ETV6-NCOA2: A Novel Fusion Gene in Acute Leukemia Associated with Coexpression of T-Lymphoid and Myeloid Markers and Frequent NOTCH1 Mutations

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Abstract

Purpose: The ETV6 gene has been reported to be fused to a multitude of partner genes in various hematologic malignancies with 12p13 aberrations. Cytogenetic analysis of six cases of childhood acute lymphoblastic leukemia revealed a novel recurrent t(8;12)(q13;p13), suggesting involvement of ETV6.

Experimental Design: Fluorescence in situ hybridization was used to confirm the involvement of ETV6 in the t(8;12)(q13;p13) and reverse transcription-PCR was used to identify the ETV6 partner gene. Detailed immunologic characterization was done, and owing to their lineage promiscuity, the leukemic blast cells were analyzed for NOTCH1 mutations.

Results: We have identified a novel recurrent t(8;12)(q13;p13), which results in a fusion between the transcriptional repressor ETV6 (TEL) and the transcriptional coactivator NCOA2 (TIF2) in six cases of childhood leukemia expressing both T-lymphoid and myeloid antigens. The ETV6-NCOA2 transcript encodes a chimeric protein that consists of the pointed protein interaction motif of ETV6 that is fused to the COOH terminus of NCOA2, including the cyclic AMP – responsive element binding protein – binding protein (CBP) interaction and the AD2 activation domains. The absence of the reciprocal NCOA2-ETV6 transcript in one of the cases suggests that the ETV6-NCOA2 chimeric protein and not the reciprocal NCOA2-ETV6 is responsible for leukemogenesis. In addition, ETV6-NCOA2 leukemia shows a high frequency of heterozygous activating NOTCH1 mutations, which disrupt the heterodimerization or the PEST domains.

Conclusions: The ETV6-NCOA2 fusion may define a novel subgroup of acute leukemia with T-lymphoid and myeloid features, which is associated with a high prevalence of NOTCH1 mutations.

Acute leukemia is subdivided into myeloid or lymphoid according to cytology and immunophenotyping. However, using these criteria, a minor proportion of acute leukemia is difficult to unambiguously assign because the blast populations do not allow classification in either a pure myeloid or lymphoid category (1). Such cases are designated as biphenotypic acute leukemia (BAL) or acute leukemia of ambiguous lineage.

In an effort to establish objective diagnostic criteria for defining such leukemias, several different immunologic classification and scoring systems, which are mostly based on the number and specificity of the lymphoid and myeloid markers expressed by the blast cells, have been introduced (1–4). Based on these categorizations, the prevalence of BAL has been determined to range from 2% to 5% in adult and childhood acute leukemia (5–7). Coexpression of myeloid and B-lymphoid antigens occurs in approximately 65% to 70%, whereas T-lymphoid and myeloid antigens account for 25% of all BAL and thus for ≤1% of acute leukemia (6).

As a basic principle, the classification of hematopoietic neoplasms should attempt to incorporate immunophenotypic, biologic, genetic, and clinical features to define specific disease...
entities. However, due to the lack of specific genetic features, BAL is defined by the application of arbitrary criteria (3). Genetic lesions such as t(9;22)(q34;q11)/BCR-ABL1, t(4;11)(q21;q23)/MLL-AF4/ AFF1, and other 11q23 abnormalities seem to commonly concur with BAL of the myeloid/B-lymphoid type, whereas the specific genetic features of myeloid/T-lymphoid BAL remain widely elusive (6–10). Thus far, two NUP98 fusions (i.e., NUP98-RAPIGDS1 and NUP98-ADD3) seem to be associated with a subset of adult T-cell acute lymphoblastic leukemia (T-ALL) with variable expression of mature T-cell and myeloid markers (11–13). Furthermore, the PICALM-MLLT10 (CALM-AF10) fusion, which is mainly associated with immature T-ALL, has also been found in leukemia with a multilineage phenotype coexpressing T-cell and myeloid antigens (14). Recently, a specific subset of acute myeloid leukemia (AML) within the population of myeloid malignancies with coexpression of T-cell genes that is characterized by silencing of the CEBPA gene and recurring mutations in NOTCH1 has been described (15).

Activating NOTCH1 mutations that disrupt the heterodimerization and/or the PEST domains are present in >50% of childhood T-ALL but only in rare cases of AML, particularly in the context of lineage switch leukemia (15–18). In contrast to the well-established role of NOTCH1 in T-ALL pathogenesis (19), controversial results have been obtained with regard to NOTCH1 signaling in myeloid development. Enforced expression of constitutively activated NOTCH1 or treatment with NOTCH ligands results in the inhibition of granulocyte differentiation and an increase of immature precursors, suggesting a potential role of NOTCH signaling in the development of myeloid leukemia (20–23). However, NOTCH1 signaling has also been shown to irreversibly reduce the self-renewal capacity of multipotent progenitors and to induce multilineage myeloid differentiation (24).

We have identified a novel genetic subtype of acute leukemia with a recurrent t(8;12)(q13;p13), which fuses the ETV6 (TEL) and NCOA2 (TIF2) genes, and a high prevalence of NOTCH1 mutations. The combination of these two genetic lesions seems to be specifically associated with acute leukemia with a mixed T-lymphoid and myeloid immunophenotype.

**Materials and Methods**

**Patients.** Bone marrow samples from children with newly diagnosed acute leukemia who were enrolled on either International Berlin-Frankfurt-Münster (BFM) or European Organization for Research and Treatment of Cancer (EORTC) protocols were obtained after informed consent of the patients or their legal guardians. Cytogenetic analysis of patients with acute leukemia enrolled in the Austrian AML-BFM 98 (n = 93, collected between 1998 and 2004; successfully karyotyped, n = 87) and the ALL-BFM 2000 (n = 432, including n = 49 T-ALLs, collected between 2000 and 2006; successfully karyotyped, n = 403) studies identified two cases carrying a t(8;12)(q13;p10-13). Subsequently, additional patient samples with this specific translocation were collected from other study centers: two from Germany and one each from France and Israel.

**Conventional and molecular cytogenetics.** Samples were processed according to standard cytogenetic techniques and karyotypes were described according to the International System for Human Cytogenetic Nomenclature (25). ETV6 rearrangements were detected using differentially labeled ETV6 exon-specific cosmids 179A6 (exon 1A), 50F4 (intron 1 and exon 2), 50F4 (exon 2), 163E7 (exons 3-5), 54D5 (exons 5-8), and 148B6 (exon 8; kindly provided by P. Marynen, Center for Human Genetics, University of Leuven, Leuven, Belgium; ref. 26). Fluorescence in situ hybridization (FISH) was done as previously described (27).

FISH patterns were evaluated using an Axioskop fluorescent microscope (Zeiss) equipped with the appropriate filter sets for 4',6-diamidino-2-phenylindole (DAPI), FITC, and Cy3. Images were taken by means of a Plan Neofluar 100×/1.3 oil immersion objective and a charge-coupled device camera (CH250, Photometrix Ltd.) using the IP Labs software (Vysis, Inc.). Using the same software, the images were split in the three color channels, and DAPI images were inverted and then again merged with the FITC and Cy3 images.

**Immunophenotyping.** Immunophenotyping was done on isolated bone marrow cells by means of flow cytometry with a panel of monoclonal antibodies. Immunologic data were determined in the facilities of the respective study centers, and thus, the antibody panels used were slightly different for the individual patient. Nevertheless, the most important markers that allow lineage assignment according to the European Group for the Immunological Classification of Leukemias (EGIL; ref. 2) criteria were analyzed: T-lymphoid markers, CD1a, CD2, cyttoplasmic CD3 (cyCD3) and/or membrane CD3 (mCD3), CD5, CD7, CD4, CD8, and TdT; myeloid markers, MPO, CD13, CD33, CDw65, and CD117; natural killer marker, CD56; and progenitor marker, CD34.

**Reverse transcription–PCR analysis.** Total RNA was isolated from cryopreserved mononuclear cell or from methanol/acetic acid–fixed cells as previously described (28) using the RNeasy Mini kit (Qiagen) and reverse transcribed with 200 units Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 100 pmol random hexamers (GE Healthcare) at 42°C for 1 h. Primer sequences are listed in Table 1. All PCRs were done using Hot Start Taq (Qiagen). Gel images were taken using a Kodak Electrophoresis Documentation and Analysis System 120 and Kodak Digital Science 1 software (Kodak). PCR products were cleaned using the QiAquick PCR Purification kit (Qiagen) and sent to MWG-Biotech for direct sequencing.

**NOTCH1 mutation analysis.** Mutations in the N-terminal and COOH-terminal heterodimerization domains enclosed by exons 26 and 27, respectively, and the PEST domain enclosed by exon 34 of NOTCH1 were identified by direct sequencing of PCR-amplified cDNA. Primer sequences are listed in Table 1. PCRs were done using Hot Start Taq with an initial denaturation step at 95°C for 15 min, followed by 35 cycles (95°C for 30 s, 64°C for 30 s, 72°C for 45 s), and a final extension at 72°C for 7 min.

**Results**

**Patient characteristics.** Clinical characteristics and cytogenetic data of the patients are summarized in Table 2. All patients displayed a t(8;12) with variable breakpoints that were difficult to assign accurately by conventional cytogenetics. In three cases (cases 1, 2, and 4), the t(8;12) was the sole karyotypic abnormality, whereas in two cases (cases 3 and 6) it occurred within complex karyotypes. In four of the patients, the morphology of the blast cells was consistent with ALL, whereas two cases were undifferentiated AML. Taking morphologic and immunologic criteria into consideration, patients were treated with the most appropriate therapy regimen. Irrespective of the treatment protocol, four of the patients (cases 1, 2, 3, and 5) are in complete remission 40 to 84 months after initial diagnosis. Case 4 was initially enrolled in the ALL IC-BFM 2002 study but responded very poorly to prednisone. Thus, the protocol was modified and more anti-AML drugs were administered and complete remission could be achieved in week 6 of therapy. In addition, this patient underwent stem cell transplantation from a matched sibling and is in continuous remission 17 months
after diagnosis. Case 6 was treated according to the AML-BFM 93 protocol, relapsed 16 months after diagnosis, and died of progressive disease.

The patients analyzed were selected based on the availability of cytogenetic data in various study centers, and thus, it is difficult to estimate the overall frequency of the t(8;12)/ETV6-NCOA2 rearrangement. However, deduced from the numbers of patients enrolled in the Austrian AML-BFM 98 and ALL-BFM 2000 studies, in which one positive patient each was found, the t(8;12) is a rare but nevertheless recurrent genetic aberration. This finding agrees with previously reported cytogenetic data in childhood T-ALL (29).

Immunophenotyping. The percentages of blast cells positive for a specific immunologic marker are given in Table 3. According to the EGIL criteria (2), all patients had T-lymphoid scores of ≥2, consistently expressed cyCD3 and CD7, and additional T-cell–specific markers, such as CD2 and CD5, to a variable extent. Except for case 3, the blast cells lacked expression of mCD3. In this leukemia, most of the cells (>50%) were devoid of mCD3 expression and as such are defined as T-II. As a consequence of the myeloid antigen expression pattern, the patients were assigned to different EGIL subtypes (Table 3). True BAL is only considered when the scores are greater than two points for both the lymphoid and the myeloid lineage (2). Nevertheless, the blast cells of each patient expressed at least one myeloid marker, either MPO (EGIL score 2; three cases), CD33 (EGIL score 1; five cases), or CD13 (EGIL score 1; two cases) in combination with T-cell markers (Fig. 1). In case 6, two main subpopulations (i.e., cyCD3+/MPOdim and cyCD3dim/MPObright) were observed (Fig. 1B) and a minor population expressing both markers. Together with the percentage of positive blast cells for the individual markers, this leukemia fulfills the EGIL criteria for BAL. In two of the samples, also expression of CD56, a marker specific for mature natural killer cells, was observed. The immature stage of the majority of the T-lymphoid/myeloid leukemias was supported by the double negativity for CD4/CD8 and, except for one case, the presence of CD34 (Table 3).

Table 2. Clinical characteristics and cytogenetic data of patients with coexpression of T-lymphoid and myeloid antigens

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (y)</th>
<th>Sex</th>
<th>FAB</th>
<th>Cytogenetics</th>
<th>Treatment protocol</th>
<th>Last follow-up from diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4</td>
<td>M</td>
<td>ALL</td>
<td>46,XY,t(8;12)(q13;p13)[15]/46,XY[5]</td>
<td>ALL-BFM 2000</td>
<td>60 mo + CR</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>F</td>
<td>AML-M1</td>
<td>46,XX,del(5)[q23q32],[r(7),t(8;12)[q12;p13],der(16)[t(1;16)[7;7q]][11]/46,XX[12]</td>
<td>ALL-BFM 98</td>
<td>44 mo + CR</td>
</tr>
<tr>
<td>3</td>
<td>14.5</td>
<td>F</td>
<td>ALL</td>
<td>46,XY,t(8;12)[q12;p12][17]</td>
<td>EORTC S9951</td>
<td>40 mo + CR</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>M</td>
<td>ALL</td>
<td>46,XY,t(8;12)[q12;p12][17]</td>
<td>ALL IC-BFM 2002, Interfant ALL 99 (modified)</td>
<td>17 mo + CR</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>M</td>
<td>AML-M1</td>
<td>47,XY,t(8;12)[q137;p11.27],[p21;4][4]</td>
<td>AML-BFM 87</td>
<td>84 mo CR*</td>
</tr>
<tr>
<td>6</td>
<td>10.9</td>
<td>M</td>
<td>ALL</td>
<td>46,XY,t(8;12)[q21],+22[4]</td>
<td>AML-BFM 93</td>
<td>21 mo DOD</td>
</tr>
</tbody>
</table>

Identification of NCOA2 as ETV6 partner gene. FISH analysis with ETV6 exon-specific probes revealed disruption of this gene (Fig. 2A). To narrow down the breakpoint in 8q, FISH with various gene-specific probes located at 8q13.1-21.1 was carried out and the most likely breakpoint was determined to occur in chromosomal band 8q13.3 (data not shown). Out of the genes located in this chromosomal region, NCO2A was considered a likely candidate gene because it has already been identified as partner gene of MYST3 (MOZ) in cases with an inv(8)(p11q13) (30). Fusion gene-specific reverse transcription-PCR experiments using primers located in exons 3 and 17 of ETV6 and NCOA2, respectively, led to the identification of chimeric ETV6-NCOA2 transcripts (Fig. 2B). Sequence analyses revealed an in-frame fusion between ETV6 exon 4 and NCOA2 exon 15 in five of the six cases (cases 1, 2, 3, 5, and 6; Fig. 2C). In case 5, also an alternatively spliced transcript lacking exon 16 of NCOA2 was detected. In the remaining case (no. 4), ETV6 exon 5 was fused in-frame to NCOA2 exon 14. Thus, the putative ETV6-NCOA2 consensus fusion protein consists of the ETV6 pointed domain (PNT) and the cyclic AMP–responsive element binding protein–binding protein (CBP) interaction (CID) and the AD2 (transactivation domain 2) domains of NCOA2 (Fig. 2D). The splice variant lacking NCOA2 exon 16 (case 5) would result in a partial deletion of the CID domain; however, the second transcript also encodes the putative consensus protein. Expression analysis for the reciprocal NCOA2-ETV6 identified two different chimeric transcripts that fused NCOA2 exon 14 to ETV6 exons 5 or 6, respectively, showing alternative splicing of ETV6 exon 5 (Fig. 2B). In case 4, despite all efforts using various primer combinations (see Table 1), no NCOA2-ETV6 transcripts could be amplified, suggesting that the ETV6-NCOA2 fusion gene is responsible for leukemogenesis.

Detection of NOTCH1 mutations. The identification of activating NOTCH1 mutations not only in T-ALL but also in rare cases of myeloid leukemia (15–18) prompted us to analyze our samples for the presence of NOTCH1 mutations. Mutation analysis of the heterodimerization and PEST domains of NOTCH1 revealed heterozygous NOTCH1 mutations in four of five samples of which sufficient material was available. Mutations were detected in one sample in the heterodimerization domain, in two in the PEST domain, and in one in both domains. As previously described (18), heterodimerization domain mutations were missense, in-frame deletions and insertions, whereas the PEST domain mutations created premature termination codons (Table 4). The two heterodimerization domain mutations found in cases 2 and 3, and the PEST domain mutations of cases 1 and 6, have been previously described (18). A PEST domain mutation similar to that detected in case 2 has as well been reported (31).

Discussion

In this study, we report six cases of childhood acute leukemia with a novel recurrent t(8;12), which results in a fusion of the repressor gene ETV6 and the transcriptional coactivator NCOA2. The ETV6-NCOA2 fusion concurs with a high prevalence of NOTCH1 activating mutations and the coexpression of cyCD3 and cytoplasmic MPO (cyMPO) in a subpopulation of the blast cells of case 2 (A) and case 6 (B). C and D, coexpression of CD7 and CD33 in case 1 (C) and case 4 (D). PE, phycoerythrin; APC, allophycocyanin.
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of T-cell–specific antigens (cycCD3 and CD7) and at least one myeloid marker (i.e., MPO, CD33, CD13, and CD65). As a result of the t(8;12) translocation, the PNT protein interaction domain of ETV6, which is involved in homodimerization and heterodimerization, is fused to the COOH terminus of NCOA2, including the CBP interaction domain and the AD2 activation domain. The same COOH-terminal domains of NCOA2 are also retained in the previously identified MYST3-NCOA2 fusion protein, which is generated by an inv(8)(p11q13) (30, 32). The absence of the reciprocal NCOA2-ETV6 transcript in one of the cases, as well as the facts that MYST3-NCOA2 is transforming and that the reciprocal NCOA2-MYST3 is not expressed, suggests that the ETV6-NCOA2 chimeric protein and not the reciprocal NCOA2-ETV6 is responsible for leukemogenesis. However, this finding needs to be confirmed in a larger cohort of ETV6-NCOA2–positive cases.

The transforming properties of MYST3-NCOA2 depend on the MYST3 MYST and the NCOA2 CBP interaction domains, whereas the MYST3 PHD and the putative AD2 acetyltransferase motifs of NCOA2 are not required for transformation (33). Expression of MYST3-NCOA2 correlates with a depletion of CBP from PML bodies and reduced cellular levels of CBP. Thus, MYST3-NCOA2 acts as a modulator of the transcriptional activity of CBP-dependent activators (34, 35). Future studies will determine whether the ETV6-NCOA2 chimeric protein has similar properties or may recruit CBP to ETV6 target genes resulting in their constitutive activation. In this respect, it is interesting to note that Ets pointed domains can also interact with the SRC1 interaction (CID) domain of CBP (36), suggesting a possible competition between ETV6-NCOA2 and normal ETV6 for CBP. Moreover, all NUP98 chimaera, including those associated with a T/myeloid phenotype (11–13), retain the COOH-terminal FG repeats, which have the potential to bind CBP (37), indicating a probable involvement of CBP in these types of leukemia.

Bona fide transcriptional coactivators are rarely involved in leukemogenic translocations. Such fusion genes, which are expressed as a consequence of chromosomal translocations, include MLL-CREBBP/CBP (38–41), MLL-EP300/p300 (41), MYST3-CREBBP (42), MYST3-EP300 (43, 44), MYST4/MORF-CREBBP (45), and MYST3-NCOA2 (30, 32). In contrast to MYST3-NCOA2 and to all other rearrangements involving transcriptional coactivators, which are exclusively associated with AML, the ETV6-NCOA2 irrespective of its occurrence in different morphologic and immunophenotypic leukemia subtypes determined by the FAB and EGIL classifications coincides with coexpression of T-lymphoid and myeloid markers.

Recently, a similar novel subtype of AML with coexpression of T-lymphoid markers characterized by CEBPA silencing through promoter hypermethylation and associated with frequent NOTCH1 mutations has been identified (15). This subgroup of acute leukemia showed several cytogenetic abnormalities, none of which was common to all cases, suggesting that the CEBPA/NOTCH1 and the ETV6-NCOA2/NOTCH1 leukemia represent distinct entities.

Mixed myeloid and lymphoid T-cell–specific or B-cell–specific leukemia has been previously described and may either represent an unphysiologic transformation-related anomaly or, alternatively, reflect the immunophenotypic features of a physiologic common myeloid-lymphoid progenitor (46). The concurrence of the ETV6-NCOA2 fusion with NOTCH1 activating mutations raises the question whether this combination of genetic lesions or ETV6-NCOA2 alone has instructive properties or at least one of them targets an uncommitted progenitor cell with a myeloid and T-lymphoid potential. In this context, the
presence of NOTCH1 mutations in lineage infidelity (17) as well as in AML with T-lymphoid features (15) suggests that these mutations may occur in a leukemic stem cell that precedes both myeloid and T-lineage commitment (17). Accumulating evidence indicates that a myeloid potential accompanies early stages of T (also B and erythroid) development and that T-cell (and B-cell) progenitors are most likely produced from a common myeloid-lymphoid progenitor through intermediate bipotent or even multipotent stages (47–52). On the other hand, some fusion proteins encoded by translocations impart, properties and the subsequent activation of NOTCH1 may direct differentiation toward the T-cell lineage. Ectopic expression of ETv6-NCOA2 will eventually reveal whether this chimeric protein reprograms progenitors or leads to expansion of a T/myeloid subset.

Although at this point the sequential or simultaneous occurrence of the two genetic events, fusion of ETv6 to NCOA2 and mutation of NOTCH1, remains indefinable, it is tempting to speculate that mutation of NOTCH1 confers self-renewal capacity to early progenitors, which are then susceptible to the accumulation of additional genetic hits such as fusion genes. Vice versa, the ETv6-NCOA2 fusion might bestow transforming properties and the subsequent activation of NOTCH1 may direct differentiation toward the T-cell lineage. Ectopic expression of ETv6-NCOA2 will eventually reveal whether this chimeric protein reprograms progenitors or leads to expansion of a T/myeloid subset.

The eminent clinical question that derives from our findings is whether this type of leukemia should be treated as ALL or AML. The currently available rather limited information indicates that the affected patients have a decent prognostic outlook when treated with either ALL or AML therapy regimens. However, only the retrospective and prospective collection, evaluation, and comparison of treatment results of a larger number of such cases will provide a conclusive answer. The identification of the ETv6-NCOA2 gene fusion and its accompanying NOTCH1 mutations is thus the first essential step to achieve this goal.

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