

# Importance of molecular confirmation in the diagnosis of myeloproliferative disorders

## *A case of a misleading constitutional translocation and a diagnosis of essential thrombocythemia with a JAK2 mutation*

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The presence of the Philadelphia (Ph) chromosome is a hallmark for the diagnosis of chronic myeloid leukemia (CML). Here, we report a cytogenetically ascertained Ph-positive case with pathological features of a myeloproliferative disorder consistent with CML. No cytogenetic improvement was observed following 7 years of treatment, despite a hematological remission. Subsequent molecular studies revealed the absence of *BCR/ABL* fusion products. The purported "Ph chromosome" was found to be derived from a constitutional t(12;22) translocation instead of an acquired t(9;22;12). An acquired point mutation, V617F, was subsequently identified in the *JAK2* gene of the patient. The diagnosis was therefore changed from CML to essential thrombocythemia, and treatment with anagrelide has been effective. This case illustrates the importance of molecular confirmation in the diagnosis of myeloproliferative disorders.

Chronic myeloid leukemia (CML) is characterized by neoplastic overproduction of granulocytes with high leukocyte counts at initial diagnosis. The diagnostic hallmark for CML is the presence of the so-called Philadelphia (Ph) chromosome, which is usually derived from a reciprocal translocation between chromosome 9, at band q34, and chromosome 22, at band q11.2. Translocations can involve submicroscopic regions of these chromosomes, resulting in Ph-negative CML, or may involve additional loci, resulting in "complex," "variant," or "masked" Ph chromosomes.<sup>1,2</sup> Such translocations result in the transfer of the Abelson oncogene (*ABL*) on chromosome 9 to the *BCR* ("breakpoint cluster region") gene on chromosome 22, and their subsequent fusion can be detected by fluorescence in situ hybridization (FISH).<sup>3</sup> Alternatively, the mRNA transcripts of *BCR/ABL* fusion can be detected by polymerase chain reaction (PCR).<sup>4</sup>

CML belongs to the family of myeloproliferative disorders characterized by clonal proliferation of hematopoietic progenitor cells. This family includes polycythemia vera, essential thrombocythemia, and idiopathic myelofibrosis.<sup>5</sup> The

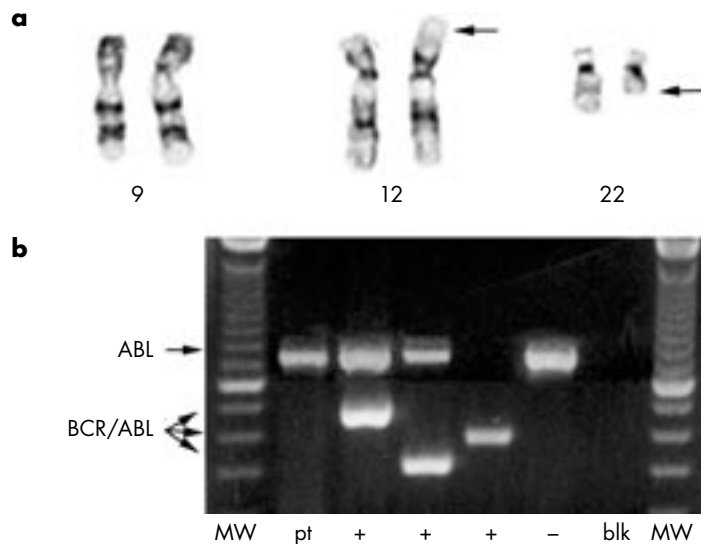
myeloproliferative disorder subtypes differ by the hematopoietic cell type that is predominantly affected. The bone-marrow morphology in CML is characterized by granulocytic and megakaryocytic hyperplasia and an increase in reticulin fibrosis, whereas megakaryocytic hyperplasia without fibrosis is found in essential thrombocythemia. Panmyelosis, with or without fibrosis, characterizes polycythemia vera. However, overlapping clinical, hematological, and morphological features sometimes obscure an exact classification. The detection or absence of the *BCR/ABL* fusion by cytogenetic or molecular methods has been used as a key diagnostic discriminator between CML and other myeloproliferative disorders.<sup>6</sup>

A single point mutation was recently identified in the JH2 pseudokinase domain of the Janus kinase 2 (*JAK2*) gene in 80%–97% of polycythemia vera patients and approximately 50% of patients

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**FIGURE 1** The patient's cells appeared to contain a Philadelphia chromosome but were negative for the expected *BCR/ABL* fusion. (a) Representative G-banded chromosomes 9, 12, and 22. The karyotype was initially interpreted as 46,XX,t(9;22;12)(q34;p11.2;p13.3). (b) Reverse transcription PCR analysis showed a complete absence of *BCR/ABL* fusion transcripts (lane pt). Three positive controls (+) were included, representing fusion transcripts of e1a2, b2a2, and b3a2, respectively. A negative control (-) and a water-blank control (blk) were also included. The internal control *ABL* was amplifiable from the patient specimen (pt). The 100-bp molecular weight marker (MW) is shown on both sides of the gel.

with essential thrombocythemia and idiopathic myelofibrosis.<sup>7-10</sup> This mutation leads to a substitution of valine by phenylalanine at amino acid position 617 (V617F) and results in dysregulated kinase activity and cytokine hypersensitivity in erythroid colonies. The discovery of this mutation promises to facilitate a new molecular classification of myeloproliferative disorders.

Here, we report a case of myeloproliferative disorder with a morphologically typical Ph chromosome that was originally interpreted as a derivative of a three-way translocation, t(9;22;12). Cell morphological features were consistent with CML. However, the purported Ph chromosome was subsequently shown to be the result of a constitutional translocation segregating in this family and did not harbor a *BCR/ABL* fusion. In addition, the V617F mutation was present in the leukocytes of this patient.

### Case report

A 77-year-old white female had

been diagnosed with CML in 1996. An automated hemogram revealed a white blood count (WBC) of  $7.9 \times 10^9/L$ , with 59% neutrophils, 22% lymphocytes, 4% monocytes, 12% eosinophils, and 2% basophils. The hematocrit was 46.9%, red blood cell (RBC) indices were normal, and the platelet count was  $1,104 \times 10^9/L$ . The peripheral blood smear showed a marked increase in platelets, some of which were abnormally large. The WBC morphology was normal, and there were no circulating immature myeloid cells. No organomegaly was evident on physical examination or imaging studies. Bone marrow biopsy showed a hypercellular marrow with a marked increase in megakaryocytes, many of which were in clusters. The megakaryocytes were abnormally large and hyperlobulated. Cytogenetic studies on cultured bone marrow cells showed an abnormality interpreted as 46,XX,t(9;22;12)(q34;q11.2;p13.3), ie, Ph chromosome-positive with a variant three-way translocation (Figure 1a). Based on

these findings, a diagnosis of CML was established, and the patient was treated with hydroxyurea.

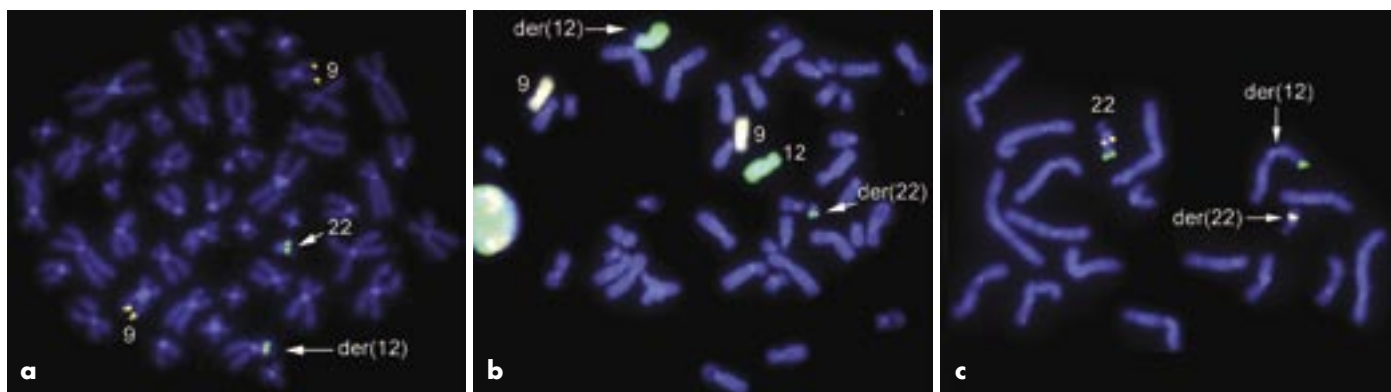
The platelet count gradually dropped to the normal range within 4 months of therapy. Interferon alpha (IFN $\alpha$ ) treatment was subsequently initiated at a dosage of  $3 \times 10^6$  U/d and then increased to  $5 \times 10^6$  U/d. However, the platelet count was not adequately controlled, and IFN $\alpha$  caused unacceptable constitutional symptoms and weight loss. Treatment with IFN $\alpha$  was therefore discontinued, and hydroxyurea therapy was resumed, resulting in excellent control of the platelet count, with mild anemia, between 1997 and 2001.

In January 2002, the patient was switched to imatinib (Gleevec) 400 mg/d.<sup>11</sup> Eight months later, however, a bone marrow biopsy again showed hypercellular marrow with increased megakaryocytes, 4%–5% myeloblasts, and no increase in eosinophils or basophils. The erythroid series showed normoblastic maturation. The platelet count was suboptimally controlled despite adjustments in the imatinib dosage. In November 2002, she was hospitalized for pulmonary edema, which was promptly reversed with discontinuation of imatinib therapy. Hydroxyurea was reinstated, with satisfactory control of the platelet counts, but the hydroxyurea induced a moderately severe anemia.

A bone marrow analysis in February 2004 showed similar morphological features and flow cytometry results, as well as normal iron staining. No bone marrow fibrosis was noticed. Repeated cytogenetic studies until February 2004 consistently showed the same "three-way translocation" in all metaphase cells examined and no evidence of clonal evolution.

### Results

Molecular studies on the bone marrow aspirate in February 2004 with reverse transcription PCR (RT-PCR) were performed for the first time to



**FIGURE 2** FISH studies ruled out a  $t(9;22;12)$  translocation and confirmed a  $t(12;22)$  translocation. (a) Hybridization with the LSI BCR/ABL Dual Color Dual Fusion Translocation probe showed no juxtaposition of the BCR (green) and ABL (red) signals. ABL signals were located on both copies of chromosome 9. One BCR signal was translocated to the short arm of chromosome 12, der(12), whereas the other one remained on the normal chromosome 22. (b) Hybridization with whole chromosome painting (WCP) probes showed complete coating of both chromosome 9 homologs by WCP 9 (orange). WCP 12 (green) painted one entire chromosome 12 and most of the other chromosome 12, except for its distal short arm, identifying it as der(12). WCP 12 was also hybridized to der(22). (c) Hybridization with the LSI TUPLE1/ARSA probe showed normal localization of the TUPLE1 signal (orange) and ARSA signal (green) on one chromosome 22. The other chromosome 22, the der(22), showed only the TUPLE1 signal (22q11.2), whereas the ARSA signal (22q13.3) appeared on the short arm of der(12).

detect *BCR/ABL* fusion transcripts. Nucleated leukocytes were isolated from the bone marrow and lysed in Trizol (Invitrogen). Total RNA was extracted and reverse transcribed. The resultant cDNA was then amplified using primers for exons 1 and 13 of the *BCR* gene in the forward direction and a reverse primer in exon 3 of the *ABL* gene. Unexpectedly, no *BCR/ABL* fusion transcript was detected (Figure 1b).

Because a false-negative result can arise with the use of these primers when there is an atypical breakpoint in the *BCR* or *ABL* gene, we performed FISH analysis using dual fusion BCR/ABL translocation probes (Vysis). This probe set allows identification of a *BCR/ABL* fusion based on the juxtaposition of fluorescently labeled *BCR* and *ABL* signals, regardless of the breakpoints involved. All cells analyzed showed normal signal patterns and no *BCR/ABL* fusion product. Examination of metaphase cells showed translocation of a *BCR* signal from the long arm of chromosome 22 to the short arm of chromosome 12 and that no *BCR* signal was seen on chromosome 9. These observations suggested a simple reciprocal translocation between chromosomes

12 and 22, ie,  $t(12;22)(p13;q11.2)$ , rather than a three-way,  $t(9;22;12)$ , translocation. Thus, the “Ph chromosome” previously ascertained by G-banding analysis was reinterpreted as a derivative chromosome 22, ie, der(22), with no *BCR/ABL* fusion (Figure 2a).

To ensure that no chromosome 9 material was involved in the translocation and that no chromosome 12 material was translocated to loci other than the long arm of chromosome 22, we employed whole chromosome painting (WCP) probes for chromosomes 9 and 12 (Vysis) in additional FISH studies on metaphase cells from this patient. The analysis demonstrated clearly that both chromosome 9 homologs were hybridized completely by the WCP 9 probe (labeled with orange) and that no orange signals were observed on any other chromosomes. WCP 12 (labeled with green), on the other hand, hybridized to one normal chromosome 12, to all but the distal short arm of the derivative chromosome 12, ie, der(12), and to the long arm of the der(22) (Figure 2b). These results were consistent with a two-way translocation,  $t(12;22)$ , with no involvement of chromosome 9.

The breakpoint of the translocati-

on chromosome 22 was further determined by FISH analysis using the LSI TUPLE1 probe (located at chromosome band 22q11, proximal to *BCR*) and the *ARSA* (arylsulfatase A) probe (located at band 22q13.3). This analysis showed that the ARSA signal was translocated onto the short arm of der(12), whereas the TUPLE1 signal remained on der(22) (Figure 2c). Combining this result with that of the *BCR/ABL* FISH results, we concluded that the breakpoint on chromosome 22 was distal to the TUPLE1 gene and proximal to the *BCR* gene at 22q11.2.

Since this translocation was consistently present in 100% of the metaphases analyzed from all samples obtained from this patient, we suspected a constitutional origin of this anomaly. An analysis of phytohemagglutinin-stimulated lymphocytes showed a translocation,  $t(12;22)$ , in all metaphase cells examined. Furthermore, the same translocation was identified in a blood sample from the patient's healthy 54-year-old son. This translocation therefore represented a hereditary constitutional change, rather than an acquired abnormality associated with the patient's myeloproliferative condition.

All clinical findings were reevaluated, and the diagnosis of CML was changed to essential thrombocythemia. The patient was treated with anagrelide 0.5 mg twice daily, starting March 2004. An automated hemogram in April 2004 showed WBC count of  $9.0 \times 10^9/L$ , a hematocrit of 39.2%, and a platelet count of  $388 \times 10^9/L$ , all within the normal range. As of this report date, the patient is asymptomatic on anagrelide therapy.

The diagnosis of essential thrombocythemia was further confirmed by mutation analysis of *JAK2*. An allele-specific PCR was performed as previously described<sup>7</sup> and amplified both the wild-type and the mutant alleles using DNA extracted from the patient's leukocytes (Figure 3). Therefore, the patient's leukocytes were heterozygous for the V617F mutation. This mutation was further confirmed by restriction enzyme digestion with *Bst*XI (data not shown).

## Discussion

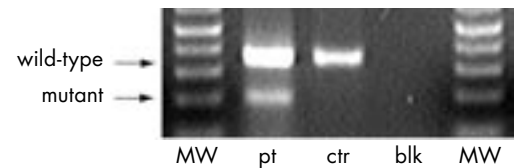
We report a case with an initial diagnosis of Ph-positive CML based on cytogenetic analysis. However, the patient was negative for a *BCR/ABL* fusion by both FISH and PCR studies and did not respond to imatinib. The purported "Ph chromosome" was actually der(22) involved in a constitutional translocation, t(12;22), and the diagnosis was subsequently revised to essential thrombocythemia.

Although cytogenetics has been the "gold standard" in diagnosing CML, limitations in resolution and sensitivity can hinder the diagnosis, and in some instances, lead to misleading results. In our patient, der(22) ascertained by G-banding looked exactly like a Ph chromosome (Figure 1a). However, the patient's cells showed no evidence of a *BCR/ABL* fusion, and therefore the diagnosis of CML could not be confirmed. In some CML cases, no Ph chromosomes are discernible by G-banding. Nevertheless, these so-called Ph-negative CML cases are

generally *BCR/ABL* positive when evaluated with FISH or RT-PCR.<sup>1</sup>

FISH analysis has greatly enhanced the detection of submicroscopic anomalies. In addition, the flexibility of using interphase nuclei instead of metaphase cells allows for screening of hundreds of cells in each specimen and hence improves the sensitivity of chromosome analysis. Although not as sensitive as PCR, FISH is particularly advantageous when breakpoints occur outside the sequences that routinely used PCR primers can detect. RT-PCR is an important measure for monitoring minimal residual diseases in patients after treatment. It also identifies specific subtypes of fusion transcripts important for identifying disease etiology. The sensitivity generally reaches  $10^{-6}$  by nested PCR.<sup>4</sup> A persistent presence of fusion transcripts usually indicates drug resistance, with a higher than normal chance of relapse, and alternative therapy should be considered. Quantification of minimal residual diseases by real-time PCR (Q-PCR) has provided significant prognostic information.<sup>12</sup> A baseline RT-PCR analysis and Q-PCR test should be performed at initial diagnosis.<sup>12</sup>

There are different opinions regarding the existence of Ph-negative CML.<sup>2,13,14</sup> In our patient, the hematological and morphological evidence was consistent with a myeloproliferative disorder. Indeed, evidence of increased granulocytic and megakaryocytic cells and eosinophilia was present in the initial bone marrow sample. However, the WBC count was not as high as the counts typically seen in CML. Platelet counts can be elevated in both CML and essential thrombocythemia patients. The diagnosis conundrum was further complicated by the existence of true Ph-positive essential thrombocythemia cases without features of CML in the blood.<sup>15</sup> These cases represent an early manifestation of chronic-phase CML, eventually progressing



**FIGURE 3** A point mutation, V617F, in the *JAK2* gene was identified in DNA extracted from the patient's leukocytes. Allele-specific PCR analysis showed the presence of both wild-type and mutant alleles (lane pt). A normal control (ctr) and a water-blank control (blk) were also included. The 1-kb molecular weight marker (MW) was shown on both sides of the gel.

to myelofibrosis and blast crisis. The distinguishing morphological feature of Ph-positive essential thrombocythemia is the small hypolobulated megakaryocytes, in sharp contrast to the large clustered hyperlobulated megakaryocytes in Ph-negative essential thrombocythemia, as was evident in this case. The absence of the *BCR/ABL* fusion gene and transcript and the presence of the V617F mutation in the *JAK2* gene, taken together with the absence of bone marrow fibrosis, hepatosplenomegaly, and evolution to an accelerated or blast crisis phase of CML over 7 years, led to revising the diagnosis in this patient from CML to essential thrombocythemia. Treatment with hydroxyurea, IFN $\alpha$ , and imatinib was complicated by cytopenias, constitutional symptoms, and fluid retention. Ultimately, treatment with anagrelide for essential thrombocythemia was well tolerated and established a hematological remission.

Frequently, constitutional genetic alterations are noticed for the first time when cytogenetic studies are done on bone marrow biopsies from patients with leukemia or lymphoma. When all of the bone marrow cells analyzed show a cytogenetically balanced rearrangement that is not typical of neoplasia, it is common practice to establish whether the translocation is constitutional or an acquired clonal aberration related to the patient's hematological disorder. Because of the reproductive significance of these familial rearrangements, oncologists

should be alerted to refer potential carrier family members to genetic counseling. The unique aspect of our case is that the constitutional translocation resulted in a der(22) identical in appearance to the Ph chromosome that characterizes CML. Therefore, the possibility that this translocation was constitutional was not initially considered. This case highlights the importance of confirming a *BCR/ABL* fusion in the diagnosis of CML and the differential diagnosis of myeloproliferative disorders.

Of particular interest is the new finding of the *JAK2* mutation in our patient. Our study demonstrated the feasibility of a timely application of this novel research finding into clinical practice. Following the initial discovery of the V617F mutation of *JAK2* in myeloproliferative disorders,<sup>7-10</sup> several groups have reported the incidence of this mutation in typical and atypical myeloproliferative disorders as well as myelodysplastic syndromes.<sup>16-18</sup> Combining the data presented by these reports, the clinical sensitivity (or detection rate) for the *JAK2* mutation is 78.4% in polycythemia vera, 34.9% in essential thrombocythemia, and 43.3% in idiopathic myelofibrosis. The V617F mutation was not identified in any cases with the *BCR/ABL* fusion or other rare tyrosine kinase fusions. Our study is therefore consistent with the results from these larger series of reports on detection of the *JAK2* mutation in myeloproliferative disorders. Complementing the detection of a *BCR/ABL* fusion, detection of the *JAK2* mutation establishes the clonal nature of a disorder and is likely to

be increasingly used in the differential diagnosis and classification of myeloproliferative disorders in the near future. Novel therapeutic approaches targeting the dysregulated kinase activity of the *JAK2* protein will likely be actively pursued as well.

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