Cytogenetic and Molecular Analysis in Philadelphia Negative CML


We studied the clinical, hematologic, cytogenetic, and molecular biologic features in four patients with Philadelphia (Ph) negative chronic myeloid leukemia (CML). In all four cases the clinical and hematologic characteristics were indistinguishable from Ph positive CML. Cytogenetic analysis showed a normal karyotype in two patients and chromosomal translocations apparently not affecting chromosome 22 in the other two cases. Southern blot analysis using probes of the bcr region, demonstrated a bcr breakpoint in all four patients. In situ hybridization with bcr, c-abl, and c-sis probes showed unusual hybridization sites for 5'-bcr and c-abl indicating complex chromosomal rearrangements affecting three different chromosomes in the four patients investigated. Using polymerase chain reaction (PCR) followed by hybridization to oligonucleotide probes specific for the bcr-abl fusion region, the expression of a chimeric bcr-abl mRNA was detected. In these patients we demonstrated that (a) CML with a breakpoint in the bcr region without cytogenetically detectable Ph chromosome is characterized by the same genomic recombination of 5'-bcr and c-abl as CML with standard Ph translocation and (b) unusual localization of 5'-bcr and c-abl sequences caused by complex Ph translocation does not interfere with transcription of the bcr-abl fusion gene.

The Philadelphia Chromosome (Ph) is found in 94% of chronic myeloid leukemia (CML) patients. In the majority of cases it originates from the standard Ph translocation t(9;22)(q34;q11). As a result of this translocation the c-abl oncogene is translocated to a specific site in a gene on chromosome 22 band q11 called breakpoint cluster region (bcr region). The part of chromosome 22 distal to the breakpoint is translocated to chromosome 9 band q34.

The 5'bcr-abl DNA on the Ph chromosome is transcribed into an 8.5 kilobase (kb) chimeric bcr-abl mRNA. Depending on the position of the breakpoint in bcr either bcr exon b2 or b3 are spliced to abl exon a2, resulting in either b2a2 or b3a2 chimeric mRNA. This 8.5 kb bcr-abl mRNA encodes an abl protein of 210 kilodalton (kD), which has in vitro enhanced tyrosine kinase activity compared with the normal abl protein. Approximately 5% of the Ph-positive CML patients have cytogenetic variants of the Ph translocation. Southern blot and in situ hybridization studies have shown that in variant Ph translocation molecular recombination of 5'-bcr and c-abl takes place in exactly the same way as in standard Ph-translocation. The remaining 6% of CML patients show no Ph chromosome and are classified as Ph-negative. Ph-negative CML patients have different clinical and hematologic features, ie, older median age (>65 years), monocytosis, thrombocytopenia, poor response to chemotherapy, rapid transformation to acute leukemia, and shorter survival than Ph-positive CML. Nevertheless a few Ph-negative CML patients present with clinical and hematologic features that are indistinguishable from Ph-positive CML, ie, median age <60 years, higher WBC with basophilia but without monocytosis, no dysplastic changes in bone marrow cells and same survival as in Ph-positive CML.

We report in this article detailed cytogenetic and molecular analysis in four patients with a disease indistinguishable from Ph-positive CML, with either a normal karyotype (two cases) or chromosomal translocations apparently not affecting chromosome 22 (two cases). Southern blot analysis and in situ hybridization studies demonstrated genomic recombination of 5'-bcr and c-abl sequences caused by complex Ph translocations. cDNA analysis using the polymerase chain reaction (PCR) showed expression of bcr-abl mRNA. These findings are identical to the findings in Ph-positive CML.

Case Reports

Patients were diagnosed and treated in three different European centers: patient no. 1 in Rome, patient no. 2 and 3 in Paris, and patient no. 4 in Amsterdam. A summary of clinical and hematologic data at diagnosis is given in Table 1.

Patient no. 1. In September 1984 CML was diagnosed that responded to hydroxyurea (2.5 g/d): splenomegaly regressed and hematologic findings returned to normal values. Hematologic remission was maintained until January 1986 when thrombocytosis appeared increasing to 1,000 × 10^9/L, but responded well to busulfan (2 mg/d). In December 1987, the patient developed a nonlymphoid blast crisis and died in June 1988 due to septic complications.

Patient no. 2. CML was diagnosed in February 1983. Clinical and hematologic remission were obtained with hydroxyurea and lasted 3 years. In April 1986 accelerated phase occurred with 20% myeloblasts in bone marrow (BM). 6-Mercaptopurine was added to hydroxyurea, and again hematologic stabilization was obtained. In October 1986 a splenectomy was performed. The spleen showed important myeloid metaplasia. In April 1987 engraftment with allogeneic BM was realized with success.

Patient no. 3. In May 1977 CML diagnosis was made and successfully treated with busulfan. Chronic phase lasted 8½ years. In January 1986 the first signs of accelerated phase appeared, characterized by bone pains, asthenia, blast cells in BM (10%) and...
Peripheral blood (10%) and thrombocytopenia (72 x 10^9/L). In September 1986, blastic transformation occurred with 55% of undifferentiated blast cells in bone marrow aspirate. The patient was treated with vincristine, daunorubicine, novantrone, and aracytidine. No remission was obtained and the patient died in October 1986 of hyperplasia, cardiac and renal failure, and hemorrhage.

Patient No. 4. CML was diagnosed in the beginning of 1980, following incidental discovery of granulocytosis without other symptoms. The patient reacted favorably to repeated courses of busulfan. In February 1987 a steady increase of myeloblasts in the peripheral blood was seen, followed within a month by a full blown myeloblastic crisis. Among other drugs treatment with alpha-2-interferon had no success. The patient died 2 months later.

MATERIALS AND METHODS

Samples. Bone marrow aspirates and blood samples were sent to Rotterdam for molecular investigations. The samples were sterile and heparinized and reached the laboratory within 24 hours after aspiration. All sampling was part of diagnostic and clinical follow-up procedures and obtained only after informed consent of the patients.

Cytogenetics. The karyotype of leukemic cells was investigated at diagnosis using standard cytogenetic procedures. Chromosomes were identified using G and/or R banding techniques and classified according to ISCN (1985).24 Because of unusual findings the cytogenetic analyses were repeated several times locally and in Rotterdam at the time of the molecular investigation. The constitutional karyotype of each patient was determined and found normal using PHA stimulated blood cultures.

DNA probes. The following probes were used in Southern blot analysis and in situ hybridization*: c-abl, 0.6 kb BamHI + 1.1 kb HindIII-EcoRI fragment; c-sis, 1.7 kb BamHI fragment; 5′-bcr, 2 kb BglII-HindIII fragment; 3′-bcr, 1.2 kb HindIII-BglII fragment. In standard Ph translocations the 5′-bcr probe recognizes the 22q− derivative and the 3′-bcr probe recognizes the 9q+ derivative.

Southern blotting was performed following standard techniques.22 In situ hybridization was performed as reported previously.25 The probes were 3H-labeled, using the method of Feinberg and Vogelstein27 to a specific activity of 10^8 cpm/μg DNA. After hybridization and autoradiography the labeled sites were scored on R or Q banded metaphases and assigned to a chromosomal band or region. Grain distribution in the in situ hybridization experiments was tested on its statistical significance using the Poisson distribution followed by the binomial method.

cDNA preparation and amplification with the PCR were performed as described by Hermans et al.28 Oligonucleotides2 2 and 3 (Fig 1) were used as primers to amplify a fragment covering the bcr-abl region. As internal positive control in the PCR, an abl fragment was amplified, using oligonucleotides 1 and 3, that is always present, irrespective of the Ph translocation. Two 40 mer oligonucleotides specific for the breakpoint junctions b2a2 and b3a2 were used as probes.

RESULTS

Cytogenetics

Patient no. 1. At diagnosis 75 metaphases were analyzed after Giemsa staining and GTG banding technique without evidence of a recognizable Ph chromosome. However, one of

![Schematic representation of 8.5 kb bcr-abl mRNA. Localization of primers used in PCR experiment is given. ▼ indicates that exon b3 may be absent or present in 8.5 kb bcr-abl mRNA. The size and localization of the two fragments amplified in the PCR experiments are presented.](www.bloodjournal.org)
the two chromosomes 22 appeared slightly shorter, one chromosome 19 appeared smaller and sub-metacentric and one chromosome 9 appeared identical to the 9q+ derivative in the standard t(9;22). R-banding studies confirmed these findings (Fig 2). A complex chromosomal translocation t(9;19;22) resulting in a masked Ph was retained as probable interpretation of the karyotype, but routine karyotyping showed two apparently normal chromosomes 22, which was the reason for inclusion of the case in this study.

**Patient no. 2.** At diagnosis 26 metaphases were analyzed and 80 metaphases in subsequent studies. All metaphases examined showed the same karyotype that was equivocal in R and G bands. There were two possible interpretations: either a simple translocation t(9;14) or a complex Ph translocation t(9;14;22) (Fig 2).

**Patient no. 3.** The BM karyotype of patient no. 3 was studied twice during the chronic phase of the disease. All 50 metaphases showed a 46,XY normal male karyotype (Fig 2). Molecular investigations were performed at the time of acceleration 8 years and 10 months after diagnosis. At that time the BM karyotypes were 46,XY in 90% and 46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY, t(1;21)(p21;q22) in 10% of the metaphases.

**Patient no. 4.** The BM karyotype of patient no. 4 was found to be normal: 46,XX in repeated investigations of blood and BM cells during the chronic phase of the disease (Fig 2). In January 1987 hematologic and clinical data indicated progression of CML, 20% of the metaphases were abnormal: 47,XX, +8,i(17q). Remarkably, the type of aberrations is the same as often described in progression of Ph positive CML.

**Southern Blot Analysis**

DNA from bone marrow and/or blood cells of the four patients was digested with BgIII, BamHI, and HindIII and hybridized to 5'- and 3'-bcr probes. All patients showed extra bands with more than one probe and more than one restriction enzyme indicating the existence of a breakpoint in the bcr region of chromosome 22 (Fig 3). The breakpoint was found in the HindIII-BgII fragment of the bcr region in patient no. 1, 2, and 4 (ie, between bcr region exon 3 and 4) and in the HindIII-BamHI fragment in patient no. 3 (ie, between bcr region exon 2 and 3). These results are similar to our observations in more than 50 CML patients with standard t(9;22).

**In Situ Hybridization Studies**

The absence of a cytogenetically recognizable Ph chromosome in the presence of a bcr breakpoint prompted investigation of the chromosomal localization of the various genes of interest using in situ hybridization. To this aim metaphase spreads of the four patients were hybridized to four different probes: (1) c-abl, (2) 5'-bcr mapping proximal to the breakpoint, (3) 3'-bcr mapping distal to the breakpoint, and (4)
filter with BamH1; it appeared that as an indicator of the distal part of chromosome 22.

Results are detailed in Table 2 and summarized in Fig. 4. All experiments were statistically significant for the probes used with the exception of the 3'-'bcr probe, which showed no statistical significance according to the Poisson distribution in patient no. 1 on chromosomes 9q+ and 22, and in patients no. 3 and 4 on chromosome 9. Though, in the case of patients no. 1 and 3, experiments with the 3'-'bcr probe could be demonstrated to be statistically significant for the chromosomes 9q+ and 9, respectively, when the binomial distribution alone was tested.

Amplification of cDNA by the PCR

The results of amplification of cDNA followed by hybridization to breakpoint specific oligonucleotides are shown in Fig 5. A 319 base pairs fragment (bp) corresponding to b2a2 joining in the bcr-abl mRNA has been found in patient no. 3, a control CML patient with standard Ph translocation and cell line BV173. A 394 bp fragment corresponding to b3a2 joining has been found in patients no. 1 and 4 and cell line K562. As expected neither b2a2 nor b3a2 joining have been detected in leukocytes of a healthy control.

DISCUSSION

In this article we report clinical, cytogenetic and molecular data obtained in four patients with CML and absence of Ph chromosome in bone marrow metaphases. In these patients clinical features at presentation and long survival (3.75 to 9.3 years) contrasted with the atypical symptomatology and rapid transformation to blast crisis usually associated with Ph-negative CML. Reclassification of these cases as myelodysplastic (CMML) or myeloproliferative syndrome does not apply to these four patients who had all characteristics of classic CML except for the Ph chromosome.

Southern blotting demonstrated a bcr breakpoint in the four cases, similar to our findings in Ph-positive CML.
Table 2. In Situ Hybridization Studies

<table>
<thead>
<tr>
<th>Patient and Karyotype</th>
<th>No. of Probes Analyzed</th>
<th>No. of Labeled Sites on Specific Bands</th>
<th>Other Chromosomes Involved</th>
<th>No. of Background Grains</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Total 9 (q33-34) 9q+ (q-distal) 22q-</td>
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<tr>
<td>Patient No. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c-abl</td>
<td>30</td>
<td>96 11 (10) 32 (22) 3 10 6</td>
<td>19/19q;1/18 (8); 46</td>
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</tr>
<tr>
<td>46, XY</td>
<td>192</td>
<td>346 10 32 (22) 24</td>
<td>19/19q;1/2/5; 103</td>
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<tr>
<td>t(9;19;22)</td>
<td>30</td>
<td>97 2 3 10 6</td>
<td>19/19q;1/7 (7); 42</td>
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<tr>
<td>3'-bcr</td>
<td>32</td>
<td>86 2 8 (7) 6</td>
<td>19/19q;1/1</td>
<td>50</td>
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<td></td>
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<tr>
<td>c-abl</td>
<td>50</td>
<td>165 20 (18) 8 35 11 no. 14:3</td>
<td>95</td>
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</tr>
<tr>
<td>46, XX</td>
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<td>158 9 10 24 2 no. 14:3</td>
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<td>t(9;14:22)</td>
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<td>156 5 4 16 13 no. 14:3</td>
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<tr>
<td>3'-bcr</td>
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<td>157 4 6 24 1 no. 14:3</td>
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<td></td>
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<tr>
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<td>230 27 (19) 4 no. 1:28 (20)</td>
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</tr>
<tr>
<td>46, XY</td>
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<td>187 9 22 no. 1:22</td>
<td>97</td>
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<tr>
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<td>210 10 18 no. 1:25 (15)</td>
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<td>3'-bcr</td>
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<td>153</td>
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<td>Patient No. 4</td>
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<td>c-abl</td>
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<tr>
<td>46, XX</td>
<td>63</td>
<td>182 9 22 no. 1:15</td>
<td>103</td>
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<tr>
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<td>75</td>
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<tr>
<td>3'-bcr</td>
<td>56</td>
<td>174 12 (5) 21 no. 1:17</td>
<td>118</td>
<td></td>
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</tbody>
</table>

*The number of labeled sites on specific parts of chromosomes or specific bands is written in parentheses.

†In patient 1 chromosome 22 and der(22) were indistinguishable in mitoses studied after in situ hybridization.

‡Number of labeled sites on the deleted arm of 19q.

§In patient 2 chromosome 22 and 14q— are indistinguishable by cytogenetics only.

‖Number of labeled sites on chromosome 1 (p34-p36).

#Statistically significant using the binomial distribution alone.

##Statistically not significant according to the Poisson and binomial distribution.

![Patient 1](image1.png)

PATIENT 1

C-ABL

3'-bcr

C/SIS

5'-bcr

C/SIS

19q

C-ABL

![Patient 2](image2.png)

PATIENT 2

3'-bcr

C/SIS

5'-bcr

C/SIS

![Patient 3](image3.png)

PATIENT 3

5'-bcr

C/SIS

3'-bcr

C/SIS

![Patient 4](image4.png)

PATIENT 4

5'-bcr

C/SIS

3'-bcr

C/SIS

Fig 4. Summary of results of in situ hybridization with 5'-bcr, 3'-bcr, c-sis, and c-abl probes in Ph-negative CML patients no. 1, 2, 3, and 4. Normal chromosomes on the left. Localization of the different probes by in situ hybridization is presented.
CYTOGENETIC AND MOLECULAR STUDIES IN Ph-CML

The results of Southern blot analysis and the PCR experiment indicated that in these Ph-negative CML patients bcr-abl fusion has taken place analogous to Ph-positive CML patients and that transcription was not influenced by the unusual chromosomal localization of 5'-bcr and c-abl in these patients. The occurrence of bcr-abl rearrangements in Ph-negative CML and the clinical significance of such findings have been studied by others.18,23,31-37

From the 50 cases with normal karyotypes that have been investigated by Southern blotting, including our own patients, 20 showed a breakpoint in the bcr region.9,32-36 In ten cases RNA or protein analysis demonstrated transcription or translation36 of the chimeric bcr-abl gene. The clinical criteria for CML diagnosis, the frequency of molecular rearrangements, and the interpretation of data are different in each study. Nevertheless most investigators13,37 concluded from their studies that in Ph-negative CML the finding of bcr-abl molecular rearrangements indicated an undetected Ph translocation and therefore classify these patients to the group of Ph-positive CML with the prognostic and therapeutic consequences attached to this diagnosis. The remaining cases (Ph negative, bcr negative) constitute the group of Ph-negative CML with atypical (ie, myelodysplastic) hematologic features and a relatively short survival. Dreazen et al19 challenged this hypothesis and claimed the presence of the bcr rearrangement in the majority of their Ph-negative CML patients and the finding of atypical features.

Our data support the former observations and indicate that in Ph-negative CML, the finding of molecular evidence for the presence of a bcr-abl fusion gene is diagnostic for classical CML. Larger prospective studies are needed to clarify the clinical relevance of molecular investigations in CML.

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