RAPID COMMUNICATION

Neutrophilic-Chronic Myeloid Leukemia: A Distinct Disease With a Specific Molecular Marker (BCR/ABL With C3/A2 Junction)

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Neutrophilic-chronic myeloid leukemia (CML-N) is a rare myeloproliferative disorder that runs a much more benign course than chronic myeloid leukemia, and for which no specific underlying molecular lesion has been described so far. We have analyzed the genomic DNA by Southern blotting and the BCR/ABL hybrid gene transcripts by reverse transcriptase-polymerase chain reaction in three patients with clinical findings of CML-N, who did have a t(9;22) chromosomal translocation. In all patients we have found a rare type of BCR/ABL rearrangement, with a breakpoint between exons c3 and c4 of the BCR gene (corresponding to BCR exons 19 and 20). This was confirmed by hybridization with an oligonucleotide probe spanning the c3/a2 region. This type of junction causes almost the entire BCR gene to fuse with ABL. The junction is in frame and it gives rise to a fusion protein of predicted 230 kD. Our data now provide a molecular diagnostic marker for CML-N, and they are consistent with the notion that the inclusion or exclusion of BCR exons in the fusion protein affects dramatically its capacity to derange myeloid proliferation and differentiation, leading to the appearance of different disease phenotypes.

CHRONIC MYELOID leukemia (CML) has become the prototype of a malignant disorder for which the molecular basis is a specific fusion gene, BCR/ABL, originating from the t(9;22) chromosomal translocation. Formal proof that this fusion gene, encoding a 210-kD fusion protein, is responsible for the disease comes from the finding that retroviral-mediated transfer of a BCR/ABL cDNA construct into mouse hematopoietic cells caused a condition similar to human CML in irradiated syngeneic recipients. The specific pathogenic significance of the fusion gene is corroborated by the remarkable finding that even in a proportion of patients with acute lymphoblastic leukemia (Ph+ ALL) there is a BCR/ABL fusion gene, but with a different breakpoint within BCR, and the cognate production of a 190-kD fusion protein. Mice transgenic for p210 and p190 hybrid constructs developed diseases differing in type, latency, and survival. The mechanism whereby the two fusion genes produce CML and ALL, respectively, is unknown.

Neutrophilic-chronic myeloid leukemia (CML-N), also known in the literature as chronic neutrophilic leukemia, is a rare myeloproliferative disorder in which the large majority of circulating myeloid cells consist of mature granulocytes. Clinically, the disease is much more benign than ‘classical’ CML: in CML-N patients the total white blood cell (WBC) count is lower, anemia is less severe, splenomegaly less prominent, and blast transformation occurs much later, if any. Here we report that three Ph+ patients with clinical findings of CML-N present a rare type of BCR/ABL rearrangement with the breakpoint on chromosome 22 between exon c3 and exon c4 of the BCR gene (BCR exons 19 and 20). This breakpoint, located distally to the so-called ‘M-breakpoint’ of CML, predicts a 230-kD fusion protein and was previously identified in two different patients who, in retrospect, also may have had a mild form of a myeloproliferative disorder. Our findings provide a molecular approach for the identification of CML-N.

PATIENTS AND METHODS

Patient 1. C.V., female, born in 1928, was found to have anemia and leukocytosis at the age of 65. Hemoglobin (Hb) level was 8.8 g/dL; WBC count 58.7 \( \times 10^9/L \), neutrophils 87%, lymphocytes 7%, monocytes 5%, metamyelocytes 1%, no myelocyte, platelet count 160 \( \times 10^9/L \). The spleen was not enlarged. Serum LDH was 760 IU/L (normal values [n.v.] 250 to 450). Bone marrow (BM) showed increased cellularity with a myeloid/erythroid ratio of 25/1; cytogenetics showed t(9;22) in 14/14 metaphases. Only supportive treatment was given, and the hematologic condition remained stable for the next 3 years. The patient died from an unrelated cause (acute myocardial infarction) in November 1995.

Patient 2. C.S., male, born in 1944, was first seen in 1985 for the evaluation of asymptomatic leukocytosis (Hb level 15.8 g/dL; WBC count 43 \( \times 10^9/L \), neutrophils 88%, eosinophils 1%, lymphocytes 7%, monocytes 2%, metamyelocytes 1%; platelet count 191 \( \times 10^9/L \), which had been discovered incidentally. The spleen was not enlarged. Neutrophil alkaline phosphatase (NAP) score was 145. BM showed myeloid hyperplasia without leftward shift; no mitoses were available for cytogenetic analysis. No treatment was planned, and blood counts remained stable for the next 3 years. In 1988 the WBC count was 43.7 \( \times 10^9/L \), the spleen was palpable 4 cm from the costal border; cytogenetics was repeated, and showed 9/36 (25%) Ph+ mitoses. Treatment with low-dose \( \alpha \)-interferon (IFN-\( \alpha \)) (3 MU/d) caused reduction of spleen size to normal and decrease of WBC count (26 \( \times 10^9/L \). Because of poor compliance, IFN was discontinued in 1991 and intermittent low-dose hydroxyurea was started. Since then, WBC counts have ranged from 20 to 30 \( \times 10^9/L \), spleen 2 to 4 cm; Hb levels and platelet counts were always normal. The patient’s clinical condition was excellent at the last follow-up in May 1996, 11 years after first presentation.

Patient 3. D.A., female, born in 1966, was first seen in 1988...
for altered blood counts on routine testing (Hb level 12.7 g/dL; WBC count 45.5 x 10^9/L, neutrophils 74%, eosinophils 4%, lymphocytes 19%, monocytes 3%, absence of circulating immature myeloid cells; platelet count 1,240 x 10^9/L). The spleen was not enlarged. BM showed excellent cellularity with a myeloid/erythroid ratio of 18:1, without leftward shift. A cytogenetic analysis showed the presence of the Ph chromosome. Small doses of hydroxyurea were administered for 7 months, causing blood count normalization; then no treatment was given for the next 3 years. In 1992 the spleen was palpable at 3 cm from the costal border with WBC count 21 x 10^9/L and platelet count 700 x 10^9/L. A treatment with low-dose IFN-α (3 MU/three times a week) caused hematologic remission. Cytogenetic analysis was repeated, and showed 60% Ph+ mitoses. At 8 years from diagnosis, the patient's clinical condition is excellent.

Cytogenetics. Cytogenetic analysis was performed on BM cells cultured in vitro for 24 hours; a G-banding technique was used to identify chromosomes.

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the hybrid gene. The method used to detect the various BCR/ABL hybrid mRNAs was based on the RT-PCR. Total RNA was extracted from BM cells by the guanidinium thiocyanate and phenol/chloroform method. In each RT-PCR analysis, 1 μg total RNA was incubated for 60 minutes at 37°C in a 40-μL reaction mixture containing 10 μmol/L Tris HCl (pH 8.3), 5 μmol/L MgCl₂, 50 μmol/L KCl, 1 μmol/L of each deoxynucleobovlute, 40 U of RNASin (Promega, Madison, WI), 2.5 μmol/L of antisense ABL primer (oligo A, see below), and 100 U of MoMLV RT (Bethesda Research Laboratories [BRL], Bethesda, MD). A 20-μL aliquot of this solution was amplified in 100-μL final volume of a PCR mixture containing 10 μmol/L Tris HCl (pH 8.3), 2 μmol/L MgCl₂, 50 μmol/L KCl, 0.2 μmol/L of each deoxynucleobovlute, 2.5 U of Taq polymerase, and 0.5 μmol/L of each specific primer (oligo A, B, C, see below). As control of both RNA integrity and PCR reaction, ABL sequences were amplified in the remaining 20-μL aliquot of RT reaction mixture. To detect the e1/a2 type of BCR/ABL transcript encoding the p190 chimeric protein, an additional amplification step with the nested primers of the first-step amplified products was used according to a recently described procedure.

The amplification procedure, performed on a temperature controller (Thermal Cycler 9600; Perkin Elmer Corp, Norwalk, CT) was as follows: after an initial denaturation step at 95°C for 5 minutes, 40 cycles were performed at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, respectively. Ten microliters of the various PCR reactions were run on agarose gel for UV analysis.

Oligonucleotides. The following primers were used for RT-PCR amplification of BCR/ABL hybrid transcripts or for hybridization with PCR products (see also Fig 1): A: exon a2 (ABL) antisense primer 5'TGTGATTATAGCCTAAGACCCGGAG3'; B: exon b1 (BCR) sense primer 5'GAAGAAGTGTTTCAGAAGGCTCTCC3'; C: exon c3 (BCR) sense primer 5'GAAGAAGTGTTTCAGAAGGCTCTCC3'; D: c3/a2 junctional probe 5'GCTGAAGGGCTTGTAGCTGCGAG3'.

Hybridization of the amplified product. The identity of the BCR/ABL junction was confirmed by hybridization of the amplified product with an oligonucleotide probe corresponding to the c3/a2 junction (oligo D, see above). Amplified products were run on agarose gel for UV analysis. Hybridization was performed on UV-exposed nitrocellulose filters, which were hybridized with 10 μL of a solution containing 50% formamide, 5x SSPE (1x SSPE is 0.15 mol/L NaCl, 0.01 mol/L NaH2PO4, 1 mmol/L EDTA), 1% sodium dodecyl sulfate (SDS) at the temperature calculated on the basis of nucleotide sequences.

Southern analysis. The M-BCR locus was also analyzed by Southern analysis using BglII, HindIII, and BamHI restriction en-

zyme digestion, and a 0.8-Kb HindIII BamHI genomic probe spanning the b2 and b3 exons, with the standard blotting and hybridization procedures described elsewhere.

RESULTS

The three patients fulfill the clinical diagnostic criteria for CML-N: (1) moderate nonprogressive chronic neutrophilic leucocytosis; (2) rare circulating immature myeloid cells without a myelocyte peak; (3) excess of mature myeloid cells in BM; (4) normal or raised NAP score; (5) absent or minimal splenomegaly; and (6) absence of an underlying disease capable of provoking a reactive leukemoid status.

Clinical findings are summarized in Table 1, which also includes the two patients reported in 1990.

Southern DNA analysis of the M-BCR region failed to show rearranged bands in all digests tested, and this result was confirmed by RT-PCR analysis using the primers specific to this region (oligonucleotides A and B described in Patients and Methods). Also, a RT-PCR analysis with primers specific for BCR/ABL transcript with the e1/a2 type of junction, which involves the m-BCR, did not give any visible amplified band in any of our patients (data not shown).

Finally, we used RT-PCR to search for the hybrid BCR/ABL gene that bears the much rarer c3/a2 junction (see Fig 1). Using a sense primer matching a sequence of exon c3 of the BCR gene (oligonucleotide C, see Patients and Methods and Fig 1), and the antisense primer matching a sequence of exon 2 of theABL gene (oligonucleotide A, see Patients and Methods), we amplified in all three patients a 244-bp band which corresponds to the c3/a2 junction (Fig 2A).

The presence of this type of junction was confirmed by hybridization with an oligonucleotide probe spanning the c3/a2 junction (oligonucleotide D, see Patients and Methods) (Fig 2B).

DISCUSSION

About 100 cases of CML-N have been reported since this condition was first described and it was recognized as a specific nosologic entity. It is possible that not all published cases of CML-N were in fact leukemic diseases, because evidence of clonality was provided only in a few instances. Conversely, a number of cases may have been misdiagnosed as CML, since it has been claimed that the presence of the Ph chromosome could rule out the diagnosis of CML-N. We do not see any reason for such a restriction: the presence of a clonality marker supports rather than excludes the diagnosis, and our molecular findings offer a good explanation for the different phenotype.

The hybrid c3/a2. Several types of junction may occur in hematopoietic malignancies carrying the t(9;22) chromosomal translocation (Table 2). However, with very few exceptions, the breakpoint within ABL always falls upstream exon 2, whereas various breakpoints are described within BCR (Fig 1), each of which giving rise to hybrid genes of specific structure and to hybrid transcripts of specific size. The c3/a2 junction, which is not revealed by probes exploring the M-BCR region, was originally found during a large molecul-
Fig 1. Scheme (not in scale) of the breakpoints (vertical arrows) so far described in the BCR gene of patients affected by Ph+ leukemias. The M-BCR and μ-BCR regions are enlarged. The 23 exons are represented as solid boxes and numbered sequentially. The μ-BCR exons are named according to Lifshitz et al. The exons located at the 5′ of breakpoints are retained in the hybrid gene on chromosome 22. In the case of breakpoints in the μ-BCR region, almost all the BCR gene sequences are retained in the corresponding hybrid gene, which encodes for a putative p230 protein. Horizontal arrows indicate the position of the primers listed in Patients and Methods.

Because we cannot exclude that a phenotype of mild myeloproliferative disorder may be caused by a molecular lesion other than BCR/ABL with the c3a2 junction, we now suggest to restrict the term ‘CML-N’ for those patients with this specific molecular lesion (so as to indicate their close relationship with CML), while the more general term ‘chronic neutrophilic leukemia’ could be maintained for those in which this marker may not be detected.

The c3/a2 junction yields a transcript that contains an additional BCR sequence of 540 bp and encodes for a chimeric protein carrying 180 additional amino acids (predicted p230) as compared with that found in typical CML. The exact role of the p230 in the pathogenesis of CML-N is still unknown, but the following observations seem relevant. (1) In patients with CML in chronic phase, we recently found, besides the p210 encoding mRNA, significant amounts of p190 mRNA (probably due to alternative splicing of the immature RNA transcribed from this gene). Although it is still unclear whether the smaller fusion protein has any relevance for the course of the disease, we stress that no trace of p190 was detected in our patients with the c3/a2 type of mRNA. (2) p210 has a lower autophosphorylation activity than p190. It would be interesting to measure autophosphorylation rate for p230; however, we have not been able to isolate p230, probably because it is produced at a very low rate in our samples which contain mainly mature cells. (3) While p190 and p210 lack the entire GTPase-activating protein (GAP) domain present at the carboxy-terminus of BCR, the predicted p230 retains the N-terminal conserved region of the GAP domain, which is essential for GTPase activity of the BCR protein. Thus, the BCR/ABL fusion protein retains a function that may

<p>| Table 1. Clinical Characteristics of Five Patients With the c3a2-type of BCR/ABL Fusion Gene |
|-----------------------------------------------|-------------|-----------|-------------|-------------|-----------|</p>
<table>
<thead>
<tr>
<th>Sex</th>
<th>ML*</th>
<th>GS*</th>
<th>CV</th>
<th>CS</th>
<th>DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>76</td>
<td>62</td>
<td>65</td>
<td>51</td>
<td>30</td>
</tr>
<tr>
<td>Spleen</td>
<td>4 cm</td>
<td>3 cm</td>
<td>4 cm</td>
<td>4 cm</td>
<td>3 cm</td>
</tr>
<tr>
<td>WBC × 10⁹/L</td>
<td>28</td>
<td>16</td>
<td>58</td>
<td>48</td>
<td>45</td>
</tr>
<tr>
<td>Platelets × 10⁹/L</td>
<td>1,020</td>
<td>870</td>
<td>160</td>
<td>228</td>
<td>1,240</td>
</tr>
<tr>
<td>t(9;22)</td>
<td>+</td>
<td>+</td>
<td>100%</td>
<td>25%</td>
<td>60%</td>
</tr>
<tr>
<td>Survival (yr)</td>
<td>&gt;5*</td>
<td>&gt;3*</td>
<td>3</td>
<td>&gt;11</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Cause of death</td>
<td>MI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MI, myocardial infarction.

* Patients studied in 1990, then lost to follow-up.

† Highest values observed during the course of the disease.
be important for the regulation of cell proliferation and maturation.

BCR/ABL fusion genes and disease phenotypes. Our findings may help to identify a correlation between the structure of the hybrid BCR/ABL gene and the hematologic phenotype: specifically, the following qualitative and quantitative differences can be noted. (1) A breakpoint occurring in the m-BCR region gives rise to a hybrid BCR/ABL gene encoding the smallest protein p190, and it is regularly associated with the ALL phenotype. (2) A breakpoint in the M-BCR produces a hybrid gene encoding the medium sized p210; it may also be found in ALL, and is typical of CML, a chronic myeloproliferative disorder with a high tendency to acute blastic transformation. (3) A breakpoint in the \( p \)-BCR produces a hybrid gene encoding the largest p230, in which almost the entire BCR sequences are conserved; it is associated with a clinical picture of CML-N, a myeloproliferative disorder characterized by a mild hematologic phenotype and a low tendency to acute blastic transformation (Table 2). The persistence of a significant pool of Ph\(^+\) progenitors after years of disease course even in absence of treatment (as we have observed in two of our three patients) is another sign of a limited proliferative advantage of the abnormal clone. In quantitative terms, the transcription of the c3/a2 hybrid gene may be low, because of the presence of at least three transcription silencer elements within the intronic sequences at the 3\(^\prime\) of exon b3 of the M-BCR region.\(^{27}\) These elements are lost in the p190-encoding BCR/ABL gene of ALL, and are partially retained in p210 of ALL and CML, and are fully retained in the p230-encoding gene of CML-N. On the basis of our findings, we conclude that: (1) CML-N is a distinct clinical entity with a specific molecular basis, similar to but distinct from that of typical CML; (2) the occurrence of a c3/a2 junction in Ph\(^+\) CML patients may have an important prognostic relevance; and (3) the detection of the c3/a2 BCR/ABL hybrid gene may help to

Table 2. The Structure of the BCR/ABL Fusion Gene Is a Determinant of the Hematologic Phenotype

<table>
<thead>
<tr>
<th>BCR/ABL Junction</th>
<th>BCR Region Involved*</th>
<th>BCR Exons Retained</th>
<th>Protein</th>
<th>BCR aa</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>e1 a2</td>
<td>m</td>
<td>1</td>
<td>p190</td>
<td>436</td>
<td>ALL</td>
<td>3</td>
</tr>
<tr>
<td>e1 a3t</td>
<td>m</td>
<td>1</td>
<td>p190</td>
<td>436</td>
<td>ALL</td>
<td>19</td>
</tr>
<tr>
<td>b2 a2</td>
<td>M</td>
<td>13</td>
<td>p210</td>
<td>902</td>
<td>CML, ALL</td>
<td>26</td>
</tr>
<tr>
<td>b2 a3t</td>
<td>M</td>
<td>13</td>
<td>p210</td>
<td>902</td>
<td>CML, ALL</td>
<td>19, 20</td>
</tr>
<tr>
<td>b3 a2</td>
<td>M</td>
<td>14</td>
<td>p210</td>
<td>927</td>
<td>CML, ALL</td>
<td>26</td>
</tr>
<tr>
<td>c3 a2t</td>
<td>( \mu )</td>
<td>19</td>
<td>p230†</td>
<td>1107</td>
<td>CML-N</td>
<td>7, this report</td>
</tr>
</tbody>
</table>

Abbreviation: aa, amino acids.
* See Fig 1.
† Rare breakpoints.
‡ Calculated from cDNA.
differentiate a clonal myeloproliferative disorder from other chronic (possibly reactive) leucocytes.

**NOTE ADDED IN PROOF**

Two additional patients with the c3/a2 type of junction have been described in the recent literature. Wada et al (Cancer Res 55:3192, 1995) reported the case of a patient with the Ph chromosome in combination with isochromosome 17, who carried two BCR/ABL hybrid transcripts (c3/a2 and c2/a2). A cell line was derived from this patient, who developed a blast crisis after 2 years. Yamagata et al (Br J Haematol 94:370, 1996) reported on a patient whom they considered as having Essential Thrombocythemia, with a BCR/ABL hybrid gene carrying a typical c3/a2 type of junction.

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**REFERENCES**

Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction) [see comments]

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