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ORIGINAL ARTICLE

MPL^{W515L} mutation in acute megakaryoblastic leukaemia

K Hussein[1], O Bock[1], K Theophile[1], K Schulz-Bischof[2], A Porwit[3], J Schlue[1], D Jonigk[1] and H Kreipe[1]

[1]Institute of Pathology, Hannover Medical School, Hannover, Germany; [2]Institute of Pathology, Katholisches Marienkrankenhaus, Hamburg, Germany and [3]Department of Pathology, Karolinska University Hospital, Stockholm, Sweden

The thrombopoietin receptor gene (MPL) is expressed in megakaryocytes and exhibits the gain of function point mutation W515K/L in ~5% of patients with primary myelofibrosis/idiopathic myelofibrosis (PMF) representing one subtype of the chronic myeloproliferative disorders (myeloproliferative neoplasm). A series of primary and secondary acute myeloid leukaemias (AML) with megakaryoblastic phenotype and myelofibrosis unrelated to PMF ($n=12$) was analysed for the $MPL^{W515K/L}$ mutation by pyrosequencing. In three cases (25%), MPL^{W515L} was found and in two of these a combination with trisomy 21 or the Philadelphia chromosome occurred. None of the secondary AML cases evolving from pre-existing PMF showed $MPL^{W515K/L}$ ($n=4$). We conclude that MPL^{W515L} occurs in a considerable proportion of acute megakaryoblastic leukaemias with myelofibrosis unrelated to PMF.
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Introduction

Acute myeloid leukaemia (AML) with megakaryoblastic differentiation represents a rare subtype of AML which tends to present with myelofibrosis and has a poor prognosis.[1,2] In childhood, most cases are associated with Down's syndrome (trisomy 21) or display the t(1;22)(p13;q13) abnormality.[2] In many instances, bone marrow biopsy is required for diagnosis due to extensive myelofibrosis. The differential diagnoses encompass acute panmyelosis with myelofibrosis and either primary or secondary myelodysplastic syndromes (MDS) with excess of blasts and myelofibrosis.[2] In addition, secondary AML evolving from chronic myeloproliferative disorders/myeloproliferative neoplasms (CMPD), particularly primary myelofibrosis (PMF) have to be excluded. Other AML subtypes, for example, MDS-related AML with multilineage dysplasia, may also be associated with myelofibrosis.

The myeloproliferative leukaemia virus proto-oncogene/thrombopoietin receptor (MPL) promotes both proliferation and differentiation of non-neoplastic megakaryocytes. It is expressed in several AML subtypes, including megakaryoblastic AML and can be involved in aberrant signal transduction in acute leukomogenesis, for example, by mediating a hypersensitivity of blast cells to thrombopoietin.[3] The thrombopoietin gene is located on chromosome 3q27–28 but appears not to be involved in the inversion 3q21q26, which can be found in AML with a megakaryoblastic phenotype associated with thrombocytosis.[4] Most recently, the first systematic search for MPL mutation in acute megakaryoblastic leukaemia has been undertaken and has revealed one single case with a MPL^{T487A} mutation.[5] MPL gain of function point mutations ($MPL^{W515K/L}$) have been described in Philadelphia chromosome-negative (Ph^-) CMPD, comprising PMF (~5% MPL mutated) and essential thrombocythemia (ET; ~1% MPL mutated).[6] More frequently than $MPL^{W515K/L}$ the Janus kinase 2 point mutation ($JAK2^{V617F}$) is detectable in ~95% of polycythemia vera and ~60% of PMF and ET cases.[7] Screening for $MPL^{W515K/L}$ and $JAK2^{V617F}$ in a representative number of 126 AML cases revealed that 23% were $JAK2^{V617F}$-positive, but only 2% of post-PMF AML or post-ET AML cases without megakaryoblastic phenotype were $MPL^{W515K/L}$ mutated.[6] Further studies on AML, mostly *de novo* cases, have been undertaken and only 5/60 (8%) megakaryoblastic AML were $JAK2^{V617F}$-mutated, whereas $MPL^{W515K/L}$ had not been tested.[3,8–10]

In this study, MPL^{W515L} (but not $JAK2^{V617F}$) was detected in about 25% of AML with a megakaryoblastic phenotype and myelofibrosis, including an MPL^{W515L}-mutated blast crisis of Ph^+ chronic myeloid leukaemia (CML), which has not previously been described.

Materials and methods

Study group

Archived, formalin-fixed and paraffin-embedded (FFPE) bone marrow trephines from 21 patients were included in the study. The study comprises 13 cases of primary or secondary AML with a megakaryoblastic phenotype in more than 50% of the blasts and myelofibrosis. In 12 cases, evidence of antecedent PMF could be excluded, whereas one case had a history of PMF. In addition, for control purposes, seven cases of primary or secondary AML with myelofibrosis, but with a blast population without any markers of megakaryocytic lineage, and one biphasic myeloperoxidase$^+$/CD61$^+$ AML with myelofibrosis, were studied. All but one sample were collected as part of the standard clinical care for evaluation of bone marrow status (diagnosed between 1990 and 2008) and were subsequently archived in the Institute of Pathology, Hannover Medical School. Consistent with the requirements of our local ethics committee and patient's approval for biopsy, storage and research use, after diagnosis had been established, archived samples were considered to be remnant material unnecessary for patient treatment. The sample from patient no. 2 was submitted by the Department of Pathology, Karolinska Hospital, Stockholm, Sweden, for consultation.

Megakaryoblastic phenotype was verified by immunostaining of megakaryocyte/platelet-specific antibodies (mouse anti-

Correspondence: Professor Dr H Kreipe, Institute of Pathology, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany.
E-mail: Kreipe.Hans@mh-hannover.de
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CD42b antibody, clone MM2/174 (Novocastra, Newcastle upon Tyne, UK); mouse anti-CD61 antibody, clone Y2/51 and mouse anti-von Willebrand factor/factorVIII antibody, clone F8/86 (both Dako, Glostrup, Denmark)) and CD34 (mouse anti-CD34 antidody, clone QBEND/10; Immunotech, Praha, Czech Republic). No corresponding peripheral blood and due to myelofibrosis ('dry tap') no bone marrow aspirate had been archived for retrospective evaluation.

Determination of MPL$^{W515K/L}$ and JAK2^{V617F} allele burden in bone marrow cells

DNA aliquots from FFPE bone marrow cells (DNeasy kit; Qiagen, Hilden, Germany) were used for PCR amplification of MPL and JAK2 fragments, which included the mutation sites and subsequent pyrosequencing (Biotage, Uppsala, Sweden) as described.[8] In brief, the specific sequencing primer binds to its corresponding PCR fragment, shortly before the mutation site, and in a second step pyrophosphate nucleotides are injected into the reaction tube. Incorporation of the wild-type or, in a subsequent reaction, the mutant nucleotide results in pyrophosphate release, which activates a connected luciferase-enzyme cascade. The resulting light signal is proportional to the amount of incorporated wild-type or mutant nucleotides and allows a quantification of alleles. All samples were analysed in duplicate (controls: JAK2^{V617F} cell line HEL, MPL-mutated PMF, JAK2/MPL-unmutated cell line HL-60). The following primer sets were used: JAK2-forward 5′-TATGATGAGCAAGCTTTCTCAC AAG-3′, JAK2-reverse 5′-biotin-AGAAAGGCATTAGAAAGCC TGTAGTT-3′, 102 bp PCR products, JAK2-sequencing 5′-GG TTTTAAATTATGGAGTATGT-3′ (GenBank AL161450); MPL-forward 5′-CCGCTCTGCATCTAGTGCT-3′, MPL-reverse 5′-biotin-CTGTAGTGTGCAGGAAACTG-3′, 79 bp PCR products; MPL-sequencing 5′-TGCTGCTGCTGAGGT-3′ (GenBank U68161). In a series of eight bone marrow samples not related to this study parallel pyrosequencing in formalin-fixed and fresh frozen tissue samples yielded similar results (data not shown).

Laser microdissection and analysis of megakaryocytic blasts

In Ph$^+$/MPL^{W515L} double-mutated case (patient no. 3), ~150 dysplastic megakaryocytes and megakaryoblasts were laser microdissected from 4 μm bone marrow sections with a SmartCutPlus-System (Olympus, Hamburg, Germany) as depicted in Figure 1 and DNA was analysed for mutant MPL by pyrosequencing. In addition, RNA from laser-microdissected megakaryocytic cells was extracted from a corresponding section (proteinase K digestion followed by phenol/chloroform extraction and isopropanol precipitation), 1 μg was pretreated with RNase-free DNase (1 U/μg RNA; RQ1; Promega, Madison, WI, USA) and transcribed into cDNA (500 ng random hexamers; Amersham Pharmacia, Picattaway, CA, USA; 200 U SuperScript II Rnase-free Reverse Transcriptase; Invitrogen, Karlsruhe, Germany).[11] Verification of sufficient cDNA synthesis was performed by amplification of control gene β-glucuronidase (β-GUS-forward 5′-CTCATTTGGAATTTTGCCGATT-3′, β-GUS-reverse 5′-CACTTTTTGGTTGTCTCTGCCG-3′; 81 bp PCR products; GenBank NM000181). As indicated by previous analysis of the corresponding bone marrow cells, the megakaryocytic cDNA sample was analysed for BCR–ABL fusion b3a2 with the following primer set: BCR-b3-forward 5′-GAGTCTCCGG GCTCTATGG-3′, ABL-a2-reverse 5′-TCAGATGCTACTGGCC GCTGAA-3′ (98 bp b3a2 fusion products; GenBank AJ131466; control cell line K 562).

Statistical analyses

Megakaryoblastic AML cases were grouped according to their MPL mutation status and were statistically analysed for differences in clinical parameters at AML presentation. One-way analysis of variance was performed (two-tailed). *P*-values $\leqslant 0.05$ were considered as statistically significant.

Results and discussion

Compared to the rather low frequency of MPL$^{W515K/L}$ in PMF (~5%),[6] AML cases with megakaryoblastic differentiation and myelofibrosis but no evidence of a pre-existing PMF revealed a higher frequency of MPL^{W515L} (3/12, 25%, Table 1). JAK2^{V617F} which affects 60–95% of Ph$^-$ CMPD,[7,8] was not detected.

Of eight studied cases with pre-existing PMF and secondary AML with myelofibrosis, two were positive for JAK2^{V617F} and devoid of MPL$^{W515K/L}$ (no. 15, no. 16). Furthermore, the MPL^{W515L} mutation was detectable in one AML secondary to MDS with myelofibrosis but without evidence of a megakaryoblastic differentiation or an antecedent PMF (no. 14). In the cohort under investigation, MPL^{W515K} was not detected.

Thus, none of the four MPL^{W515L}-positive AML cases with myelofibrosis had evidence of a previous PMF preceding the AML and three of these cases had a megakaryoblastic differentiation. Based on our limited cohort, the frequency of MPL^{W515L}-positive cases in AML with myelofibrosis seems to be fourfold higher than in PMF (4/21, 19%).

None of the remaining seven cases with primary or secondary AML and myelofibrosis and with a blast population not belonging to the megakaryocytic lineage revealed MPL$^{W515K/L}$ mutation.

None of the four MPL^{W515L}-positive AML cases had evidence of a previous PMF or ET preceding AML. In patient no. 1, megakaryoblastic AML was the initial disease presentation,

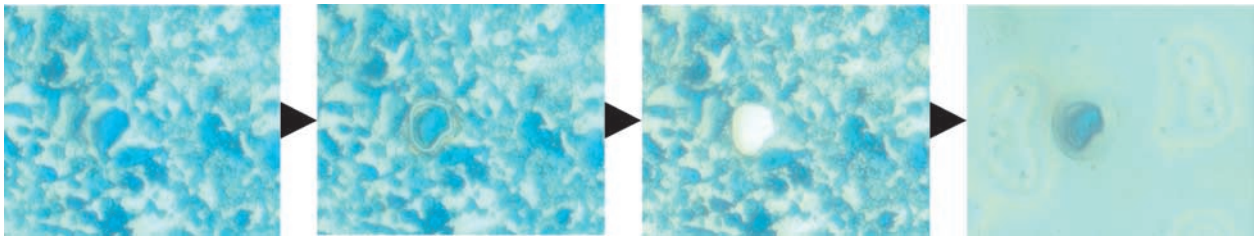

Figure 1 Laser microdissection of dysplastic megakaryocytes and megakaryoblasts in MPL^{W515L} acute myeloid leukaemia (AML). The laser microdissection of a megakaryocytic cell from a bone marrow section of patient no. 3 is depicted (methylene blue stain, original magnification × 400; SmartCutPlus-System based on a CKX41 inverse microscope, Olympus).

Table 1 MPL^{W515L} in acute megakaryoblastic leukaemia with myelofibrosis

Case (No.)	*Gender (age; follow-up period)*	*Diagnosis (relevant details)*	*Myelo-fibrosis*	*MPL^{W515L} % mutant alleles in bone marrow cells*	*JAK2^{V617F} % mutant alleles in bone marrow cells*	*Karyotypes and mutations*	*HB (g/l)*	*WBC ($\times 10^9$/l)*	*PLT ($\times 10^9$/l)*	*LDH (U/l)*	*BM/PB blast cells (%)*
1	♂ (74 years)	Megakaryoblastic AML (no splenomegaly)	+	62	WT	ND	93	3.4	132	394	>20/5
2	♂ (10 years)	Megakaryoblastic AML (Down's syndrome)	+	35	WT	47,XY, +21	82	2.2	78	ND	>20/6
3	♂ (21 years)	CML	−	WT	WT	Ph$^+$	ND	ND	ND	ND	5–10/ND
	+210 months	Megakaryoblastic AML (hydroxyurea therapy during the first 5 years, allogeneic stem cell transplantation 7 years after diagnosis)	+	18 (MK 12)	WT	BM cells b3a2-positive, MK b3a2-negative	83	12.5	225	1625	10/29
4	♀ (73 years)	CML with marked myelofibrosis	+	WT	WT	Ph$^+$	109	25.8	41	516	<5/30
	+2 months	Megakaryoblastic AML (postchemotherapy)	+	WT	WT	Ph$^-$, FLT3/ITR$^+$	96	2.34	137	559	⩽5/1
5	♀ (3 years)	Megakaryoblastic AML	+	WT	WT	Complex karyotype (legend)	61	3.7	6.9	ND	>20/ND
6	♂ (63 years)	Megakaryoblastic AML	+	WT	WT	ND	112	7.7	35	ND	>20/28
7	♀ (64 years)	Megakaryoblastic AML	+	WT	WT	ND	70	11.2	94	3876	>20/ND
8	♀ (67 years)	Megakaryoblastic AML	+	WT	WT	ND	92	2.4	72	ND	>20/ND
9	♀ (70 years)	Megakaryoblastic AML	+	WT	WT	ND	9	6.74	17	ND	>20/33
10	♀ (65 years)	Megakaryoblastic AML (post-MDS)	+	WT	WT	ND	95	6.2	105	204	80/60
11	♂ (65 years)	Megakaryoblastic AML (post-MDS)	+	WT	WT	ND	88	1.2	75	1130	50/18
12	♂ (82 years)	Megakaryoblastic AML (post-MDS)	+	WT	WT	ND	83	1.7	37	ND	20/9
13	♀ (64 years)	Cellular stage PMF (no splenomegaly)	−	ND	ND	ND	148	9.2	667	ND	<5/ND
	+153 months	megakaryoblastic AML	+	WT	WT	ND	ND	ND	ND	ND	>90/ND
14	♂ (53 years)	AML (post-MDS, HIV)	+	33	WT	ND	113	2.4	8	209	30/ND
15	♂ (56 years)	Cellular stage PMF	−	WT	75	ND	70	18.1	160	ND	<5/ND
	+92 months	Biphasic MPO$^+$/CD20$^+$ acute leukaemia (postsplenectomy)	+	WT	63	ND	83.7	89.7	29	ND	30/ND
16	♀ (58 years)	AML (post-PMF with mild myelofibrosis)	+	WT	41	ND	109	ND	ND	ND	30/ND
	+6 months	AML (postchemotherapy)	+	WT	58	46,XX	91	3	12	ND	90/ND
17	♂ (57 years)	AML (post-PMF with marked myelofibrosis)	+	WT	WT	ND	90	0.5	28	ND	>30/ND
18	♂ (42 years)	Biphasic MPO$^+$/CD61$^+$ AML	+	WT	WT	ND	70	11.3	89	395	>20/ND
19	♀ (70 years)	AML	+	WT	WT	ND	90	6.74	17	969	>20/ND
20	♀ (69 years)	AML	+	WT	WT	ND	61	2.8	18	ND	>20/15–20
21	♀ (80 years)	AML	+	WT	WT	ND	97	16	300	ND	20/20

Abbreviations: ♀, female; ♂, male; −, no myelofibrosis; +, myelofibrosis; AML, acute myeloid leukaemia; BM, bone marrow blast; CML, chronic myeloid leukaemia; FLT3/ITR$^+$, FMS-Like-Tyrosine kinase-3 internal tandem repeat; HB, haemoglobin; HIV, human immunodeficiency virus; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; MK, laser-microdissected megakaryoblasts; ND, not determined/data not available; PB, peripheral blood blasts; PLT, platelet counts; PMF, primary myelofibrosis; WBC, white blood cell counts; WT, wild-type at mutation site.
Haematological parameters, particularly platelet counts, were not significantly altered by an underlying MPL^{W515L} mutation. Karyotype no. 5: 47,XX t(X;9)(p10;p10), +21 [7]/46,XX [10].

whereas in patient no. 2, the megakaryoblastic leukaemia had developed against the background of a Down's syndrome and patient no. 3 presented initially with a Ph$^+$ CML without myelofibrosis. MPL mutations were not detected in a total of 19 previously analysed cases of Down's syndrome-related myeloproliferative leukaemias.[5,12] Most recently, Malinge *et al.*[5] identified a MPL^{T487A} gain of function mutation in one of 20 non-Down's syndrome children with megakaryoblastic AML but no MPL$^{W515K/L}$-positive case.

Our findings may indicate that either clonal evolution had taken place, adding the MPL^{W515L} mutation to an already aberrant leukaemic stem cell, or a second leukaemic clone characterized by MPL^{W515L} mutation had emerged. To clarify this issue, dysplastic megakaryocytes and megakaryoblasts from case no. 3 were isolated by laser microdissection. This patient had developed relapse and blast crisis of Ph$^+$ CML after allogeneic stem cell transplantation and was chosen because two genetic aberrations could be compared. Bone marrow histology revealed myelofibrosis and 80% infiltration by megakaryoblasts (CD42b$^+$/von Willebrand-factor$^+$/CD34$^+$). Ph$^+$ and MPL^{W515L} were detectable in cDNA and DNA, respectively, isolated from all bone marrow cells. After microdissection of megakaryoblasts it became obvious that this blast population did not share the Ph$^+$ abnormality but exhibited only MPL^{W515L} mutation. Although loss of the Philadelphia chromosome during clonal evolution of blast crisis cannot be excluded, development of a second malignant clone rather than clonal evolution of the existing one, appeared to be responsible for the megakaryoblastic MPL^{W515L}-positive blast crisis. Coexistence of mutations of growth regulatory genes, which are usually sufficient to transform haematopoietic cells alone have been described before in CMPD. It remains a matter of debate, whether one double-mutated or two independent clones are the basis of neoplastic growth in these types of CMPD.[13,14]

In conclusion, (1) MPL^{W515L} mutation can be detected in bone marrow samples of megakaryoblastic AML with myelofibrosis unrelated to classical PMF and (2) MPL^{W515L} can occur in megakaryoblastic blast crisis of CML.

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