Molecular mechanisms associated with leukemic transformation of MPL-mutant myeloproliferative neoplasms

Philip A. Beer,[1,2,3] Christina A. Ortmann,[1,2,3] Frank Stegelmann,[4] Paola Guglielmelli,[5] John T. Reilly,[6] Thomas S. Larsen,[7] Hans C. Hasselbalch,[7] Alessandro M. Vannucchi,[5] Peter Möller,[4] Konstanze Döhner,[4] and Anthony R. Green[1,2,3]

[1]Cambridge Institute for Medical Research and [2]Department of Haematology, University of Cambridge, Hills Road, Cambridge, UK; [3]Department of Haematology, Addenbrooke's Hospital, Hills Road, Cambridge, UK; [4]University Hospital of Ulm, Department of Internal Medicine III, Albert-Einstein-Allee 23, Ulm, Germany; [5]Department of Haematology, University of Florence, Viale Morgagni, 85, 50134 Florence, Italy; [6]Department of Haematology, Royal Hallamshire Hospital, Sheffield, UK; [7]Department of Haematology and Pathology, Odense University Hospital, Denmark

ABSTRACT

Somatic activating mutations in *MPL*, the thrombopoietin receptor, occur in the myeloproliferative neoplasms, although virtually nothing is known about their role in evolution to acute myeloid leukemia. In this study, the *MPL* T487A mutation, identified in *de novo* acute myeloid leukemia, was not detected in 172 patients with a myeloproliferative neoplasm. In patients with a prior *MPL* W515L-mutant myeloproliferative neoplasm, leukemic transformation was accompanied by *MPL*-mutant leukemic blasts, was seen in the absence of prior cytoreductive therapy and often involved loss of wild-type *MPL* by mitotic recombination. Moreover, clonal analysis of progenitor colonies at the time of leukemic transformation revealed the presence of multiple genetically distinct but phylogenetically-related clones bearing different *TP53* mutations, implying a mutator-phenotype and indicating that leukemic transformation may be preceded by the parallel expansion of diverse hematopoietic clones.

Key words: MPL, JAK2, TP53, TET2, myeloproliferative neoplasm, acute myeloid leukemia, essential thrombocythemia, primary myelofibrosis, hydroxycarbamide.

Citation: Beer PA, Ortmann CA, Stegelmann F, Guglielmelli P, Reilly JT, Larsen TS, Hasselbalch HC, Vannucchi AM, Möller P, Döhner K, and Green AR. Molecular mechanisms associated with leukemic transformation of MPL-mutant myeloproliferative neoplasms. Haematologica 2010;95(12):2153-2156. doi:10.3324/haematol.2010.029306

Introduction

Acquired mutations in *MPL*, encoding the thrombopoietin receptor, are found in the myeloproliferative neoplasms (MPN) essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1-5] Exon 10 alterations affect the juxtamembrane (W515L/K/A/R) or transmembrane (S505N) domains, resulting in ligand-independent receptor activation.[6,7] An exon 9 T487A mutation, reported in a single case of *de novo* acute myeloid leukemia (AML), produced an ET-like phenotype in a mouse model[8] although its prevalence in human MPN is unknown.

Virtually nothing is known about molecular events associated with disease progression in *MPL*-mutant MPN. Although mutations in *TET2* and *MPL* may coexist,[9] their clonal relationship has not been reported. A mutant allele burden exceeding 50% occurs in patients with *MPL*-mutant PMF or rarely ET[1-5] and often reflects duplication of the mutant *MPL* allele by mitotic recombination.[10-12] AML following a *JAK2* V617F-positive MPN commonly lacks the *JAK2* mutation,[13-15] and although *MPL* mutations have been observed in unfractionated post-MPN AML bone marrow samples,[2] the *MPL* status of the prior MPN and of purified blast cells was not established.

We have studied the role of *MPL* mutations in early and leukemic phase MPN, focusing on the prevalence of mutations in exon 9, the role of *MPL* and additional mutations in leukemic transformation and mechanisms by which the wild-type *MPL* allele is lost.

Design and Methods

Screening for *MPL* exon 9 mutations was performed on a cohort of 172 patients attending a single MPN clinic in Cambridge, UK. Three patients who developed AML following an *MPL*-mutant MPN were identified on an ad hoc basis from clinics in Cambridge, Ulm and Florence. Patients were diagnosed with ET, post-ET myelofibrosis or PMF according to published criteria.[16,17] A diagnosis of AML transformation required 20% blasts or more in blood and/or bone marrow. Local Research Ethics Committee approval was obtained and studies were carried out in accordance with the principals of the Declaration of Helsinki. Cell fractionation and progenitor colony assays were performed as described.[15] Leukemic blasts, purified by CD34-immunomagnetic selection, were 90% or more pure by mor-

Acknowledgments: we would like to thank Drs Penny Wright and Wendy Erber for helpful comments on bone marrow histology and the Addenbrooke's Haematology Disorders Sample Bank for processing and managing patient samples.
Funding: work in the authors' laboratories is supported by the UK Medical Research Council, Leukaemia and Lymphoma Research, the Kay Kendal Leukaemia Fund, the NIHR Cambridge Biomedical Research Centre, the Leukemia and Lymphoma Society of America, and the 2008 MIUR Project funded to AMV.
Manuscript received on June 21, 2010. Revised version arrived on July 27, 2010. Manuscript accepted on August 24, 2010.
Correspondence: Anthony R. Green, Cambridge Institute for Medical Research, Hills Road, Cambridge CB2 0XY, UK. Phone: + international +44.1223.762665. Fax: international +44.1223.762670. E-mail: arg1000@cam.ac.uk

phological criteria. Mutations in MPL (exons 9 and 10), *N/KRAS* (codons 12, 13 and 61), *CEBPA* (exon 1), *RUNX1* (all coding exons), *GATA2* (exon 4), *NPM* (exon 12), *WT1* (exons 7 and 9), *TP53* (exons 4 - 8), *CBL* (exons 8 and 9), *IDH1* (exon 2), *IHD2* (exon 4) and *TET2* (all coding exons) were assessed by direct sequencing. *MPL* copy number was assessed by real-time PCR using control regions on 13q and 9p.

Results and Discussion

Expression of the AML-associated *MPL* exon 9 T487A allele in mouse bone marrow cells produced an ET-like disease *in vivo* that was indistinguishable from a similar mouse model of *MPL* W515L,[8] an allele associated with human ET and PMF. Of note, *JAK2* V617F mutations have been observed in occasional patients with *de novo* AML, indicating that MPN-associated mutations may be seen in *de novo* acute leukemia, and/or occasional patients may present in blastic phase of a previously undiagnosed MPN.[15] To ascertain whether the *MPL* T487A allele or other changes in the MPL extracellular-juxtamembrane domain are associated with chronic phase ET or with PMF, *MPL* exon 9 was sequenced in granulocyte DNA from 172 patients (Table 1). No mutations were detected. These data indicate that *MPL* exon 9 mutations occur rarely, if at all, in human MPN.

Progression to acute leukemia is observed in a proportion of patients with a *JAK2*-mutant, *MPL*-mutant or mutation negative MPN, and in ET the presence or absence of an *MPL* mutation does not appear to modulate this risk.[4] To investigate the role of *MPL* mutations in leukemic transformation, we studied 3 patients with AML following an *MPL* W515L-positive MPN (Table 2). All 3 patients were negative for the *JAK2* V617F mutation. In patients 1 and 2, bone marrow studies performed at AML progression showed granulocytic hyperplasia, dysplastic megakaryocytes, reticulin fibrosis of 3 or more (graded on a 0-4 scale) and clusters of $CD34^+$ cells, in keeping with the AML subtype 'acute panmyelosis with fibrosis'[7,18] (Figure 1A). In patient 3, AML was diagnosed by more than 95% blast cells in the peripheral blood. Patients 1 and 3 had received hydroxycarbamide but patient 2 had not received cytoreductive therapy. As patients with a *JAK2* V617F-positive MPN may develop leukemia that lacks the *JAK2* mutation,[13-15] leukemia *MPL* mutation status was determined using purified blasts, free from contamination by the preceding MPN. In patient 1, initially diagnosed with ET, leukemic blasts were heterozygous for the *MPL* W515L mutation, whereas in patients 2 and 3, with preceding PMF and post-ET myelofibrosis, respectively, only the mutant *MPL* allele was detected (Figure 1B). The absence of wild-type allele in patients 2 and 3 might reflect acquisition of a second mutation, deletion of the wild-type allele or mitotic recombination.

To distinguish between these possibilities, we studied W515L-homozygous leukemic blasts from patients 2 and 3 together with granulocytes from a W515L-positive *JAK2* V617F-negative PMF patient (patient 4) with a mutant allele proportion of over 0.9. In all 3 patients, informative SNPs (genotyped by direct sequencing) at both the telomeric end of the *MPL* locus and 39Mb distal (close to the 1p telomere) showed loss of heterozygosity in leukemic blasts (patients 2 and 3) or granulocytes (patient 4) (*data not shown*), excluding acquisition of a second *MPL* mutation. To distinguish deletion of the wild-type allele from mitotic recombination, *MPL* copy number was assessed by real-time PCR, which demonstrated two copies of *MPL* in all cases (Figure 1C). These findings demonstrate that homozygosity for an acquired *MPL* W515L mutation had arisen by mitotic recombination in these 3 patients, confirming previous studies in which acquired uniparental disomy affecting the MPL locus had been detected by SNP array technology.[10-12] Together these findings mirror the situation with other signaling pathway mutations, such as *JAK2* V617F and *FLT3*-

Table 1. Cohort of patients with a myeloproliferative neoplasm screened for mutations in *MPL* exon 9.

	JAK2 V617F positive	*MPL* exon 10 positive	Mutation negative*	Total
PMF	43	8	45	96
ET	19	1	56	76
Total	62	9	101	172

Negative for JAK2 V617F and mutations in MPL exons 9 & 10

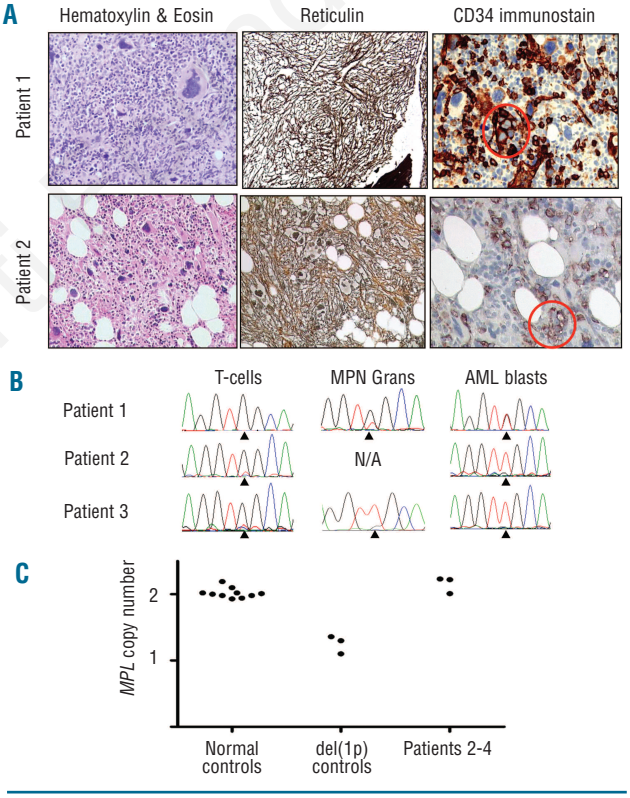

Figure 1. Acute myeloid leukemia following an MPL-mutant myeloproliferative neoplasm may be heterozygous or homozygous for the MPL W515L mutation, with homozygosity arising by mitotic recombination. (A) Bone marrow trephine biopsies from patients 1 and 2, acquired at time of leukemic transformation, showing prominent dysplastic megakaryocytes, reticulin fibrosis and clusters of $CD34^+$ cells (examples circled in red). All images original magnification x400. (B) Sequencing of *MPL* exon 10 in T cells, granulocytes from the MPN phase of disease (MPN Grans) and purified leukemic blasts (AML blasts), showing W515L-heterozygous AML in patient 1 and W515L-homozygous AML in patients 2 and 3. (C) Real-time PCR *MPL* copy number assay on three W515L-homozygous patient samples (leukemic blasts from patients 2 and 3; granulocytes from patient 4) with loss of heterozygosity at telomeric end of the *MPL* locus (rs498166 or rs499163) and close to the 1p telomere (rs7537577 or rs1870509): the presence of two copies of *MPL* in all 3 cases establishes mitotic recombination as the mechanism by which the wild-type MPL allele is lost. Analysis of 10 normal individuals and 3 cell lines (NB4, HT3 and LAN1) known to harbor a single copy of the MPL locus[19] are shown as controls.

ITD, where mitotic recombination results in duplication of the mutant allele, implying a selective advantage is conferred by either increased mutant gene dosage or loss of the wild-type allele.

Leukemic blasts were screened for mutations in *N/KRAS, CEBPA, RUNX1, GATA2, NPM, WT1, TP53, CBL, IDH1/2* and *TET2*. Mutations were identified in *TP53* and *TET2* in patient 1 but no additional lesions were found in patients 2 and 3. In patient 1, the *TET2* mutation was predominant in bone marrow cells obtained three years prior to leukemic transformation, whereas the *MPL* mutation was present at a relatively low level (Figure 2A). Sequencing is not highly quantitative, but the magnitude of the observed difference suggests that the *TET2* mutation preceded acquisition of the MPL mutation. Erythroid and granulocyte-macrophage colonies (n=41 and n=21, respectively), confirmed by cytological analysis, all harbored both *MPL* and *TET2* mutations, demonstrating that the mutations arose within the same clone. In addition, 13 of 62 colonies harbored mutations in *TP53*. Remarkably, a total of four different *TP53* mutations were identified, all of which are recurrent, functionally-significant cancer-associated alleles.[20] Progression to acute leukemia was associated with loss of wild-type *TP53* in one subclone (Figure 2A).

Detection of *TP53* mutations within erythroid and granulocyte-macrophage colonies indicates that terminal differentiation may proceed in the presence of mutant p53 where the wild-type allele is retained. Furthermore, the presence of multiple p53-mutant clones, all involving C:G-to-T:A transitions (Figure 2A), implies a mutator-phenotype prior to the development of an AML-associated differentiation block. One possible mechanism invokes a mutagenic effect of mutant *MPL*, *TET2* or other unidentified genes, as report-

Table 2. Clinical and laboratory features of MPL W515L-positive patients at presentation and at time of progression to acute myeloid leukemia.

At presentation	Patient 1	Patient 2	Patient 3
Age / sex	61 F	69 M	51 F
Diagnosis	ET	PMF	ET -> MF
Hemoglobin (g/dL)	13.2	11.7	14.8
White cell count (×10⁹/L)	11.6	10.3	6.8
Platelet count (×10⁹/L)	813	611	505
Palpable splenomegaly	No	2cm	No
Bone marrow fibrosis¶	0	3	0
Bone marrow karyotype	Normal	ND	Normal
At AML transformation			
Disease duration (yrs)	5	9	14
Prior therapy	HC	None	HC
Hemoglobin (g/dL)	8.0	6.9	9
White cell count (×10⁹/L)	32	9.1	80.3
Circulating blast count (×10⁹/L)	8.64	4.5	78
Platelet count (×10⁹/L)	15	362	61
Palpable splenomegaly	No	10cm	10cm
Bone marrow fibrosis¶	3	4	Not done
Bone marrow karyotype	Complex†	Complex‡	Not done
Additional mutations	*TP53* R248Q *TET2* Q1532fs	None detected	None detected

ET: essential thrombocythemia; PMF: primary myelofibrosis; MF: myelofibrotic transformation; AML: acute myeloid leukemia; HC: hydroxycarbamide. ¶reticulin fibrosis on a 0-4 scale; †44-49,XX,5,+6,del(6)(q?21q?23),+8,t(11;21)(q13;q22),+der(11)t(11;21),del(13)(q?14q?22), del(13)(q?14q?22),der(15;17)t(15;17)(q?15;p?12),-17,+1-4mar; ‡del(20)(q11q13),add(9)(q13),-12,+mar

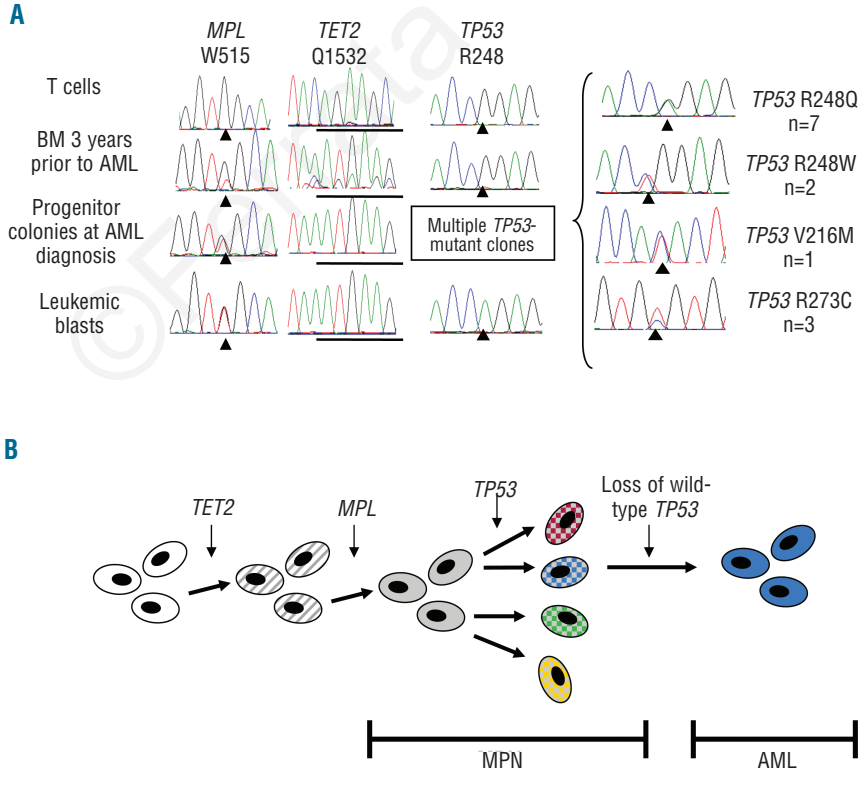

Figure 2. Leukemic progression associated with the proliferation of divergent, TP53-mutant clones. (A) Analysis of sequential samples from patient 1, demonstrating acquisition of a *TET2* mutation prior to a mutation in *MPL*, the proliferation of erythroid and granulocyte-macrophage colonies harboring different heterozygous mutations in *TP53*, and loss of wild-type *TP53* in leukemic blasts. (B) Model of disease progression in patient 1, characterized by the parallel expansion of multiple genetically distinct but phylogenetically-related clones bearing heterozygous *TP53* mutations, with loss of wild-type *TP53* in one of these subclones associated with progression to acute leukemia. BM: bone marrow cells, MPN: myeloproliferative neoplasm, AML: acute myeloid leukemia.

ed in models of oncogenic ERBB2 and BCR-ABL1 which resulted in a bias towards transversion or transition mutations, respectively.[21,22] In this patient, no other acquired synonymous or non-synonymous mutations were identified in 11.5Kb of DNA sequence from leukemic blasts, although the mutation prevalence in solid tumors (one mutation every 10^5 - 10^6 bases) suggests that a genome-wide approach would be necessary to elucidate the true mutation frequency. In addition, it is possible that alterations of *MPL*, *TET2* or unidentified gene(s) within the parental clone impart a strong selective pressure for the acquisition of *TP53* mutations. Alternatively, diverse clones may arise following exposure to an exogenous agent. Hydroxycarbamide (received by this patient) has been linked to abnormalities of 17p (which harbors the TP53 locus),[23,24] although a specific mutation signature has not been reported.

Taken together, our data demonstrate the parallel expansion of genetically distinct but phylogenetically-related clones prior to leukemic transformation (Figure 2B). Of note, clonal diversity (assessed by loss of heterozygosity and *TP53/CDKN2A* mutation) in patients with Barrett's esophagus has been associated with an increased risk of progression to adenocarcinoma,[25] suggesting that in this disease expansion of competing clones may also presage progression to a fully malignant phenotype.

In conclusion, this study used paired MPN/AML samples to demonstrate that progression to AML is part of the natural history of *MPL* W515L-associated disease, may occur in the absence of prior cytoreductive therapy and may involve loss of the wild-type *MPL* allele by mitotic recombination. Moreover, studies of progenitor colonies revealed the expansion of divergent but phylogenetically-related clones during progression from *MPL*-mutant MPN to AML.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. 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.
2. Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood. 2006;108(10):3472-6.
3. Guglielmelli P, Pancrazzi A, Bergamaschi G, Rosti V, Villani L, Antonioli E, et al. Anaemia characterises patients with myelofibrosis harbouring Mpl mutation. Br J Haematol. 2007;137(3):244-7.
4. Beer PA, Campbell PJ, Scott LM, Bench AJ, Erber WN, Bareford D, et al. MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. Blood. 2008;112(1):141-9.
5. Vannucchi AM, Antonioli E, Guglielmelli P, Pancrazzi A, Guerini V, Barosi G, et al. Characteristics and clinical correlates of MPL 515W>L/K mutation in essential thrombocythemia. Blood. 2008;112(3):844-7.
6. Staerk J, Lacout C, Sato T, Smith SO, Vainchenker W, Constantinescu SN. An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor. Blood. 2006;107(5):1864-71.
7. Ding J, Komatsu H, Iida S, Yano H, Kusumoto S, Inagaki A, et al. The Asn505 mutation of the c-MPL gene, which causes familial essential thrombocythemia, induces autonomous homodimerization of the c-Mpl protein due to strong amino acid polarity. Blood. 2009;114(15):3325-8.
8. Malinge S, Ragu C, Della-Valle V, Pisani D, Constantinescu SN, Perez C, et al. Activating mutations in human acute megakaryoblastic leukemia. Blood. 2008;112(10):4220-6.
9. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360(22):2289-301.
10. Buxhofer-Ausch V, Gisslinger H, Berg T, Gisslinger B, Kralovics R. Acquired resistance to interferon alpha therapy associated with homozygous MPL-W515L mutation and chromosome 20q deletion in primary myelofibrosis. Eur J Haematol. 2009;82(2):161-3.
11. Szpurka H, Gondek LP, Mohan SR, Hsi ED, Theil KS, Maciejewski JP. UPD1p indicates the presence of MPL W515L mutation in RARS-T, a mechanism analogous to UPD9p and JAK2 V617F mutation. Leukemia. 2009;23(3):610-4.
12. Kawamata N, Ogawa S, Yamamoto G, Lehmann S, Levine RL, Pikman Y, et al. Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray. Exp Hematol. 2008;36(11):1471-9.
13. Campbell PJ, Baxter EJ, Beer PA, Scott LM, Bench AJ, Huntly BJ, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. Blood. 2006;108(10):3548-55.
14. Theocharides A, Boissinot M, Girodon F, Garand R, Teo SS, Lippert E, et al. Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. Blood .2007;110(1):375-9.
15. Beer PA, Delhommeau F, Lecouedic JP, Dawson MA, Chen E, Bareford D, et al. Two routes to leukemic transformation following a JAK2 mutation-positive myeloproliferative neoplasm. Blood. 2010;115(14):2891-900.
16. Barosi G, Ambrosetti A, Finelli C, Grossi A, Leoni P, Liberato NL, et al. The Italian Consensus Conference on Diagnostic Criteria for Myelofibrosis with Myeloid Metaplasia. Br J Haematol. 1999;104(4):730-7.
17. Harrison CN, Bareford D, Butt N, Campbell P, Conneally E, Drummond M, et al. BCSH guidelines for investigation and management of patients presenting with a thrombocytosis. Br J Haematol. 2010;149(3):352-75.
18. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of Tumours of Haematopoietic and Lymphoid Tissues: Lyon: IARC Press; 2008.
19. Forbes SA, Tang G, Bindal N, Bamford S, Dawson E, Cole C, et al. COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer. Nucleic Acids Res. 2010;38(Database issue):D652-7.
20. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat. 2007;28(6):622-9.
21. Liu S, Liu W, Jakubczak JL, Erexson GL, Tindall KR, Chan R, et al. Genetic instability favoring transversions associated with ErbB2-induced mammary tumorigenesis. Proc Natl Acad Sci USA. 2002;99(6):3770-5.
22. Stoklosa T, Poplawski T, Koptyra M, Nieborowska-Skorska M, Basak G, Slupianek A, et al. BCR/ABL inhibits mismatch repair to protect from apoptosis and induce point mutations. Cancer Res. 2008;68(8):2576-80.
23. Sterkers Y, Preudhomme C, Lai JL, Demory JL, Caulier MT, Wattel E, et al. Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. Blood. 1998;91(2):616-22.
24. Thoennissen NH, Krug UO, Lee DH, Kawamata N, Iwanski GB, Lasho T, et al. Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome-negative myeloproliferative neoplasms. Blood. 2010;115(14):2882-90.
25. Maley CC, Galipeau PC, Finley JC, Wongsurawat VJ, Li X, Sanchez CA, et al. Genetic clonal diversity predicts progression to esophageal adenocarcinoma. Nat Genet. 2006;38(4):468-73.