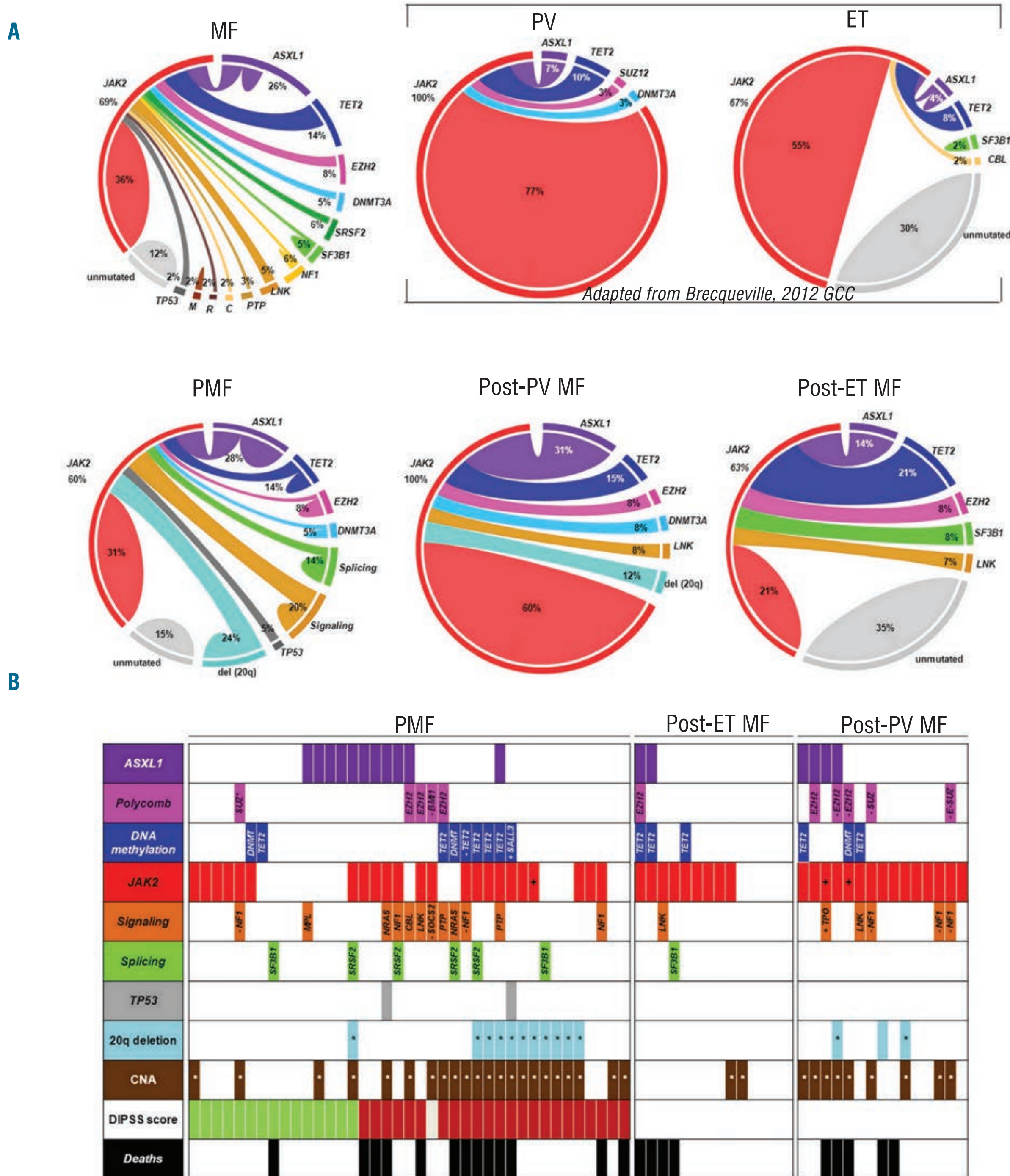

Figure 3. Frequency and distribution of gene mutations. (A) Circos plot showing frequencies of gene mutations in MF, in PV and ET according to a previous study,[23] in PMF, in post-PV MF and post-ET MF. The ribbon representing the splicing pathway is composed of *SF3B1* and *SRSF2* mutations; that representing 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*.

del(11q) increased (50-70% and -0.8, respectively) and a trisomy 9 was present (*Online Supplementary Table S7*).

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 (*Online Supplementary Figure S3*). Indeed, several patients already carried *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) (*Online Supplementary Figure S3*). 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, eight months later acquired 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's condition worsened during the disease course (HD-1352) and the patient 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 (*Online Supplementary Table S7*).

Blast phase MF transformation

Among MF transformed to AML (n=5), 2 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 2 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 *SRSF2*- 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) (*Online Supplementary Figure S4*); the patient died rapidly (*Online Supplementary Table S7*).

Among 17 BP-MF, we detected CNAs in 82% of the cases (14 of 17) (*Online Supplementary Table S8*). 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 of 67) of CNAs and included deletions in 12p (*ETV6, CDKNA1B/p27*), 13q14.2 (*RB1*), 15q21.3 (*TCF12*), 17q11 (*NF1*), 18p11 (*PTPN2*), and 21q22 (*RUNX1*) (*Online Supplementary Figure S8* and *Online Supplementary Figure S4*). 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 of 9), *ASXL1* (25%, 3 of 12), *TP53* (23%, 3 of 13) and *EZH2* (22%, 2 of 9) (*Online Supplementary Table S9*).

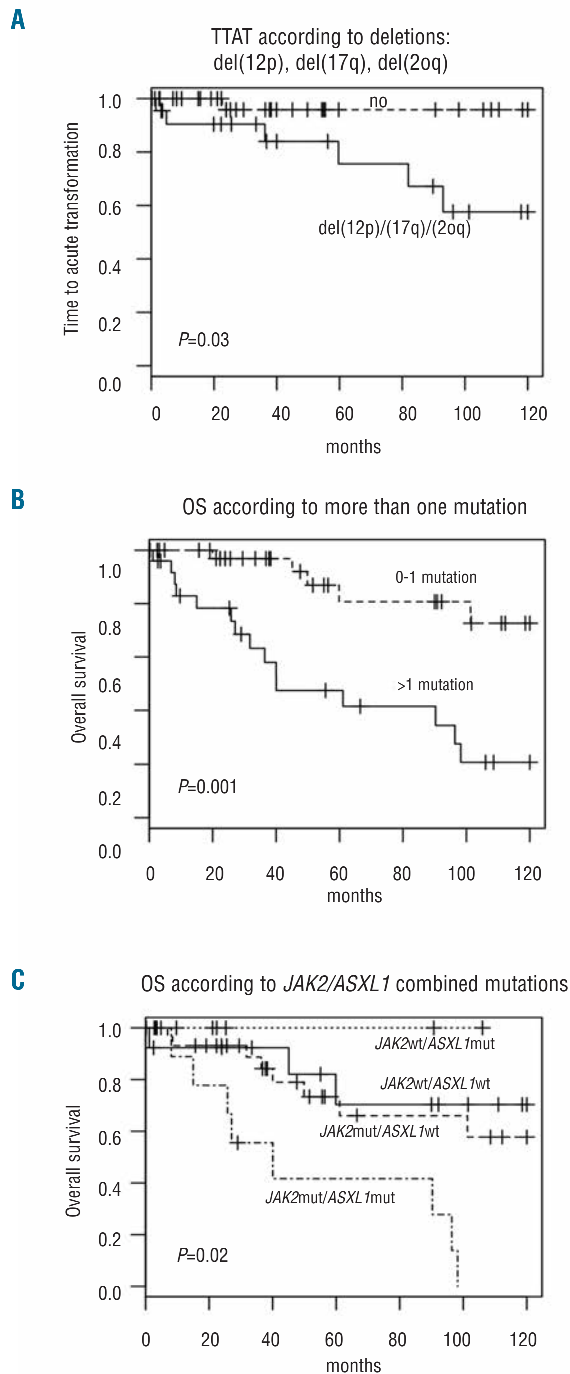

Figure 4. Kaplan-Meier estimates. (A) Time to acute transformation (TTAT) according to deletions: del(12p), del(17q), del(20q). (B) Overall survival (OS) according to more than one mutation. (C) OS according to *JAK2/ASXL1* combined mutations. del: deletion; mt: mutated; wt: wild-type.

Prognostic impact of CNAs and gene mutations

We studied TTAT according to CNAs and genes frequently mutated (>5%) (*Online Supplementary Table S10*). In univariate analysis, there was no difference in TTAT 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) (*Online Supplementary Table S10*).

The 5-year OS was severely decreased in patients with more than one mutation (81% *vs.* 47%; *P*=0.001) (Figure 4B), with at least one mutation in epigenetic-associated genes (*ASXL1, EZH2, TET2, DNMT3A*) (63% *vs.* 73%; *P*=0.03) and with at least one mutation in a splicing-associated gene (*SF3B1, SRSF2*) (42% *vs.* 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 and *P*=0.003, respectively). This was not the case for *TET2* (*P*=0.59). *SRSF2*-mutated patients had a poor OS (*P*=0.01) (*Online Supplementary Table S8*). In multivariate analyses, we included *ASXL1*, *EZH2* and *SRSF2* mutations as covariates; *EZH2* and *SRSF2* mutations predicted poor OS (*P*=0.04 and *P*=0.007, respectively) (*Online Supplementary Table S8*). 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 shown the presence of non-recurrent mutations in novel genes.[38]

Primary and secondary MF

There was no difference in 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 course 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 (*Online Supplementary Table S5*). 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 any 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 4 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 leukocyte 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 (*Online Supplementary Figure S6*). *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, down-regulated 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 died, 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-making 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 879 PMF cases showed that *ASXL1* mutations had prognostic relevance independent of the DIPPS-plus model.[27] Due to the small number of patients in our study, 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 already had *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 or 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-making. In agreement with an important recently published 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-making and the design of a new therapeutic association between JAK2 inhibitors and epigenetic drugs according to mutational status.

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References

1. Tefferi A, Thiele J, Vardiman JW. The 2008 World Health Organization classification system for myeloproliferative neoplasms: order out of chaos. Cancer. 2009; 115(17):3842-7.
2. Mesa RA, Verstovsek S, Cervantes F, Barosi G, Reilly JT, Dupriez B, et al. Primary myelofibrosis (PMF), post polycythemia vera myelofibrosis (post-PV MF), post essential thrombocythemia myelofibrosis (post-ET MF), blast phase PMF (PMF-BP): Consensus on terminology by the international working group for myelofibrosis research and treatment (IWG-MRT). Leuk Res. 2007;31(6):737-40.
3. Cervantes F, Dupriez B, Pereira A, Passamonti F, Reilly JT, Morra E, et al. New prognostic scoring system for primary myelofibrosis based on a study of the International Working Group for Myelofibrosis Research and Treatment. Blood. 2009;113(13):2895-901.
4. Passamonti F, Cervantes F, Vannucchi AM, Morra E, Rumi E, Pereira A, et al. A dynamic prognostic model to predict survival in primary myelofibrosis: a study by the IWG-MRT (International Working Group for Myeloproliferative Neoplasms Research and Treatment). Blood. 2010; 115(9):1703-8.
5. Gangat N, Caramazza D, Vaidya R, George G, Begna K, Schwager S, et al. DIPSS plus: a refined Dynamic International Prognostic Scoring System for primary myelofibrosis that incorporates prognostic information from karyotype, platelet count, and transfusion status. J Clin Oncol. 2011;29(4):392-7.
6. Hussein K, Van Dyke DL, Tefferi A. Conventional cytogenetics in myelofibrosis: literature review and discussion. Eur J Haematol. 2009;82(5):329-38.
7. Tam CS, Abruzzo LV, Lin KI, Cortes J, Lynn A, Keating MJ, et al. The role of cytogenetic abnormalities as a prognostic marker in primary myelofibrosis: applicability at the time of diagnosis and later during disease course. Blood. 2009;113(18):4171-8.
8. James C, Ugo V, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;21(6-7):669-70.
9. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med. 2006;3(7):e270.
10. Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood. 2009;113(24):6182-92.
11. Delhommeau F, James C, Trannoy S, Massé A, Kosmider O, Pharm D, et al. Mutation in TET2 in Myeloid Cancers. New Engl J Med. 2009;360(22):2289-301.
12. Carbuccia N, Murati A, Trouplin V, Brecqueville M, Adélaïde J, Rey J, et al. Mutations of ASXL1 gene in myeloproliferative neoplasms. Leukemia. 2009;23(11): 2183-6.
13. Pardanani A, Lasho TL, Finke CM, Mai M, McClure RF, Tefferi A. IDH1 and IDH2 mutation analysis in chronic- and blast-phase myeloproliferative neoplasms. Leukemia. 2010;24(6):1146-51.
14. Jäger R, Gisslinger H, Passamonti F, Rumi E, Berg T, Gisslinger B, et al. Deletions of the transcription factor Ikaros in myeloproliferative neoplasms. Leukemia. 2010;24(7): 1290-8.
15. Oh ST, Simonds EF, Jones C, Hale MB, Goltsev Y, Gibbs KD, et al. Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. Blood. 2010;116 (6):988-92.
16. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet. 2010;42(8):722-6.
17. Brecqueville M, Cervera N, Adélaïde J, Rey J, Carbuccia N, Chaffanet M, et al. Mutations and deletions of the SUZ12 poly-

comb gene in myeloproliferative neoplasms. Blood Cancer J. 2011;1(8):e33.
18. Brecqueville M, Cervera N, Gelsi-Boyer V, Murati a, Adélaïde J, Chaffanet M, et al. Rare mutations in DNMT3A in myeloproliferative neoplasms and myelodysplastic syndromes. Blood Cancer J. 2011;1(5):e18.
19. Lasho TL, Finke CM, Hanson CA, Jimma T, Knudson RA, Ketterling RP, et al. SF3B1 mutations in primary myelofibrosis: clinical, histopathology and genetic correlates among 155 patients. Leukemia. 2012; 26(5):1135-7.
20. Lasho TL, Jimma T, Finke CM, Patnaik M, Hanson CA, Ketterling RP, et al. SRSF2 mutations in primary myelofibrosis: significant clustering with IDH mutations and independent association with inferior overall and leukemia-free survival. Blood. 2012;120(20):4168-71.
21. Stein BL, Williams DM, O'Keefe C, Rogers O, Ingersoll RG, Spivak JL, et al. Disruption of the ASXL1 gene is frequent in primary, post-essential thrombocytosis and post-polycythemia vera myelofibrosis, but not essential thrombocytosis or polycythemia vera: analysis of molecular genetics and clinical phenotypes. Haematologica. 2011;96 (10):1462-9.
22. Abdel-Wahab O, Pardanani A, Patel J, Wadleigh M, Lasho T, Heguy A, et al. Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms. Leukemia. 2011;25(7):1200-2.
23. Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, et al. Mutation Analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in Myeloproliferative Neoplasms. Genes Chromosomes Cancer. 2012;51(8): 743-55.
24. Tefferi A, Mesa RA, Schroeder G, Hanson CA, Li CY, Dewald GW. Cytogenetic findings and their clinical relevance in myelofibrosis with myeloid metaplasia. Br J Haematol. 2001;113(3):763-71.
25. Guglielmelli P, Biamonte F, Score J, Hidalgo-Curtis C, Cervantes F, Maffioli M, et al. EZH2 mutational status predicts poor survival in myelofibrosis. Blood. 2011;118(19): 5227-34.
26. Tefferi A, Jimma T, Sulai NH, Lasho TL, Finke CM, Knudson RA, et al. IDH mutations in primary myelofibrosis predict leukemic transformation and shortened survival: clinical evidence for leukemogenic collaboration with JAK2V617F. Leukemia. 2012;26(3):475-80.
27. Vannucchi AM, Lasho TL, Guglielmelli P, Biamonte F, Pardanani A, Pereira A, et al. Mutations and prognosis in primary myelofibrosis. Leukemia. 2013. In press.
28. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-51.
29. Gelsi-Boyer V, Trouplin V, Roquain J, Adélaïde J, Carbuccia N, Esterni B, et al. ASXL1 mutation is associated with poor prognosis and acute transformation in chronic myelomonocytic leukaemia. Br J Haematol. 2010;151(4):365-75.
30. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003;34(2):148-50.
31. Dong M, Blobe GC. Role of transforming growth factor-beta in hematologic malignancies. Blood. 2006;107(12):4589-96.
32. Vannucchi AM, Bianchi L, Paoletti F, Pancrazzi A, Torre E, Nishikawa M, et al. A pathobiologic pathway linking thrombopoietin, GATA-1, and TGF-beta1 in the development of myelofibrosis. Blood. 2005;105(9):3493-501.
33. Gui T, Sun Y, Gai Z, Shimokado A, Muragaki Y, Zhou G. The loss of Trps1 suppresses ureteric bud branching because of the activation of TGF-signaling. Dev Biol. 2013;377(2):415-27.
34. Plo I, Nakatake M, Malivert L, De Villartay J-P, Giraudier S, Villeval J-L, et al. JAK2 stimulates homologous recombination and genetic instability: potential implication in the heterogeneity of myeloproliferative disorders. Blood. 2008;112(4):1402-12.
35. Shikauchi Y, Saiura A, Kubo T, Niwa Y, Yamamoto J, Murase Y, et al. SALL3 interacts with DNMT3A and shows the ability to inhibit CpG island methylation in hepatocellular carcinoma. Mol Cell Biol. 2009;29(7):1944-58.
36. Brecqueville M, Adélaïde J, Bertucci F, Finetti P, Chaffanet M, Murati A. Alterations of polycomb gene BMI1 in human myeloproliferative neoplasms. Cell Cycle. 2012;11(16):3141-2.
37. Oguro H, Yuan J, Tanaka S, Miyagi S, Mochizuki-Kashio M, Ichikawa H, et al. Lethal myelofibrosis induced by Bmi1-deficient hematopoietic cells unveils a tumor suppressor function of the polycomb group genes. J Exp Med. 2012;209(3):445-54.
38. Merker JD, Roskin KM, Ng D, Pan C, Fisk DG, King JJ, et al. Comprehensive whole-genome sequencing of an early-stage primary myelofibrosis patient defines low mutational burden and nonrecurrent candidate genes. Haematologica. 2013. In press.
39. Schaub FX, Jäger R, Looser R, Hao-Shen H, Hermouet S, Girodon F, et al. Clonal analysis of deletions on chromosome 20q and JAK2-V617F in MPD suggests that del20q acts independently and is not one of the predisposing mutations for JAK2-V617F. Blood. 2009;113(9):2022-7.
40. Jeromin S, Haferlach T, Grossmann V, Alpermann T, Kowarsch A, Haferlach C, et al. High frequencies of SF3B1 and JAK2 mutations in refractory anemia with ring sideroblasts associated with marked thrombocytosis strengthen the assignment to the category of myelodysplastic/myeloproliferative neoplasms. Haematologica. 2013;98 (2):e15-7.
41. Hahm C, Mun YC, Seong CM, Chung WS, Huh J. Additional genomic aberrations identified by single nucleotide polymorphism array-based karyotyping in an acute myeloid leukemia case with isolated del(20q) abnormality. Ann Lab Med. 2012;32(6):445-9.
42. Braun T, De Botton S, Taksin A-L, Park S, Beyne-Rauzy O, Coiteux V, et al. Characteristics and outcome of myelodysplastic syndromes (MDS) with isolated 20q deletion: a report on 62 cases. Leuk Res. 2011;35(7):863-7.
43. Aziz A, Baxter EJ, Edwards C, Cheong CY, Ito M, Bench A, et al. Cooperativity of imprinted genes inactivated by acquired chromosome 20q deletions. J Clin Invest. 2012;123(5):2169-82.
44. Valleron W, Laprevotte E, Gautier E-F, Quelen C, Demur C, Delabesse E, et al. Specific small nucleolar RNA expression profiles in acute leukemia. Leukemia. 2012;26(9):2052-60.
45. Boudry-Labis E, Roche-Lestienne C, Nibourel O, Boissel N, Terre C, Perot C, et al. Neurofibromatosis-1 gene deletions and mutations in de novo adult acute myeloid leukemia. Am J Hematol. 2013;88(4):306-11.
46. Wall M, Rayeroux KC. ETV6 deletion is a common additional abnormality in patients with myelodysplastic syndromes or acute myeloid leukemia and monosomy 7. Haematologica. 2012;97(12):1931-3.
47. Andreasson P, Johansson B, Arheden K, Billström R, Mitelman F, Höglund M. Deletions of CDKN1B and ETV6 in acute myeloid leukemia and myelodysplastic syndromes without cytogenetic evidence of 12p abnormalities. Genes Chromosomes Cancer. 1997;19(2):77-83.
48. Haferlach C, Bacher U, Schnittger S, Alpermann T, Zenger M. ETV6 Rearrangements Are Recurrent in Myeloid Malignancies and Are Frequently Associated with Other Genetic Events. Genes Chromosomes Cancer. 2012;51(4):328-37.
49. Thol F, Friesen I, Damm F, Yun H, Weissinger EM, Krauter J, et al. Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. J Clin Oncol. 2011;29(18):2499-506.
50. Schnittger S, Eder C, Jeromin S, Alpermann T, Fasan A, Grossmann V, et al. ASXL1 exon 12 mutations are frequent in AML with intermediate risk karyotype and are independently associated with an adverse outcome. Leukemia. 2013;27(1):82-91.