Clonal eosinophils are a morphologic hallmark of ETV6/ABL1 positive acute myeloid leukemia

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Background and Objectives. The ETV6 gene undergoes rearrangements with tyrosine kinases in hematologic malignancies and solid tumors. ETV6/ABL1 chimeric proteins have been detected both in lymphoid and myeloid disorders. Our objective was to study two new cases of ETV6/ABL1-positive acute myeloid leukemia (AML) and to focus on bone marrow morphology and on molecular cytogenetics of eosinophilic cells.

Design and Methods. Fluorescence in situ hybridization (FISH) was performed in two AML cases with different translocations, i.e. t(8;12)(p21;p13) and t(9;12) (q34; p13). We used probes for the short arm of chromosome 12, for ABL1 and BCR, for centromeric regions, and for whole chromosome arms. Polymerase chain reaction (PCR) was carried out by applying primers selected for the ETV6 gene.

Results. In both cases, bone marrow morphology was characterized by trilineage dysplasia and increased abnormal eosinophils. FISH showed the 5′ETV6 translocated to chromosome 11 in patient #1, and to chromosome 9 in patient #2. A 3′ PCR identified chimeric products resulting from fusion between ETV6 exon 4 or exon 5, and ABL1 exon 2. Accordingly, an ETV6/ABL1 fusion signal was detected on der(8) in patient #1, and on der(9) in patient #2. Using interphase FISH abnormal bone marrow eosinophils were proved to belong to the neoplastic clone, carrying the ETV6 rearrangement.

Interpretation and Conclusions. Our findings provide new information on the heterogeneity of conventional cytogenetics in ETV6/ABL1 positive leukemias, and indicate the putative target cell in this AML is an immature precursor capable of terminally differentiating towards eosinophils.

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leukocytosis (40 × 10^9/L) with the following differential count: neutrophils 23%, lymphocytes 4%, monocytes 4%, eosinophils 42%, basophils 2%, myelocytes 3%, metamyelocytes 16%, and blasts 6%. AML-M2 subtype was diagnosed as the bone marrow showed 64% blasts of small-medium size and a low nuclear/cytoplasmic ratio. Immunophenotyping was positive for CD13 (91%) and CD33 (89%). Complete remission was achieved with the GIMEMA protocol LAM99. The patient underwent haploidentical stem cell transplantation from his mother and, 20 months later, he is still alive and in complete hematologic and cytogenetic remission.

Patient #2
A 48-year old man was referred to the Hematology and Oncology Unit, Bologna Hospital, because of asthenia and weight loss. The peripheral blood count was: Hb 6.5g/dL; platelets 20 × 10^9/L, white cell count 7.8 × 10^9/L (differential: neutrophils 55%, lymphocytes 16%, monocytes 8%, basophils 1%, promyelocytes 1%, myelocytes 9%, metamyelocytes 8%, and blasts 2%). Refractory anemia with excess of blasts (RAEB) was diagnosed in the presence of 15% blasts but one month later the patient developed AML-M1. Treatment with cytosine-arabinoside, idarubicin, and etoposide was started but the patient died of respiratory distress.

Cytogenetics
Short-term cultures from bone marrow samples were performed at diagnosis, in both cases. Metaphases were analyzed after G-banding with Wright stain. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN 1995).

FISH
FISH was done as previously described using a panel of biotin-16-dUTP labeled DNA probes for chromosomes 12p ordered from telomere to centromere: 4H9A-170G6-407G6-543P15-433J6. The ETV6 gene was studied with the following cosmids: t(9;22)(q34;q11) translocation, were probing the t(9;22) and at der(22)(q11) in cells carrying the t(9;22)(q34;q11) translocation, were applied in a double color experiment. The 3′ABL1 was studied separately with cosmid ABL18 (kindly provided by Dr. Birg, University of Marseille, France). Whole chromosome paints (Oncor, Appligene, Gaithersburg, MD, USA) and centromeric probes for chromosomes 8, 9, and 12, (D8Z2, pHUR98, and pSb12H8, respectively), labeled with digoxigenin or biotin, were also used in patient #1. Six abnormal metaphases were studied by FISH and G-banding for each probe. FISH data were collected on an Olympus fluorescence microscope (Provis, Olympus) equipped with a cooled CCD camera Sensys (Photometrics, Tucson, AZ, USA) run by PathVysion software (Vysis, Stuttgart, Germany). In patient #1, a mixture of cosmid 179A6 and 148B6 was tested by interphase FISH on nuclei and on cytospins from mononuclear intact bone marrow cells. Two hundred nuclei/cells were analyzed in each experiment. Before FISH evaluation only selected fields with homogeneous hybridization were evaluated on cytospins.

PCR
Patient #1. Total RNA was isolated by the use of Trizol Reagent. cDNA was obtained by retrotranscription of 1 µg of total RNA with MMLV RNase H (Life Technologies, Invitrogen, Paisley, United Kingdom) and a modified oligo(dT) primer as described in the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). Briefly, after first strand synthesis there is an elongation step that produces a SMART 5′ priming site. 5′ and 3′ RACE were done by using the anchor primer (5′ CTA ATA CGA TTC ACT ATA AGG G 3′), linked both to the oligo(dT) and the SMART priming site, and ETV6-ex8 (5′ CCC GCT GAC GTG GAC GAC TGT GTC TT 3′) or ETV6-ex5 (5′ GTC TCT TCT CCC CCT CCG CCT GAA G 3′) primers, respectively. The reaction was performed using the Advantage 2 Polymerase Mix: 2 min at 94°C, 30 cycles of 30 sec at 94°C, 3 min at 68°C, followed by 7 min at 68°C.

Nested primers to increase the PCR specificity were: ETV6-ex8i (5′ TGG TTC TGT CAT CAG TCA T 3′), ETV6-Ex5i (5′ GAA GAG CAC GCC ATG CCC ATT T 3′) and nested-Anchor (5′ AAG CAC GTG TAA CAA CGC AGC AGA GT 3′). The PCR products were purified, ligated into the PCR 2.1 vector (TOPO TA cloning kit, Invitrogen) and sequenced.

The ETV6/ABL1 chimeric transcript was amplified with ETV6-ex4 (5′ GCC GGA GGT CAT ACT GCA TCA G 3′) and ABL-ex3 (5′ GCA TTT TGT TGG GCT TCA CAC CAT TCC 3′) primers.

Patient #2. Total RNA was extracted from bone marrow using Chomczynsky and Sacchi’s method. The amount of extracted RNA was determined by optical density at 260 nm; integrity was checked by loading 1 µg on 2% agarose gel. cDNA was prepared from 1 µg of total RNA using random examiners (5 µM) and the M.MLV reverse kit (Life Technologies); 5 µL of the reverse transcriptase reaction...
was used for PCR in a total volume of 50 µL using 10× Boehringer buffer (Roche Diagnostics, Basel, Switzerland), 200 µM of each dNTP, 20 pmol of each primer and 1 U of Taq DNA polymerase. ETV6/ABL chimeric transcript was amplified in two ways, first with ETV6-ex4 (5′ TGC TGA CCA AAG AGG ACT TTC 3′) and ABL-ex3 (5′ GTT TGG GCT TCA CAC CAT TCC 3′); and second with ETV6- ex5 (5′ TGC ACC CTC TGA TCC TGA AC 3′) and the ABL-ex3 primers. PCR conditions were: 5 min at 95°C, 35 cycles of 30 sec at 94°C, 1 min at 65°C and 1 min at 72°C. PCR products were analyzed on a 2% agarose gel containing ethidium bromide. PCR purified products were sequenced on a ABI PRISM 377 sequencer (PE Applied Biosystem, Foster City, CA, USA) using the same specific primers as above and the Big Dye Terminator Sequencing Kit (PE Applied Biosystem).

Results
Cytogenetics
Patient #1. All the cells [15/15] showed an abnormal karyotype with the t(8;12)(p21;p13) translocation as the only chromosomal abnormality at diagnosis. All metaphases [30/30] were normal after induction therapy. Patient #2. The karyotype at diagnosis was: 46,XY,t(9;12)(q34;p13) [18/20]/51,XY,+8,+9,t(9;12)(q34;p13),+12,+14,+17 [2/20].

FISH
Patient #1. In the ETV6 gene, the 12p13 breakpoint, was narrowed between the 5th and 8th exon since cosmid 163C7 was translocated to the der(8) and cosmid 148B6 was kept on der(12). The probe for the BCR/ABL1 fusion gene (BCR in red and ABL1 in green) gave two red signals on both normal 22, two green signals on both apparently normal 9 and a third green signal on der(8)t(8;12). FISH with cosmids 163E7 (red) and cosmid ABL18 (green) showed a red signal on normal 12, a green signal on normal 9 and a fusion signal on der(8)t(8;12) (Figure 1). Paints 8 and 12 confirmed the reciprocal exchange of material between these two chromosomes. Paint 9 hybridized with both 9 chromosomes and with a small DNA segment inserted between chromosomes 8 and 12 on der(8)t(8;12).

Patient #2. The probe for BCR-ABL1 fusion gene gave two red signals on both 22 and two green signals on both 9 as expected from samples not carrying the BCR/ABL1 rearrangement. A double color experiment with cosmids 179A6 and 148B6 (red) and cosmid ABL18 (green) resulted in one green signal on normal 9, two red signals on normal 12 and on der(12), and a fusion signal on der(9).

Morphology and FISH
Morphologic bone marrow findings showed similarities, including myeloid dysplastic features and hypergranular neutrophilic elements, in these two cases. Abnormal mature and immature eosinophils with coarse granules were noted. Some elements with pronounced basophilic granules were also observed in both cases (Figures 2a, 2b).

Cosmid 179A6 plus cosmid 148B6 gave three signals of fluorescence in 90% of interphase nuclei. On cytospins, 50% of 200 eosinophils (Figure 2c) and 50% of 200 other mononuclear hematopoietic cells
presented three fluorescence signals. In addition, 4 of the 20 mature eosinophils which were detected on bone marrow smears showed two fluorescence signals and the other 16 showed three signals, as expected in cells with ETV6 rearrangement.

PCR identification of the ETV6-ABL fusion

Patient #1. No chimeric transcript was identified with the 5' RACE. 3' RACE detected several abnormally spliced ETV6 products and a transcript with ETV6 exon 5 fused with intron 1a of the Abl gene. As the breakpoint at the 5' splicing site of intron 5 destroyed the intron, ETV6 exon 5 could not splice out the intronic sequences. Using RT-PCR, we confirmed the ETV6/ABL1 fusion with ETV6 exon 4 and ABL exon 3 primers. A chimeric transcript with the fusion site located between ETV6 exon 4 and ABL1 exon 2 was detected. No evidence was obtained for the involvement of other genes in this translocation.

Patient #2. Using RT-PCR and sequencing of purified PCR products, we confirmed the involvement of only ETV6 and ABL1 genes in the t(9;12)(q43;p13). The first five exons of ETV6 and exon 2 of ABL1 provided the fusion transcript.

Discussion

The activation of ABL1 by its fusion with ETV6 was first described by Papadopoulos et al. in a pediatric case of common ALL-L1. Six more cases of ETV6/ABL1-positive leukemias have been reported: four Philadelphia-negative CML, one AML, and one T-ALL. ETV6/ABL1 recombination may escape conventional cytogenetic analysis as only one of the five cases reporting karyotype showed the expected reciprocal translocation involving 9q34/ABL1 and 12p13/ETV6 (Table 1). In the other four cases cytogenetic results were heterogeneous with three showing different structural rearrangements and one a normal karyotype. Molecular breakpoints cluster between ABL1 ex2 and ETV6 ex4 or ex5. In three patients both fusion transcripts, i.e. ETV6ex4/ABL1ex2 and ETV6ex5/ABL1ex2, were present, suggesting an alternative splicing mechanism at transcriptional level. Both these chimeric proteins contain the helix-loop-helix (HLH) domain of ETV6 and the tyrosine kinase domain of ABL1. Therefore, the HLH domain of ETV6, like BCR in the BCR/ABL1 rearrangements, is required for tyrosine kinase activation and cytoskeletal localization of ABL1. A reciprocal fusion transcript ABL1/ETV6 has never been found. Data on the hematologic features of ETV6/ABL1 positive cases (Table 1) emphasize the presence of an abnormal eosinophil proliferation as a morphologic hallmark in all. Malig-
lymphoid disorders has been studied in a subgroup of B-cell ALL with a reciprocal t(5;14) disrupting the promoter of the IL3 gene, on 5q31, and the switch region of the IgH locus, on 14q32. The high eosinophil blood count has been defined as a reactive phenomenon caused by neoplastic B-cells releasing stimulating cytokines. In two AML subgroups with established genetic lesions, i.e. inv(16)(p13q22) and t(8;21) (q22;q22), eosinophils have been shown to bear the typical genetic change.19,20 The nature of eosinophils in ETV6/ABL1 positive ALL remains to be determined. The question arises as to whether eosinophils belong to the neoplastic clone or whether they are a cellular reaction to blastic cell products. In the first AML case of this study, FISH on intact bone marrow cells clearly showed ETV6 was disrupted in eosinophils as in the other mononuclear hematopoietic cells (Figure 2c). Thus the putative target cell in ETV6/ABL1-positive AML is an immature precursor which is capable of terminally differentiating towards eosinophils. Several reports provide evidence that ETV6 plays an active role in the commitment of hematopoietic myeloid precursors to eosinophilic differentiation.

Eosinophilia is common feature of myelodysplastic/myeloproliferative malignancies associated with other ETV6 translocations, i.e., t(5;12)(q33;p13),21 t(9;12)(q22;p12),21 and t(10;12)(q21;p13).21 Eosinophilic differentiation is typical in a M3 cell line bearing ETV6/ARG fusion from a t(1;12)(q25;p13) translocation in addition to the t(15;17).3 Moreover, in avian hematopoietic cells, specific members of the Ets family, to which the ETV6 gene belongs, cooperate with GATA 1 and C/EBPα in determination of eosinophilic differentiation.22 Molecular studies showed that the ETV6/ABL1 recombination is rare in hematologic malignancies as it was not found in 186 adults and 30 children with ALL, or in 67 cases of chronic myeloproliferative disorders (33 chronic myelomonocytic leukemias, 22 chronic myeloproliferative disorders, and 12 Ph-negative CMLs).24 Our two cases emerged from a study designed to use molecular cytogenetics to investigate AML breakpoints. Undoubtedly, molecular diagnosis of rare lesions, which are cryptic to conventional cytogenetic analysis, is intriguing but very expensive in large scale multicenter studies. This work shows that AML patients with eosinophilia, but without inv(16) and t(8;21), are good candidates in whom to search for this rare ETV6/ABL1 fusion gene by FISH and/or PCR.

Contributions and Acknowledgments

RLS was the principal investigator in FISH experiments and in writing the paper. MT, EO, EB performed the molecular studies. NT performed the cytogenetic investigations. BC performed FISH on interphase nuclei and on intact bone marrow cells. GF critically reviewed the bone marrow smears. PGP supervised the molecular studies. MFM was involved in the patients’ clinical management. CM was responsible for the conception of the study and the paper.

We acknowledge Prof. GL Castoldi’s expert analysis of the bone marrow sample from patient #1. The authors wish to thank Dr. Geraldine Anne Boyd for assistance in the preparation of the manuscript.

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Table 1. Clinical, hematologic, cytogenetic, and molecular findings in reported ETV6/ABL1 positive leukemias.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Eosinophilia BM and/or PB</th>
<th>Karyotype</th>
<th>Fusion transcripts</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/F</td>
<td>ALL common</td>
<td>n.r.</td>
<td>n.r.</td>
<td>ETV6ex4/ABLex2</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>81/M</td>
<td>AML</td>
<td>n.r.</td>
<td>*t(9;12;14)(q34;p13;q22)</td>
<td>ETV6ex5/ABL1ex2</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>32/M</td>
<td>Ph-CML</td>
<td>+</td>
<td>46,XYt(12;14)(q11;p13)</td>
<td>ETV6ex6/ABL1ex2</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>49/n.r.</td>
<td>aCML</td>
<td>+</td>
<td>n.r.</td>
<td>ETV6ex5/ABL1ex2</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>59/M</td>
<td>Ph-CML</td>
<td>+</td>
<td>[46,XYdel(6)(p13q22)][9;12]{q34p13}</td>
<td>ETV6ex4/ABL1ex2</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>4/M</td>
<td>T-ALL</td>
<td>+</td>
<td>47,XXc,del(8)(p12q21)47,XXc</td>
<td>ETV6ex4/ABL1ex2</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>53/M</td>
<td>Ph-CML</td>
<td>+</td>
<td>46,XY</td>
<td>ETV6ex4/ABL1ex2</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
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<td>46,XYt(8;12)(p12;p13)</td>
<td>ETV6ex4/ABL1ex2</td>
<td>p.c.</td>
</tr>
<tr>
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<td>48/M</td>
<td>RAEB</td>
<td>+</td>
<td>46,XYt(9;12)(q34;p13)</td>
<td>ETV6ex5/ABL1ex2</td>
<td>p.c.</td>
</tr>
</tbody>
</table>

f, female; M, male; BM, bone marrow; PB, peripheral blood; Ref, reference; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; aCML, atypical CML; RAEB, refractory anemia with excess of blasts; RT-PCR, reverse transcriptase polymerase chain reaction; * partial description of the complex karyotype; + whole chromosome paints 9 and 12 do not evidence involvement of these chromosomes; n.r., not reported; + presence of eosinophilia; ex, exon; p.c., present case.
Disclosures
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

References