Technical Advance

Detection of the Activating JAK2 V617F Mutation in Paraffin-Embedded Trephine Bone Marrow Biopsies of Patients with Chronic Myeloproliferative Diseases

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The discovery of the activating V617F mutation in the JAK2 tyrosine kinase in a high proportion of patients with Ph[−] chronic myeloproliferative diseases (CMPD) represents a diagnostic breakthrough for these disorders. Trephine bone marrow biopsy is an essential part of the diagnostic workup of CMPD and represents a valuable archival source of DNA. Therefore, we studied 152 paraffin-embedded trephines with CMPD and related disorders for the presence of the V617F mutation, using both allele-specific polymerase chain reaction (PCR) and nested PCR with subsequent digestion with *Bsa*XI. Only 6 of 152 (4%) samples were not evaluable because of poor DNA quality. The V617F mutation was detected in 27 of 28 (96%) cases of polycythemia vera, 17 of 23 (74%) cases of essential thrombocythemia, 28 of 45 (62%) cases of chronic idiopathic myelofibrosis, six of eight (75%) cases of CMPD unclassified, and two of four (50%) cases of myelodysplastic/myeloproliferative syndrome. Ph⁺ chronic myelogenous leukemia (four cases), reactive (secondary) erythrocytosis (14 cases), and thrombocytosis (one case) as well as normal controls (19 cases) all lacked the V617F mutation. Based on results of *Bsa*XI digestion and sequencing, 24 of 54 (44%) evaluable V617F⁺ cases were considered homozygously mutated. Thus, detection of the V617F JAK2 mutation is feasible in paraffin-embedded trephine biopsies and represents a major advance in the diagnostic evaluation of CMPD. (*J Mol Diagn* 2006, 8:299–304; DOI: 10.2353/jmoldx.2006.050128)

Chronic myeloproliferative diseases (CMPDs) are a group of related hematopoietic stem cell proliferations characterized by an increase in circulating mature and immature blood cells, frequent extramedullary hematopoiesis, and associated bone marrow (BM) changes.^{1,2} The latter include hyperplasia of one or more hematopoietic cell lines, dysplastic features predominantly of the megakaryocytic lineage, fibrosis, and changes of BM microvasculature. CMPDs include chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and chronic idiopathic myelofibrosis (CIMF), as well as rarer entities such as idiopathic hypereosinophilic syndrome/chronic eosinophilic leukemia, and chronic neutrophilic leukemia.

The diagnosis of CMPD relies on a combination of clinical, morphological, and, in part, genetic features. Although strict diagnostic criteria have been defined, discrimination of the various entities may be problematic, and separation from reactive hematopoietic hyperplasia can be difficult, especially in early disease stages. Because assessment of marrow architecture, distribution and maturation of the different hematopoietic cell lineages, and the amount of marrow fibrosis are of paramount importance for correct classification and prognostic information, the recent World Health Organization classification of myeloid disorders considers a trephine BM biopsy as essential for the diagnosis of CMPD.¹

Whereas CML is characterized by the *BCR-ABL* fusion leading to constitutive activation of the ABL tyrosine kinase, no defining molecular alterations had been found in the other common CMPDs. The recent identification of the recurrent V617F mutation in exon 12 of janus kinase 2 (JAK2) in a high proportion of patients with PV, ET, and CIMF represents a major breakthrough in our understanding of CMPD and at the same time provides an important diagnostic marker.^{3–8} The V617F mutation is

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acquired, because it is lacking in nonhematopoietic cells of patients carrying the mutation, and must have important functional consequences because it is found homozygously due to uniparental disomy of chromosome 9p in almost half of the patients, indicating a selective advantage for cells carrying two mutated alleles.

Given the diagnostic and biological significance of the V617F mutation in CMPDs, the aim of our study was to assess whether detection of this mutation is feasible in formalin-fixed, decalcified, and paraffin-embedded BM trephine biopsies. This would allow the retrospective analysis of archival paraffin-embedded BM biopsies and could help to better define the morphological features of CMPDs carrying the V617F mutation. Therefore, a large series of trephine BM biopsies from patients with confirmed or suspected CMPD and controls were studied for the presence of the V617F mutation using mutation-specific polymerase chain reaction (PCR) as well as restriction enzyme digestion and direct sequencing of PCR products.

Materials and Methods

Patients

Trephine BM biopsies with a diagnosis of CMPD, clinically suspected CMPD, and control samples were retrieved from the files of the Institute of Pathology, Technical University Munich, Munich, Germany. The specimens had been fixed in buffered formalin for 12 to 24 hours, decalcified in ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin. Approximately half of the samples were decalcified with ultrasonic energization (Medite, Burgdorf, Germany), reducing the time for decalcification from 48 to 8 hours. All slides, including serial sections stained using hematoxylin and eosin, Giemsa, NASD-chloroacetate esterase, Gomori's reticulin, and iron stains, were reviewed by three of the authors (F.F., M.K., and W.M.P.). Clinical information including peripheral blood counts, spleen size at the time of biopsy, and the presence or absence of a *BCR/ABL* translocation was retrieved from the clinical files of the patients or obtained through the treating hematologists. Information concerning the *BCR/ABL* status was available for all patients with CMPD other than PV.

Classification of CMPD was performed following the criteria of the 2001 World Health Organization classification of tumors of hematopoietic and lymphoid tissues.^{1,2} Marrow fibrosis was graded from 0 to 3 according to a recent consensus proposal.⁹ If a discrepancy was noted with the originally rendered histological diagnosis on review, a consensus diagnosis was reached through joint evaluation of the histological slides and pertinent clinical data.

DNA Extraction and Control Amplification

Two to three 10- μ m sections were taken from the whole tissue block, deparaffinized in graded alcohols and xylene, and digested with proteinase K (1 mg/ml) in standard Tris-EDTA buffer overnight. After proteinase K inac-

tivation by heating at 96°C for 15 minutes, a 1:10 dilution of the DNA extract was used as template to amplify a 268-bp β -globin fragment for control of DNA quality as reported previously.^{10,11} If no or only a weak product of correct size was detected on a 3% agarose gel by ethidium bromide staining, the PCR was repeated with undiluted DNA extract. If this second PCR rendered no or weak amplification products, the case was excluded from further analysis.

Detection of the V617F JAK2 Mutation

For detection of the V617F mutation, two alternative strategies were used. The first strategy consists of an allele-specific multiplex PCR with one common reverse and two separate forward primers.³ The first forward primer amplifies a 364-bp fragment of both mutant and wild-type alleles and thus serves as amplification control. The second forward primer is specific for the mutated allele and contains an additional mismatch near the 3' end. This primer generates a 203-bp product only in the presence of the V617F mutation.³ Two μ l of DNA extract served as template in a total volume of 25 μ l containing 1.5 mmol/L MgCl₂, 20 pmol/L of each primer, 0.2 mmol/L dNTPs, and 1 U of *Taq* polymerase (Amersham, Little Chalfont, UK). Forty cycles of amplification with an annealing temperature of 60°C were performed. To test the sensitivity of the mutation-specific PCR, we made a serial dilution of DNA extracted from the HEL erythroleukemia cell line, which contains a homozygous JAK2 V617F mutation, in control DNA with wild-type JAK2. Less than 3% of mutated DNA was reproducibly detected.

The second approach is based on a nested PCR, which amplifies the region containing the mutation⁸ and subsequent restriction enzyme digestion with *Bsa*XI. For the first round of amplification, 35 cycles with an annealing temperature of 55°C were performed, using 2 μ l of DNA as template, followed by 25 cycles of amplification with an annealing temperature of 60°C, using 2 μ l of the first round product as template. Concentrations of PCR reagents were the same as described above. Five μ l of the second round product were digested with *Bsa*XI (New England Biolabs, Hitchin, UK) in the appropriate buffer at 37°C for 3 hours. If only wild-type DNA is present, two fragments of 170 and 203 bp in size are generated, whereas mutated DNA remains undigested. All PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The primer sequences are listed in Table 1.

Appropriate positive and negative controls were included in every run, and special precautions were used to prevent carry-over of products from the nested PCR. In addition, amplicons generated by the nested PCR were directly sequenced in selected cases to confirm the presence of the mutation and to estimate the amount of the mutated allele, using an ABI 3130 capillary sequencer (Applied Biosystems, Darmstadt, Germany).

Table 1. JAK2 Primer Sequences

Allele-specific PCR (3)	5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAAG-3'
Forward (internal control)	5'-AGCATTTGGTTTTAAATTATGGAGTATATT-3'
Forward (V617F specific)	5'-CTGAATAGTCCTACAGTGTTCAGTTTCA-3'
Reverse	
Nested PCR (8)	
Outer forward	5'-GGTTTCCTCAGAACGTTGATGG-3'
Outer reverse	5'-TTGTTTGGGCATGTAAACCTTC-3'
Inner forward	5'-TGCTGAAAGTAGGAGAAAGTGCAT-3'
Inner reverse	5'-TCCTACAGTGTTCAGTTTCAAAA-3'
Sequencing primer	5'-AGTCTTTCTTTGAAGCAGCAA-3'

Results

Pathological Findings

In total, 152 BM biopsies were entered into the study. Six samples (4%) failed to amplify both the β -globin fragment as well as the 364-bp control fragment of the allele-specific PCR and were excluded from further analysis. The remaining 146 samples included 127 biopsies from patients with either confirmed CMPD or a clinical suspicion of CMPD and 19 normal controls. A consensus diagnosis was reached after review of all histological slides and pertinent clinical data. The final diagnoses as well as age and sex distribution are listed in Table 2. Eight cases were diagnosed as CMPD, unclassified. This group mainly corresponded to early-stage CMPD in which a definitive assignment to one of the major entities was not possible on morphological and clinical grounds. The four cases in the MDS/MPS, unclassified group contained one case of atypical CML negative for the BCR/ABL translocation and three cases with anemia and leukocytosis and/or thrombocytosis and BM morphology showing overlapping features between CMPD and MDS according to the World Health Organization criteria. Secondary erythrocytosis (14 cases) and reactive thrombocytosis (one case) were diagnosed in patients with a sustained elevation of peripheral blood counts in whom a BM biopsy was performed to exclude a CMPD and where neither the further clinical course nor BM histology showed evidence of a CMPD.

Detection of the JAK2 V617F Mutation

All 146 cases were successfully analyzed for the V617F mutation with the allele-specific PCR. The results are

summarized in Table 3. The frequency of the mutation was highest in the PV group, in which 27 of 28 (96%) cases carried the mutation, followed by ET with 17 of 23 (74%) and CIMF with 28 of 45 (62%) (Figure 1A). The mutation was also detected in six of eight cases of unclassified CMPD and two of four cases of MDS/MPS, including the BCR/ABL-negative atypical CML. None of the cases with Ph+ CML, secondary erythrocytosis or thrombocytosis or the normal controls carried the mutation. In the CMPD cases carrying the mutation, the intensity of the band originating from the mutated allele was of equal or even stronger intensity than the control band in the majority of samples, indicating a high percentage of mutated cells. The nested PCR with subsequent restriction enzyme digestion was performed successfully in 82 of 108 (76%) examined cases, thus showing a higher dropout rate than the allele-specific PCR (Figure 1B). The correlation between the two techniques was excellent. All cases positive by restriction fragment length (RFL) analysis were also positive by allele-specific PCR, and only two ET samples positive by allele-specific PCR did not show presence of an undigested PCR product. These two cases showed reproducible but consistently weak mutation-specific bands, indicating a minor percentage of cells carrying the V617F mutation. A tentative assignment to homozygous or heterozygous status of the V617F mutation was based on the results of RFL analysis. If no digested product was visible or the digested bands were significantly weaker than the undigested band, cases were considered as homozygously mutated, although this may underestimate the frequency of homozygosity. Sequencing of the nested PCR product was performed in a number of samples and confirmed the presence or absence of the V617F mutation in concordance with PCR results in all cases analyzed. Furthermore, there was an excellent correlation between the preponderance of the mutated versus unmutated allele by sequencing as compared to the results of RFL analysis, supporting the assessment of the zygosity status by *BsaXI* digestion (Figure 2). Based on the results of *BsaXI* digestion and sequencing, 24 of 54 (44%) evaluable cases positive for the V617F mutation were considered to be homozygous. The homozygosity rate was highest for PV (62%), whereas none of the ET cases was homozygous. The correlation of important clinical findings with JAK2 mutational status is shown in Table 4.

Table 2. Final Diagnoses and Age and Sex Distribution

Diagnosis	No.	Age (mean, range)	Sex
PV*	28	65 (43 to 81)	14 M:14 F
CIMF	45	68 (47 to 88)	21 M:24 F
ET	23	62 (23 to 87)	9 M:14 F
CMPD unclass.	8	67 (43 to 81)	4 M:4 F
MDS/MPS	4	70 (57 to 82)	2 M:2 F
All Ph- CMPD	108	66 (23 to 88)	50 M:58 F
CML Ph+	4	57 (35 to 79)	1 M:3 F
Sec EC/TC	15	59 (37 to 87)	7 M:8 F
Normal controls	19	57 (26 to 71)	12 M:7 F
Total	146	64 (23 to 88)	70 M:76 F

*Including spent phase with secondary myelofibrosis.
Sec EC/TC, secondary erythrocytosis/thrombocytosis.

Table 3. Results of 146 Cases Analyzed for V617F Mutation

Diagnosis	Allele-specific PCR Pos/total (% pos)	Nested PCR with <i>Bsa</i> XI digestion	
		Total/ampl	Pos/homo (% homo)/neg
PV	27/28 (96%)	28/22	21/13 (62%)/1
CIMF	28/45 (62%)	41/32	19/9 (47%)/13
Fibrosis 0 to 1	7/10 (70%)	9/7	5/1 (20%)/2
Fibrosis 2 to 3	21/35 (60%)	32/25	14/8 (57%)/11
ET	17/23 (74%)	22/16	9/0 (0%)/7*
CMPD unclass.	6/8 (75%)	7/5	4/2 (50%)/1
MDS/MPS†	3/4 (75%)	3/1	1/0 (0%)/0
All Ph— CMPD	81/108 (75%)	101/76	54/24 (44%)/22
Ph+ CML	0/4	1/1	0/0 (0%)/1
Sec EC/TC	0/15	6/5	0/0 (0%)/5
Normal controls	0/19	n.d.	n.d.
Total	81/146 (55.5%)	108/82 (76%)	

Pos, positive for the JAK2 V617F mutation; homo, homozygous for the V617F mutation; % homo, homozygous cases as percentage of all mutated cases; ampl, successfully amplified in the nested PCR; Sec EC/TC, secondary erythrocytosis/thrombocytosis; n.d., not done.

*Two of the negative cases were positive by allele-specific PCR.

†Including the Ph— atypical CML.

Discussion

This study demonstrates the presence of the activating JAK2 V617F mutation in a high percentage of paraffin-embedded BM biopsies from patients with CMPD other than Ph+ CML. These results not only confirm the results of previous studies but document that trephine BM biopsies are suitable for the detection of this important molecular alteration, thus opening a window of opportunity to study the vast collections of archival material in a retrospective manner.

Although BM trephines undergo processing procedures that are potentially damaging to nucleic acids, a variety of molecular studies targeting both DNA and RNA have been applied successfully to archival BM biopsies.¹² Critical factors for the success of molecular studies are the types of fixation and decalcification as well as the extraction technique used. The trephines in this study have been fixed in buffered formalin and decalcified in EDTA, which renders good results both for immunohistochemistry and molecular studies. Brief acid decalcifica-

Figure 1. Detection of the JAK2 V617F mutation in paraffin-embedded BM trephine biopsies. **A:** Allele-specific multiplex PCR. The upper band of 364 bp confirms the presence of amplifiable DNA. A second band of 203 bp of varying intensity is clearly visible in cases of Ph— CMPD showing the mutation. **Lanes 1, 5, and 9:** PV. **Lanes 2 and 3:** Reactive erythrocytosis. **Lanes 4, 6, 8, and 10:** CIMF. **Lane 7:** ET. C indicates the negative control with DNA derived from a normal donor, N is a no template control. **B:** The same 10 cases analyzed by nested PCR of JAK2 exon 12 with subsequent digestion with *Bsa*XI. Cases 2, 3, and 6 negative for V617F by allele-specific PCR show a lack of undigested PCR product, confirming the absence of the mutation. The ratio of the undigested upper band versus the digested fragments gives an estimate for the relative abundance of mutated DNA in the template.

Figure 2. Comparison of *Bsa*XI digestion (**A**) with sequencing chromatograms (**B**) derived from the same PCR products. **Left lane and top chromatogram:** CIMF without evidence of the mutation and normal wild-type DNA sequence. **Middle lane:** Heterozygous case of ET with predominance of digested PCR product and predominance of the wild-type allele (G>T) in sequencing. **Right lane and bottom chromatogram:** Homozygous case of PV with a majority of undigested PCR product and almost complete dominance of the mutated allele in the chromatogram.

Table 4. Correlation of Clinical Features and JAK2 V617F Mutation in the Common CMPD

Diagnosis and JAK2 status	WBC $\times 10^9$ /L mean (range)	Platelet $\times 10^9$ /L mean (range)	Hemoglobin g/L mean (range)	Splenomegaly*
PV†				
Homozygous	17.9 (7.0 to 49.3)	362 (152 to 620)	145 (68 to 204)	90%
Heterozygous	11.9 (6.9 to 16.3)	464 (91 to 798)	167 (120 to 202)	83%
Negative	9.1	342	188	no
CIMF				
Homozygous	12.6 (5.2 to 22.4)	412 (77 to 762)	130 (83 to 154)	83%
Heterozygous	9.7 (3.9 to 15.8)	608 (23 to 1028)	122 (86 to 160)	100%
Negative	9.9 (2.1 to 53)	530 (28 to 1800)	101 (35 to 136)	75%
ET				
Heterozygous	11.4 (6.8 to 21.5)	930 (393 to 1620)	131 (100 to 156)	62%
Negative	9.0 (3.5 to 11.4)	781 (220 to 1400)	126 (89 to 152)	60%

WBC, white cell count at time of trephine biopsy, platelet count.

*Increased spleen size as determined by abdominal ultrasound.

†Including spent phase of PV.

tion is expected to render similar results. In this study, we demonstrate in a large series of CMPD that trephine BM biopsies are an excellent source for the reliable identification of the JAK2 V617F mutation and compare two different methods for the detection of this important genetic alteration. Despite the relatively large control fragment of 364 bp, the dropout rate of 4% for the allele-specific PCR compares very favorably with results of other molecular studies in BM trephines.¹² In contrast to allele-specific PCR, nested PCR followed by restriction enzyme digestion was not successful in 24% of our cases. This makes the more straightforward, as well as more sensitive, mutation-specific PCR the preferred technique in our hands. Except for a limited number of cases studied by sequencing, no systematic analysis of the JAK2 V617F mutation has been performed in trephine biopsies to date.¹³

The frequency of the JAK2 V617F mutation detected in our BM trephines of patients with CMPD is generally in good agreement with the data of the recently published studies, but some differences are worth mentioning.^{3,5,7,8,13–16} The mutation rate of 74% in ET in our series is higher than in most other studies (23 to 57%).^{3,7,8,14} Some of this discrepancy can be ascribed to the mutation-specific amplification strategy used by us,³ which is more sensitive than RFL analysis or sequencing used in previous studies. Accordingly, the percentage of ET cases with the V617F mutation rose from 12 to 57% in the series from Baxter et al³ when this mutation-specific primer set was used. Other possibilities to explain the higher mutation frequency in ET are a stricter selection of cases by including BM morphology following the new World Health Organization classification as diagnostic criterion and the usage of BM cells rather than cells from the peripheral blood in our study. If the V617F mutation is limited only to a fraction of hematopoietic precursors, rather than affecting all myeloid cells, this could lead to higher detection rates in BM cells compared to PB cells. Of note, the two cases of our series with a negative result by RFL analysis but positive allele-specific PCR, indicative of a low number of cells carrying the mutation, belong to the ET group, similar to the results obtained by Baxter et al.³

Another interesting aspect of our study is the distribution of homozygous versus heterozygous cases in the different diagnostic groups. Although our approach may be less precise than loss of heterozygosity analysis or pyrosequencing,^{3,7,8,14} a significant excess of undigested PCR product is clearly indicative of homozygosity. This was proven by sequencing in a number of cases that confirmed an excess of the mutated allele. In accordance with previous reports, we found absence of homozygosity in ET and a low rate in prefibrotic cases of CIMF. Although not our primary focus, correlation of JAK2 mutational data with clinical findings seems to reflect some of the features identified in larger series, such as lower leukocyte counts in ET without JAK2 V617F mutation (Table 4) or a lower frequency of homozygosity in newly diagnosed PV (data not shown).^{4,8}

The frequency of the JAK2 V617F mutation in other myeloid disorders has not yet been explored in detail, but it is rare in acute myeloid leukemia and myelodysplastic syndromes.^{13,15} In contrast, it seems to be a relatively frequent event in cases with unclassified CMPD or MDS/MPS—occurring in 9 of 12 cases in our series—with the exception of CMML, which was not included in our study.^{13–15,17} A more detailed analysis of the clinical and morphological features of cases of atypical CMPD with or without the V617F mutation will help to better classify these cases.

Although identification of the activating JAK2 mutation represents a major step for the understanding of CMPD, many questions remain to be investigated. To date, it is still unclear which mechanisms are responsible for the wide range of clinical features as exemplified by the different CMPD subtypes in carriers of the V617F mutation. Besides the possibility to retrospectively study archival material, the investigation of trephine biopsies is potentially useful in many other aspects. Fibrosis is a prominent feature of many cases of CMPD and prevents aspiration of marrow cells for analysis, making the biopsy an alternative source for DNA if peripheral blood is unavailable. In addition, a more detailed investigation of the morphological and phenotypical characteristics of CMPD in correlation with the presence or absence of the V617F

mutation will provide new insights in the biology and evolution of these disorders.

Note:

Since submission of this article, the detection of the JAK2 V617F mutation in trephine bone marrow biopsies has been described in an article by Bock et al.¹⁸

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