

Genomic characterization of spleens in patients with myelofibrosis

Despite the central role of splenomegaly in the pathophysiology of myelofibrosis (MF), evidence regarding the genomic landscape within these spleens is limited. In a study reporting the splenic cytogenetics of 92 patients with MF who underwent splenectomy, 52 (56%) had abnormal splenic karyotypes. A comparison between spleen and bone marrow (BM) karyotypes was available in 68 patients, with concordant results in 85% and discordant in 15%. In 9 out of the 10 patients with discordant findings, additional abnormalities occurred in the spleens.¹ Subsequent studies showed presence of *JAK2V617F* in the spleens of MF patients, and reported rare cases of *JAK2V617F* occurring in the spleen but not the BM.^{2,3} Since the publication of these studies, technologies that provide significantly higher resolution of genomic alterations have emerged. To the best of our knowledge, chromosomal microarrays and next generation sequencing (NGS) have not been applied to study MF spleens. We performed conventional cytogenetics, FISH, array-comparative genomic hybridization with single nucleotide polymorphisms microarray (aCGH+SNP), and NGS on spleen cells from 14 patients with MF who had undergone splenectomy from 2011 until 2015, and assessed the degree of genomic diversity. We hypothesized that specific genomic aberrations might be unique to the spleen, and can potentially contribute to disease progression.

We previously reported the clinical characteristics and post-splenectomy outcomes of 12 of the 14 patients.⁴ Our cohort included patients with primary MF and post-polythemia vera or post-essential thrombocythemia MF who fulfilled the 2008 WHO diagnostic criteria.⁵ Median age was 65 years (range 51-79). Indications for splenectomy were refractory cytopenias in 10 cases, symptomatic splenomegaly not responding to ruxolitinib in 2, and preparation for stem-cell transplantation in 2. Median post-splenectomy survival was 14 months but ranged widely from 34 days to over four years. Following ethical approval, single-cell suspensions from spleen specimens were prepared as previously described.⁶ BM or peripheral blood (PB) samples were collected within two months prior to and/or following splenectomy. The genomic studies are summarized in Table 1.

Metaphase cytogenetics and interphase-FISH were performed using standard methodology. Cytogenetic results were concordant between spleens and BM/PB in 12 (86%) patients, including 7 with a normal karyotype and 5 with abnormal karyotypes. Chromosomal abnormalities occurred at comparable frequencies in both tissue specimens. Discordant findings were observed in 2 (14%) patients (ns. SP01 and SP09), both harboring a complex karyotype in which the spleen contained additional abnormalities that were not detected in the PB. In patient SP01, who had pentasomy-21 in spleen only, trisomy-21 was subsequently observed in a post-splenectomy PB karyotype. In another patient (n. SP02), who at the time of splenectomy had a concordant normal karyotype, del(20q) was detected in a single (non-clonal) spleen metaphase cell. This patient developed a dominant del(20q) clone (85% of metaphase cells) in a BM study performed 2.5 years post splenectomy, suggesting that this clone originated in the spleen. Since the spleen cells from each patient always contained at least the same abnormalities as the BM/PB, it seems likely that MF originated in the BM and later migrated to the spleen.

However, the additional abnormalities detected in the spleens suggest that clonal evolution can occur within the MF spleen and that these cells then re-enter the BM/PB post splenectomy. This hypothesis is supported by evidence of leukemic transformation arising in the spleens of patients with myeloproliferative neoplasms (MPN).⁷ Other explanations, such as the emergence of previously undetectable clones in the BM, are also possible.

Array-CGH+SNP was performed on genomic DNA extracted from spleen cells available from 13 patients, using GenetiSure Unrestricted-2X400K array (Agilent Technologies, Santa Clara, CA, USA). This platform can identify copy-number alterations (CNAs) and copy-neutral loss of heterozygosity (CN-LOH) involving 2,300 cancer-related genes with an average of 6 probes per exon, affording a resolution of <500bp for CNAs and 2.5Mb for CN-LOH. CNAs were called if they were >500bp and contained a minimum of five probes. Regions of CN-LOH were called if they were ≥5Mb. For each CNA detected we reviewed the Database of Genomic Variants (DGV) (<http://dgv.tcag.ca/dgv>) to exclude normal copy-number variants.

Array-CGH+SNP revealed gain of 168 regions, loss of 43 regions and 37 regions of CN-LOH (Figure 1A). Genomic alterations were detected in all patients, including those with a normal karyotype. In patient n. SP01, aCGH+SNP revealed chromothripsis that was cytogenetically described as der(5q), included 78 alterations, and correlated with a *TP53* mutation as shown by NGS. Chromothripsis was recently linked to *TP53* alterations in acute myeloid leukemia with complex karyotypes.⁸ When patient n. SP01 was excluded from the analysis, the mean number of genomic alterations per patient was 14 (range 7-20) including 9.4 gains, 2.1 losses, and 2.6 CN-LOH. These frequencies are higher than previously reported in array studies of advanced MF, likely due to the higher resolution of this platform. Array analysis confirmed all cytogenetic abnormalities except for balanced t(8;12) in SP05, and del(20q) in 5% of metaphase cells (below the sensitivity of array testing) in patient n. SP14. However in patient n. SP14, aCGH+SNP identified deletion of *ETV6* (12p13.2), confirmed by FISH in more than 60% of both spleen and PB cells, that was not detected by metaphase cytogenetics. Additional clinically relevant abnormalities revealed solely by array were regions of CN-LOH involving mutated genes, such as CN-LOH of 9p in 4 *JAK2*-mutated patients.

While many of the novel genomic alterations detected by array occurred in single cases, eight regions of gain were each identified in a remarkably high proportion of patients. Four of these recurrent regions occurred within *RHOC* (1p13.2), *NCOR2* (12q24.31), *RASA3* (13q34), and *TAF15* (17q12), genes reported in association with myeloid malignancies⁹⁻¹² (Table 2). *RHOC* encodes a GTPase up-regulated in numerous solid cancers, and was previously implicated in leukemogenesis.⁹ *NCOR2* encodes SMRT, a member of a protein complex that can modify chromatin structure or bind to nuclear receptors to repress transcription. Remarkably, mice carrying inactivating mutations in SMRT developed a syndrome resembling MF that included BM fibrosis and splenomegaly. SMRT inactivation resulted in reduced repression of the retinoic acid receptor, leading to increased cytokine gene expression, particularly thrombopoietin and TGFβ, resembling pathological pathways that occur in MF patients.¹⁰ *RASA3* encodes for a negative regulator of the RAS and RAP1 pathways, and inhibitor of RAP1-dependent platelet activation. Inactivation of

Table 1. Genomic characterization of spleens in 14 patients with myelofibrosis.

Patient #	Diagnosis and DIPSS	Age at splenectomy and sex	Karyotype	aCGH+SNP*	NGS
SP01	PMF, Int-2	58, F	complex karyotype additional: add(10q), del(20q), +21x3	complex array including chromothripsis 5q	JAK2 31% TP53 43% ZRSR2 20%
SP02	post-PV MF, Int-2	70, F	normal [del(20q) in 1 metaphase cell]	CN-LOH 4q CN-LOH 9p del(21q)	JAK2 78% TET2 35% U2AF1 51%
SP03	PMF, Int-1	64, M	normal	CN-LOH 4q CN-LOH 13q	triple negative SRSF2 22% TET2 49%
SP04	PMF, Int-2	79, M	del(20)(q11.21q13.32)	del(20)(q11.21q13.32)	JAK2 85% TET2 37%
SP05	PMF, Int-2	68, M	t(8;12)(q13;q15)	no known CNA or CN-LOH	CALR 14.8% TP53 45% EZH2 45%
SP06	PMF, Int-2	65, M	normal	CN-LOH 8q	JAK2 13% ASXL1 11% RUNX1 10% NRAS 5.4% EZH2 26%
SP07	PMF, Int-2	63, M	normal	NA	JAK2 42% SRSF2 25%
SP08	post-PV MF, Int-2	65, F	del(20)(q11.23q13.33) and subclonal +8	del(20)(q11.23q13.33) CN-LOH 5q	JAK2 41% ZRSR2 25%
SP09	post-PV MF, Int-2	64, F	complex karyotype additional: t(1;7), ins(1;19), del(12p), -13, i(17q), der(20)t(13;20)	complex array CN-LOH 9p	JAK2 69%
SP10	PMF, Int-2	75, M	normal	CN-LOH 21q	JAK2 28% TP53 32% SF3B1 40%
SP11	post-PV MF, Int-2	53, F	normal	CN-LOH 9p	JAK2 55% TET2 6.8%
SP12	PMF, Int-2	73, M	del(13)(q13.1q21.2)	del(13)(q13.1q21.2) CN-LOH 9p	JAK2 91% SRSF2 45% RUNX1 45%
SP13	post-ET MF, Int-2	52, F	normal	CN-LOH 4q CN-LOH 7q	CALR 19.4% ASXL1 33% TET2 63% EZH2 74% SETBP1 36%
SP14	PMF, Int-1	51, M	del(20q)(q11q13) del(12p)(p13.2) [FISH]	del(12p)(p13.2)	JAK2 40% ASXL1 39.4% SRSF2 39% KRAS 38%

DIPSS: Dynamic International Prognostic Scoring System; aCGH+SNP: array comparative genomic hybridization and single nucleotide polymorphisms microarray; NGS: next generation sequencing; PMF: primary myelofibrosis; Post-PV MF: post-polycythemia vera myelofibrosis; Post-ET MF: post-essential thrombocythemia myelofibrosis; CNA: copy number alteration; CN-LOH: copy-neutral loss of heterozygosity; F: female; M: male; NA: not available. Text appearing in bold indicates cases with abnormalities found in the spleen but not the peripheral blood or bone marrow. *Only selected regions known to be recurrent in myeloid malignancies are shown. For complete results of our aCGH+SNP analysis, see Figure 1.

RASA3 in mouse hematopoietic cells led to megakaryocyte dysplasia and resulted in cytopenias, BM fibrosis, splenomegaly and decreased survival.¹¹ TAF15 is involved in the fusion-gene product of a rare translocation reported in acute leukemia.¹²

In validation analyses, the recurrent regions were observed in a cohort of 173 patients with myeloid malignancies (Table 2), albeit at lower frequencies, but not in any of 42 individuals without disease. Gain within NCOR2 was reported in the DGV in 2 individuals (<1%), but was not excluded from our analysis due to its high frequency in our cohort. Furthermore, 3 patients with this gain harbored mutations in NCOR2 by NGS, located distal from the region of gain. Focal gains or duplications

occurring in either coding or non-coding sequences within a gene can lead to its decreased or increased expression or to aberrant function.¹³ From these studies, we suggest that these novel abnormalities might play a role in disease progression.

Simultaneous aCGH+SNP was performed on matched spleen and PB specimens from 4 patients (Figure 1B). Abnormalities of known clinical significance occurred in both spleen and PB. However, multiple discordant CNAs and regions of CN-LOH in all 4 patients were observed, with a higher number of abnormalities occurring in the spleens.

To complete the genomic characterization of MF spleens, high-throughput sequencing with a targeted

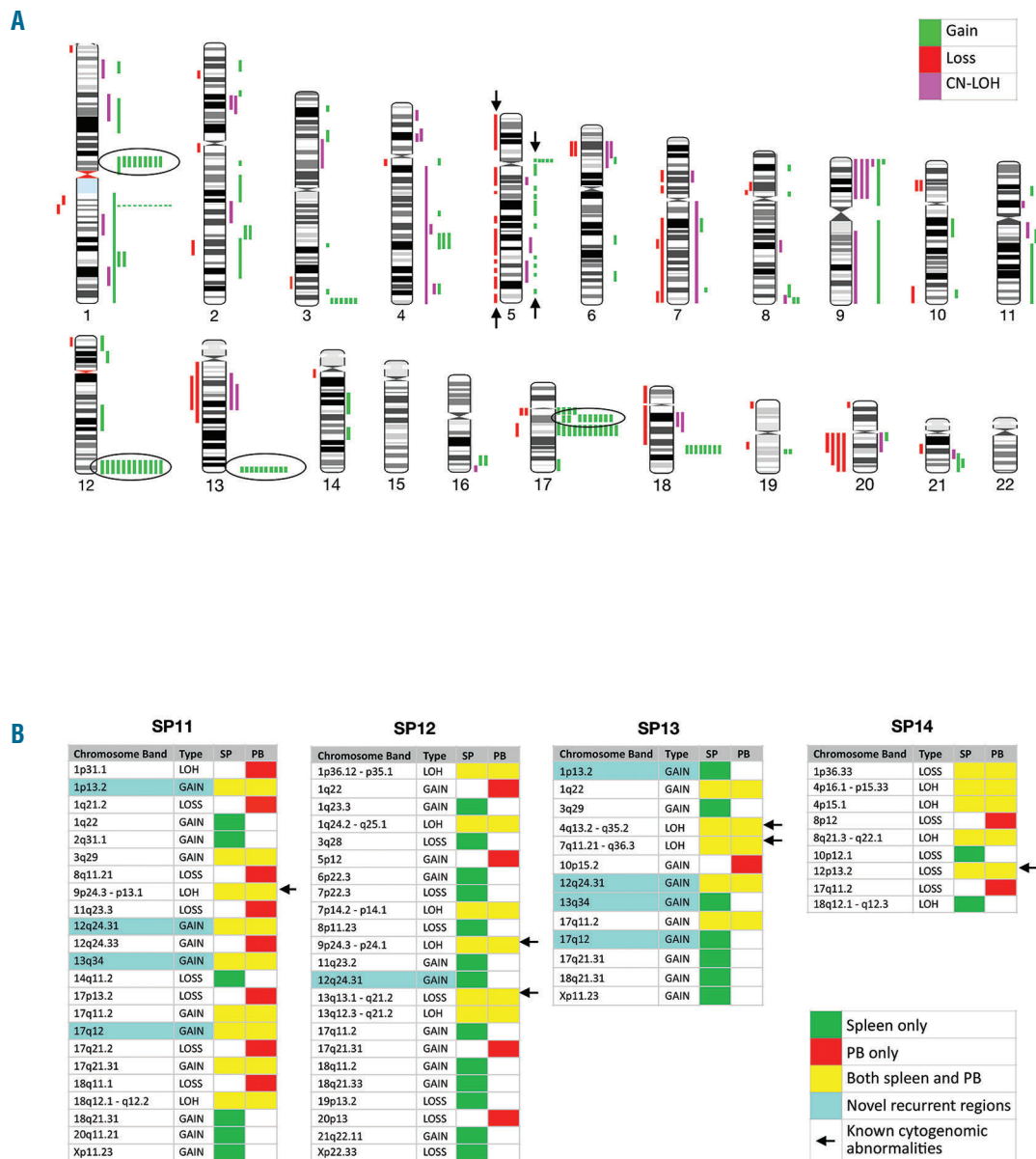


Figure 1. aCGH+SNP analysis. (A) Chromosome ideogram of all alterations detected in myelofibrosis (MF) spleens from 13 patients. Bars depict the physical position of the aberration but not the size. All bars shown between arrows represent genomic changes within chromothripsis of chromosome 5 in SP01. The novel recurrent regions of gains mapping to 1p13.2 (RHOC), 12q24.31 (NCOR2), 13q34 (RASA3) and 17q12 (TAF15) are shown in circles. (B) Comparison of matched spleen and peripheral blood (PB) specimens from 4 patients. Green boxes show alterations detected in spleens only; red in PB only; yellow in both tissues. Aqua highlights the novel recurrent regions of gain discussed above. Of note, in SP11 all four recurrent regions were detected in both spleen and PB, while in SP12 and SP13 most were detected only in the spleen (except for gain mapping to 12q24.31 in SP13). Arrows point to abnormalities of known clinical significance, including del(13q), del(12p13.2) involving ETV6, and CN-LOH of 9p, 4q and 7q involving JAK2, TET2 and EZH2, respectively.

Table 2. Regions of recurrent chromosomal gains and involved genes.

Chromosomal region	Size of region (bp)	Average log ₂ ratio*	Involved gene	Exon / intron	# of patients in study cohort (%)	# of patients in validation cohort (%) [§]
1p32.2	786	0.5	RHOC	Intron	9 (69%)	7 (4%)
12q24.31	1,895	1.0	NCOR2	Exon 49	12 (92%)	17 (10%)
13q34	1,958	1.3	RASA3	Exons 18, 17	11 (85%)	17 (10%)
17q12	1,272	0.6	TAF15	Exons 13-15	9 (69%)	16 (9%)

bp: base pairs; RHOC: Ras homolog family member C; NCOR2: Nuclear receptor corepressor 2 (also known as SMRT, Silencing Mediator of Retinoic acid and Thyroid hormone receptor); RASA3: Ras p21 protein activator 3; TAF15: TATA-box binding protein associated factor 15. *A cutoff signal-intensity log₂ ratio (between patient and reference DNA) of ≥ 0.18 for gain or ≤ -0.18 for loss was used. [§]The validation cohort consisted of 173 patients with myeloid malignancies including acute myeloid leukemia (n=61), chronic myeloid leukemia (n=4), myelodysplastic syndromes (n=61) and myelofibrosis (n=47) of all risk categories.

deep sequencing assay of 585 cancer-related genes (HemePACT) was performed as previously described.¹⁴ NGS identified an average of 2.8 oncogenic mutations per spleen. Aside from MPN driver mutations detected in 13 of the 14 patients, additional acquired mutations that have been identified in MPNs¹⁵ were detected in most patients. The most common additionally mutated genes were *TET2* (in 5 patients) followed by *SRSF2* (in 4 patients), and *ASXL1*, *EZH2* and *TP53* (in 3 patients). The high incidence of mutations in epigenetic modifiers and splicing factors is typical of an advanced MF patient population. NGS was used to compare matching spleen and PB specimens in 4 patients, harboring a total of 14 mutations. The concordance of mutations and their variant allele frequencies was strikingly high. However, an *SRSF2* mutation was detected only in the spleen of SP12.

The high concordance of genomic aberrations between spleens and BM/PB, and the comparable frequency of both chromosomal abnormalities and mutation allele burden; suggest an overall similar clonal architecture existing within these tissues. In spite of the overall similarity, discordant findings were detected by each modality. In almost all of these instances, additional abnormalities were found in the patients' spleens. These findings are consistent with earlier studies comparing MF spleens to BM/PB specimens^{1-3,6,7} and suggest a higher genomic complexity in the spleen.

In conclusion, these studies indicate that there are genomic differences between MF hematopoietic cells that reside in the spleen and BM or PB, which might be the consequence of different tumor microenvironments. Array-CGH+SNP detected novel recurrent gains within *RHOC*, *NCOR2*, *RASA3* and *TAF15* that may play a role in disease pathogenesis, and these merit further investigation.

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