Genomic analysis of myeloproliferative neoplasms in chronic and acute phases

Myeloproliferative neoplasms (MPNs) are chronic, clonal hematopoietic disorders that include polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF). A substantial proportion transforms into acute myeloid leukemia (AML) with a dismal prognosis.[1] Mutually-exclusive hotspot driver mutations in *JAK2, CALR,* and *MPL* genes are present in 90% of PV, ET and PMF. The remaining 10% of cases are called triple negatives (TN). Mutations in other genes (e.g., *ASXL1, DNMT3A, EZH2, IDH1/2, SH2B3, SRSF2, TET2*) are also recurrent in MPNs.[2-5] To better define the genetic events involved in MPN evolution and transformation,[2,3,5,6] we studied 57 MPNs in chronic phase and 38 post-MPN AMLs from the IPC/CRCM Tumor Bank (authorization #AC-2007-33). Patients were appropriately informed and asked to consent in writing, in compliance with French and European regulations.

MPN diagnosis was established according to the revised criteria of the World Health Organization.[7] The 57 chronic cases were separated into two groups: 40 without acute evolution, arbitrarily called "steady" MPNs or sMPNs (median follow up was 17.9 years (11–28)); and 17 that transformed to AML, called tMPNs (median time between diagnosis of chronic and acute phases was 8.5 years (1– 29)). The latter group had 17 matched post-MPN AML samples among the 38 cases of post-MPN AML. Genomic analysis was carried out for most patients in chronic phase during disease course, not at diagnosis. Median time between diagnosis and sample was 12.9 years (2–24 years) for sMPNs, and 5.7 years (0-25) for tMPNs (*Online Supplementary Table S1*). Post-MPN AML

Table 1. Frequency of gene alterations (mutations and CNVs) grouped according to the affected cellular function: comparison between sMPNs and tMPNs and comparison between total MPNs and total post-MPN AML cohorts.

Driver genes and cellular functions affected by mutations and CNV abnormalities	sMPNs (n=40)	tMPNs (n=17)	*P*	OR and CI	Total MPNs (n=57)	Total post-MPN AML (n=38)	*P*	OR and CI
JAK2 V617F	19 (47.5%)	14 (82.4%)	**0.020**	0.20 [0.032; 0.87]	33 (57.9%)	22 (57.9%)	1	1 [0.40; 2.50]
CALR	14 (35%)	0 (0%)	**0.0053**	Not app	14 (24.6%)	5 (13.2%)	0.20	2.13 [0.64; 8.35]
MPL W515	1 (2.5%)	1 (5.9%)	0.51	0.42	2 (3.5%) [0.0051; 34.20]	1 (2.6%)	1	1.34 [0.068 ; 81.46]
RNA Splicing (*SRSF2, SF3B1, U2AF1, PRPF8, ZRSR2*)	1 (2.5%)	9 (52.9%)	**$2.30*10^{-5}$**	0.025 [0.0005 ; 0.22]	10 (17.5%)	17 (44.7%)	**0.0054**	0.27 [0.092;0.74]
Chromatin modification (*ASXL1, EZH2,…*)								
without aCGH	3 (7.5%)	6 (35.3%)	**0.015**	0.155 [0.022; 0.86]	9 (15.8%)	12 (31.6%)	0.082	0.41 [0.13; 1.22]
with aCGH						18 (47.4%)	**0.0012**	0.21 [0.071; 0.60]
Tumor suppressor (*NPM1; RB1; TP53*)								
without aCGH	3 (7.5%)	2 (11.8%)	0.63	0.61 [0.063; 8.04]	5 (8.8%)	15 (39.5%)	**0.00058**	0.15 [0.038; 0.50]
with aCGH	5 (12.5%)		1	1.07 [0.15; 12.43]	7 (12.3%)		**0.0029**	0.22 [0.066; 0.66]
TP53	2 (5%)	2 (11.8%)	0.57	0.40 [0.027; 6.01]	4 (7.0%)	13 (34.2%)	**0.0010**	0.15 [0.032; 0.54]
DNA methylation (*DNMT3A, TET2, IDH1/2*)								
without aCGH	4 (10%)	4 (23.5%)	0.22	0.37 [0.059; 2.28]	8 (14.0%)	19 (50%)	**0.00020**	0.17 [0.053; 0.48]
with aCGH						20 (52.6%)	**$7.9*10^{-5}$**	0.15 [0.048; 0.43]
Signaling pathway (*CBL, NF1, FLT3, RAS,…*)								
without aCGH	1 (2.5%)	4 (23.5%)	**0.024**	0.088 [0.0017; 0.99]	5 (8.8%)	13 (34.2%)	**0.0029**	0.19 [0.047; 0.64]
with aCGH						15 (39.5%)	**0.00058**	0.15 [0.038; 0.50]
Transcription regulation (*RUNX1, CUX1,…*)								
without aCGH	4 (10%)	5 (29.4%)	0.11	0.27 [0.046; 1.50]	9 (15.8%)	13 (34.2%)	**0.048**	0.36 [0.12; 1.07]
with aCGH		6 (35.3%)	0.051	0.21 [0.037; 1.070]	10 (17.5%)	18 (47.4%)	**0.0027**	0.24 [0.083; 0.66]
Cohesin complex (*STAG2, RAD21,…*)								
without aCGH	1 (2.5%)	1 (5.9%)	0.51	0.42 [0.0051; 34.20]	2 (3.5%)	3 (7.9%)	0.39	0.43 [0.034; 3.93]
with aCGH						5 (13.2%)	0.11	0.24 [0.022; 1.59]
aCGH Type I	33 (82.5%)	12 (75%)**	0.71	1.56 [0.28; 7.54]	45 (80.4%)**	11 (28.9%)	**$1.26*10^{-6}$**	9.73 [3.49; 29.52]
aCGH Type II	7 (17.5%)	4 (25%)**			11 (17.5%)**	12 (31.6%)	0.23	0.53 [0.18; 1.53]
aCGH Type III	0 (0%)	0 (0%)**	Not app	Not app	0**	15 (39.5%)	**$1.66*10^{-7}$**	Not app
Median number of mutation per patient	1.38	3.18	**0.0064**	Not app	1.91	3.92	**$2.94*10^{-6}$**	Not app

*sMPNs, "steady" MPNs; tMPNs, "transformed" MPNs (CI, confidence interval at 95%; CNV, copy number variation; OR, odd ratio; Not app, not applicable; **available for 16 of 17 samples of tMPNs and 56 out of 57 samples of MPN.*

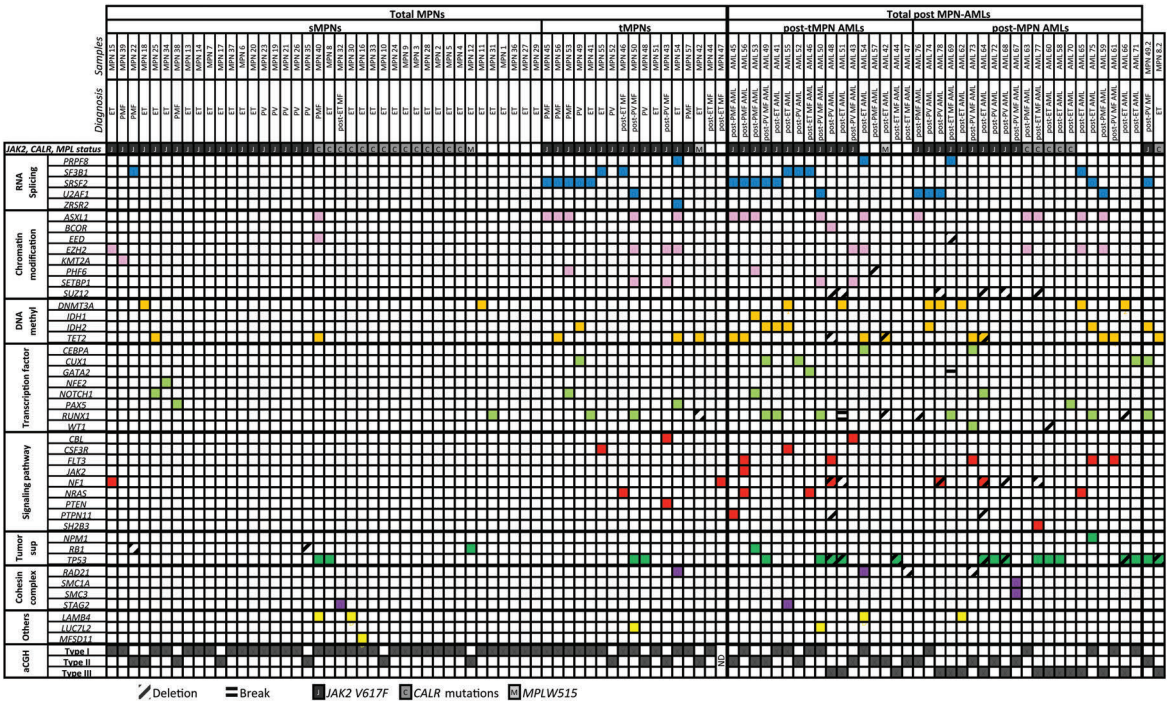

Figure 1. Mutational spectrum and copy number aberrations in myeloproliferative neoplasms (MPNs) and post-MPN acute myeloid leukemias (AMLs). Driver mutations of MPNs are designated by squares shaded in grey and J (JAK2 V617F), C (*CALR* mutations), M (MPLW515); and white squares correspond to triple negatives. Genes studied are grouped according to the cellular function they are involved in. Colored squares indicate mutations. sMPNs: "steady" MPNs; tMPNs: MPNs that transformed to AML; ET: essential thrombocytosis; PV: polycythemia vera; MF: myelofibrosis; ND: not determined.

samples were obtained at diagnosis aside from 2 samples obtained during accelerated phase. History of the diseases is detailed in *Online Supplementary Table S2.*

DNAs from peripheral blood leukocytes (n=87) or bone marrow aspirates (n=10) were extracted and prepared for sequencing as described.[8,9] For each sample, a library of all coding exons and intron-exon boundaries of 79 genes, selected on the basis of their known involvement in the pathogenesis of myeloid malignancies (*Online Supplementary Table S3*), was constructed with HaloPlex target enrichment system (Agilent technologies, Santa Clara, USA) and sequenced on an Illumina MiSeq (*Online Supplementary Methods*). Copy number aberrations (CNAs) were identified by using genome-wide, high-density 244K CGH Microarrays (Hu-244A, Agilent Technologies, Massy, France) as described.[8] Fisher's exact tests were used for qualitative variables with discrete categories, and Student's *t*-test for continuous variables. $P<0.05$ was considered statistically significant.

In the 56 MPNs at chronic phase and 38 post-MPN AMLs analyzed by aCGH, we found CNAs in 40 samples. We classified aCGH profiles into three types. In type I profiles, called "normal-like", no CNA was detected. Type II (intermediate profiles, between one and three CNAs) showed interstitial deletions/breaks/gains that affected few or single genes within a chromosomal region, such as deletions in 1p31 (*NF1A*), 4q24 (*TET2*), 8q21 (*RAD21*), 12p13 (*ETV6, CDKN1B*), Xq25 (*STAG2*), 17q11 (*NF1, SUZ12*), 20q12-q13 (*L3MBTL1, PTPN1, PTPRT*), 21q22 (*RUNX1*) or isolated gain or loss of one chromosome or chromosome arm (+8, -7, 1q+). Type III comprised complex profiles with more than three CNAs. In the 57 MPNs and 38 post-MPN AMLs analyzed by NGS, we retained 139 single-nucleotide variants (SNVs) and small insertions or deletions (indels) affecting 45 genes with at least one mutation in 92.8% of samples (*Online Supplementary Tables S2 and S4*).

Both sMPNs and tMPNs had a majority of "normal-like" aCGH profiles (Figure 1 and *Online Supplementary Table S2*). Rare abnormalities such as del12p13, del20q12-q13 and +1q were observed; they may be associated with worse prognosis,[4,10,11] and should be taken into account at diagnosis. tMPNs patients were older than sMPNs at diagnosis (60.4 years-old *vs.* 46.6, $P=0.0012$) and more mutated than sMPNs (median number 3.18 *vs.* 1.38) (*Online Supplementary Tables S1*). tMPNs were more JAK2V617F-mutated (82.4% *vs.* 47.5%), less *CALR*-mutated (0% *vs.* 35%), and as often TN (11.8% *vs.* 10%). The frequency of alterations (mutations and CNVs) grouped according to the affected cellular function is depicted in Table 1. Genes involved in RNA splicing (*SRSF2* $P=0.001$), chromatin modification (*ASXL1* $P=0.007$), and signaling pathways were more affected in tMPNs (52.9%, 35.3% and 23.5% of the cases, respectively) than in sMPNs (2.5%, 7.5% and 2.5% of the cases, respectively) (Figure 1, Table 1).

In the 38 post-MPN AMLs, the median time for acute transformation (AT) was 10.6 years, and the median age at diagnosis was 70.5 years (*Online Supplementary Table S1*). Fifteen of the 38 cases (39.5%) had a type III aCGH profile, 12 (31.6%) a type II, and 11 (28.9%) a type I; 57.9% were JAK2V617F-mutated, 13.2% were *CALR*-mutated, 1 was MPL-mutated, and 26.3% were TN. The median number of mutations in post-MPN AMLs was higher than in sMPNs (3.92 *vs.* 1.38, $P=3.33*10^{-9}$) but the same as in tMPNs (3.92 *vs.* 3.18, $P=0.26$) (Figure 1, Table 1). The median number of mutations per case was differ-

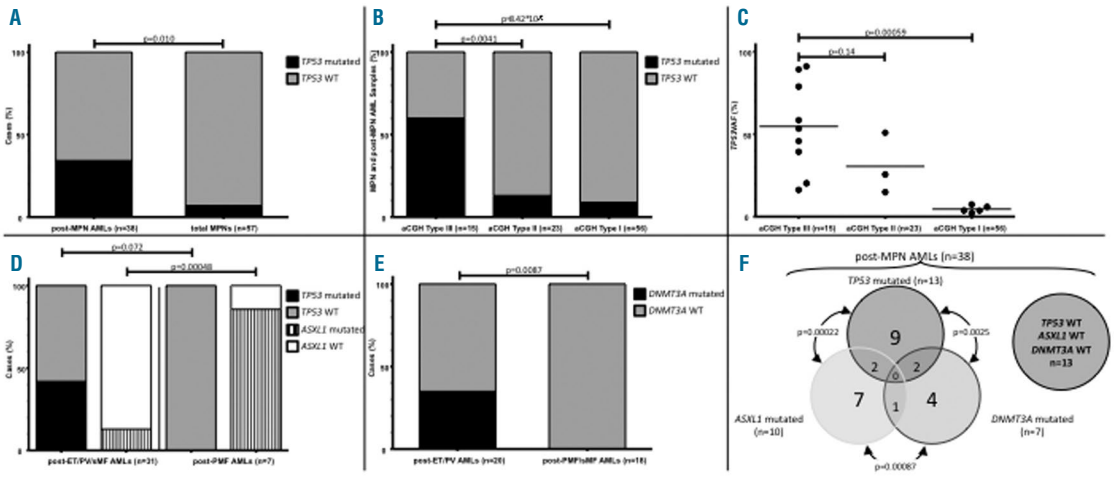

Figure 2. *TP53*, *ASXL1* and *DNMT3A* mutations in chronic MPNs and/or post-MPN AMLs. (A) *TP53* mutations were more frequent in post-MPN AMLs than in MPNs; (B) *TP53* mutations were associated with aCGH type III complex profiles; (C) High *TP53* VAF was associated with aCGH type III; (D) *TP53* mutations were more frequent in post-ET/PV/sMF AML, whereas *ASXL1* mutations were associated with post-PMF AML; (E) *DNMT3A* mutations were associated with post-ET/PV AML; (F) *TP53*, *ASXL1* and *DNMT3A* genes are not often co-mutated in post-MPN AML. ET, essential thrombocytemia; PV, polycythemia vera; PMF, primitive myelofibrosis; sMF, secondary myelofibrosis.

ent between JAK2-, CALR-, MPL-mutated, and TN cases (4.5 mutations, 2.8, 2 and 3.4, respectively). *CALR*-mutated cases had fewer other mutations than *JAK2*-mutated cases (P=0.04). Three of the 5 CALR-mutated post-MPN AMLs had a *TP53* mutation and an aCGH type III profile (60%). *In vivo* cooperativity of *TP53* alterations with JAK2V617F has already been reported,[5] but not yet with *CALR* mutations. Half of the AMLs were mutated in genes involved in DNA methylation: *TET2 (21.1%), DNMT3A (18.4%) and IDH1/2 (15.8%)*. Seventeen cases (44.7%) were mutated in RNA splicing genes: *SRSF2* (15.8%), *U2AF1* (13.2%), *SF3B1* (10.5%), and *PRPF8* (5.3%). *TP53* mutations were found in 13 cases (34.2%) (Figure 2A, see *Online Supplementary Figure S1* for *TP53* mutations in our study and in others). Thirteen cases (34.2%) were mutated in genes involved in cell signaling: *FLT3* (13.2%), *NRAS* (7.9%), and *NF1* (7.9%). *NF1*-mutated cases also had *NF1* deletions. Thirteen cases (34.2%) had mutations in transcription regulators with frequent damaging mutations of *RUNX1* (13.2%). Twelve cases (31.6%) were mutated in chromatin regulators: *ASXL1* (26.3%) and *EZH2* (13.2%). (Figure 1, Table 1, *Online Supplementary Table S2*).

When we compared the mutational status of the 57 MPNs to that of the 38 post-MPN AMLs, we found that mutations in genes involved in DNA methylation, tumor suppressors, signaling, RNA splicing and transcription factors were more frequent in post-MPN AMLs. Also, whilst chronic phases were associated with "normal-like" aCGH profiles (45/56, 80.4%), the 38 AMLs had more type III profiles (15/38, 39.5%) (Table 1).

Seventeen matched chronic phases were available for the 38 post-MPN AML patients. For these pairs, median time between diagnosis of chronic and acute phases was 8.5 years (*Online Supplementary Table S1*). The acute phases had an average gain of one mutation (4.24 *vs.* 3.18), and a worse aCGH profile (35.3% type I, 47.1% type II, 17.6% type III) than the chronic phases (75% type I, 25% type II) (P=0.037). VAF evolution between chronic and acute phase for each patient is represented in *Online Supplementary Figure S3*. For most mutations in chronic phase, the median VAF was around 40%, and there was no increase of VAF in acute phases (VAF variation <20%), suggesting that these mutations were present in the founding clone (as previously demonstrated[6]) (*Online Supplementary Figure S2*). In some cases, an increase of VAF was supported by a loss of heterozygosity (*EZH2*: AML43; *TP53*: AML48, 51; *TET2*: AML42). We also observed the apparition of *TP53* mutations with an average VAF of 40.2% in three cases (AML44, 49, 51) (*Online Supplementary Figure S3A*). Considering all cases, when the *TP53* VAF was >10%, the patients generally displayed a complex aCGH profile and complex karyotype, suggesting that mutated *TP53* confers genomic instability. Conversely, when *TP53* VAF was <10%, the cytogenetic analysis could not detect complex abnormalities (Figure 2B and 2C).

These results suggest that genome alterations and mutations in *TP53*, DNA methylation, and transcription factors are simultaneous with (and potentially responsible for) evolution to AML, whereas alterations in RNA splicing, chromatin modification, and signaling pathways, which are found in chronic phases before AT, may increase AT risk (*Online Supplementary Figure S3*). These results are in agreement with recent studies of myelodysplastic syndromes[12] and AML patients.[13] In two recent NGS studies of 197 MPNs in chronic phase[6] and 33 post-MPN AMLs[5], the distribution of mutations according to the cellular function affected was similar to that of our study. Here, we also compared the mutational status of chronic phase MPNs with stable course to chronic phase MPNs that transformed to AML, which showed a different profile (Table 1).

Post-PV/ET or post-secondary MF AMLs (13/31) had more *TP53* mutations than post-PMF AMLs (0/7). Conversely, they had less *ASXL1* mutations (4/31 *vs.* 6/7), and this was independent of their *JAK2/CALR/MPL* mutational status (Figure 2D). *DNMT3A* mutations were

more frequent in post-ET/PV AMLs than in post-MF (secondary or PMF) AMLs (7/20 *vs.* 0/18) (Figure 2E). In total, 25 out of the 38 post-MPN AMLs were *ASXL1* and/or *TP53, ASXL1* and/or *DNMT3A, TP53* and/or *DNMT3A*, with very rare co-mutated cases (Figure 2F). These results suggest the existence of three mechanisms of AT depending on the MPN subtype, with *TP53* or *DNMT3A* mutation in post-ET/PV AML and *ASXL1* mutations in PMF.

Knowledge of mutations, including early detection of small abnormal clones, could modify therapeutic choices. In particular, the detection of mutations at diagnosis (e.g., *ASXL1, DNMT3A*, splice, *TP53*) and during disease course, may help identify an MPN with risk of AT and help adapt the treatment.

Frédéric Courtier,[1,2,5] Nadine Carbuccia,[1,2] Séverine Garnier,[1,2] Arnaud Guille,[1,2] José Adélaïde,[1,2] Nathalie Cervera,[1,2] Véronique Gelsi-Boyer,[1,2,4,5] Marie-Joelle Mozziconacci,[1,2,4] Jérôme Rey,[1,3,5] Norbert Vey,[1,3,5] Max Chaffanet,[1,2,5] Daniel Birnbaum[1,2,5] and Anne Murati[1,2,4,5]

[1]Centre de Recherche en Cancérologie de Marseille, (CRCM), Inserm, U1068, CNRS UMR7258, ; [2]Institut Paoli-Calmettes (IPC), Département d'Oncologie Moléculaire, ; [3]IPC, Département d'Hématologie and [4]IPC, Département de Biopathologie, Marseille and [5]Aix-Marseille Université, UM 105, Marseille, F-13284, France

Acknowledgments: the authors would like to thank the patients whose samples were included in the study.

Funding: this work was supported by Inserm, Institut Paoli-Calmettes and grants from the Laurette Fugain association (2013/05) and the Site de Recherche Intégrée sur le Cancer (SIRIC grant INCa-DGOS-INSERM 6038). We thank our collaborative group, the French Intergroup of Myeloproliverative neoplasms (FIM) for helpful discussions.

Correspondence: muratia@ipc.unicancer.fr
doi:10.3324/haematol.2016.152363

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.

References

1. Cervantes F, Tassies D, Salgado C, Rovira M, Pereira A, Rozman C. Acute transformation in nonleukemic chronic myeloproliferative disorders: actuarial probability and main characteristics in a series of 218 patients. Acta Haematol. 1991;85(3):124-127.
2. Abdel-Wahab O, Pardanani A, Patel J, et al. Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms. Leukemia. 2011;25(7):1200-1202.
3. Beer PA, Delhommeau F, LeCouedic JP, et al. Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. Blood. 2010;115(14):2891-2900.
4. Brecqueville M, Rey J, Devillier R, et al. Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase. Haematologica. 2014; 99(1):37-45.
5. Rampal R, Ahn J, Abdel-Wahab O, et al. Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. Proc Natl Acad Sci U S A. 2014;111(50):E5401-E5410.
6. Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. Blood. 2014;123(14):2220-2228.
7. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.
8. Bertucci F, Finetti P, Guille A, et al. Comparative genomic analysis of primary tumors and metastases in breast cancer. Oncotarget. 2016; 7(19):27208-27219.
9. Brecqueville M, Rey J, Bertucci F, 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):743-755.
10. Harutyunyan A, Klampfl T, Cazzola M, Kralovics R. p53 lesions in leukemic transformation. N Engl J Med. 2011;364(5):488-490.
11. Klampfl T, Harutyunyan A, Berg T, et al. Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood. 2011;118(1):167-176.
12. Pellagatti A, Roy S, Di GC, et al. Targeted resequencing analysis of 31 genes commonly mutated in myeloid disorders in serial samples from myelodysplastic syndrome patients showing disease progression. Leukemia. 2016;30(1):248-250.
13. Lindsley RC, Mar BG, Mazzola E, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. Blood. 2015; 125(9):1367-1376.