Biallelic Alterations of Both ETV6 and CDKN1B Genes in a t(12;21) Childhood Acute Lymphoblastic Leukemia Case

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ABSTRACT

Recently, a new recurrent t(12;21)(p13;q22) has been identified in a B-cell lineage childhood acute lymphoblastic leukemia (ALL). The translocation results in a fusion of two known genes, ETV6/TEL (12p13) and AML1 (21q22), previously shown to be involved in the pathogenesis of myeloid disorders. We report results of cytogenetic fluorescence in situ hybridization and molecular studies of a B-cell childhood common ALL with a cryptic 12;21 translocation. Aberrations identified in this case involve both chromosomes 12 and include not only the ETV6-AML1 gene fusion and two different microdeletions of ETV6 but also the hemizygous loss of CDKN1B, D12S119, and KRAS2 loci and a putative rearrangement of the second CDKN1B allele as a result of an inv(12)(p13q24). Moreover, it was shown that the AML1-ETV6 reciprocal chimeric transcript was not present in the malignant cells, and hence may not play a major role in leukemogenesis. In addition, the putative loss of wild-type function of CDKN1B and ETV6 could indicate a synergistic effect of both genes in the pathogenesis of this leukemia case.

INTRODUCTION

Chromosomal abnormalities involving the short arm of chromosome 12, observed in a broad spectrum of hematological disorders (1), appear to be particularly common in a type of childhood ALL (2, 3). These aberrations consist of both deletions and reciprocal or nonreciprocal translocations of 12p with various partner chromosomes, and according to recently published FISH and molecular data, they remain underestimated by classical cytogenetics (4—6). For example, a t(12;21)(p13;q22) that was recently identified as a recurrent translocation in a type of childhood B-cell ALL escaped routine banding analysis and was initially discovered by FISH analysis (7). Two groups independently demonstrated that the molecular consequence of the (12;21)(p13;q22) translocation is the fusion of two known genes, namely ETV6, mapped to 12p13, and AML1, located at 21q22, previously shown to be involved in chromosomal translocations characteristic of myeloid malignancies (8, 9). The resulting chimeric protein consists of the helix-loop-helix domain of ETV6 and the entire AML1 gene, including its DNA binding and transactivation domains.

The ETV6 gene, encoding an ETS-like putative transcription factor, was initially identified by its fusion with the platelet derived growth factor receptor B in chronic myelomonocytic leukemia associated with a t(5;12)(q33;p13) (10). In addition, other chimeric transcripts, namely ETV6-ABL (11) and ETV6-MNL (12), plus a translocation ETV6-10q24 (13), have been identified in ALL, AML, and myelodysplastic syndrome, respectively, indicating that the gene is especially prone to alterations that may affect cells of myeloid as well as lymphoid origin. Moreover, detection of loss of heterozygosity at 12p13 and FISH analysis of 12p abnormalities in patients with various hematological disorders revealed that ETV6 and the CDKN1B gene encoding the cyclin-dependent kinase inhibitor p27KIP1 are situated in the smallest critically deleted region (5, 6) and that both genes are frequently deleted in a type of childhood ALL (14).

The second gene rearranged by t(12;21), AML1, was cloned from a breakpoint of a t(8;21)(q22;q22) translocation that has been observed in about 40% of karyotypically abnormal cases of AML-M2 (15). The translocation results in a fusion of the DNA binding runt domain of the AML1 gene and the entire ETO/MTG8 gene, which encodes a putative transcription factor. Several translocations involving the AML1 gene have been identified in myeloid leukemias and in all of them the resulting chimeric genes contain the AML1 promoter and runt domain fused to other partner genes including the EAP, MDS1, and EVII genes on chromosome 3 (16). Therefore, t(12;21) is unique among AML1 variant translocations because it involves the entire AML1 gene and affects leukemias of lymphoid origin.

In the recently published series of studies of ALL, the ETV6-AML1 fusion has been documented in 16—36% of pediatric patients (17, 18), so making it the most common abnormality in this subtype of leukemia. Moreover, Shurtleff et al. (17) reported that ETV6-AML1-expressing ALLs constitute a clinically distinct entity affecting children ages 1—10 years with B lineage, nonhyperdiploid leukemia lymphoblasts, and favorable prognosis. One of the striking molecular findings in these patients is a frequent deletion of the nontranslocated ETV6 allele resulting in a loss of wild-type ETV6 function in the leukemic cells. The consistency of the involvement of the ETV6-AML1 rearrangement in childhood ALL is emphasized in the present paper in which we report a cryptic t(12;21)(p13q22) masked by a dic(12;13)(p11q10) and detected by FISH. In addition, the ETV6-AML1 gene fusion in this patient was associated with rather complex aberrations involving both chromosome 12s, which were further investigated by FISH and gene rearrangement studies.

PATIENTS AND METHODS

Case. The patient, a 15-year-old girl, was admitted in June 1995 because of bone pain, fatigue, and fever. Her previous medical history was unremarkable. Clinical examination revealed a pale skin and a slight hepatosplenomegaly. Hematological data were as follows: hemoglobin, 9.9 g/dl; platelets, 111 x 10^12; and white blood cells, 9.9 x 10^9/L with 9% neutrophils, 37% lymphocytes, and 54% lymphoblasts. Bone marrow was hypercellular with a proportion of 85% of blast cells expressing CD34, CD19, CD22, CD10, HD, CD33, and CD13. The diagnosis of common ALL, classified as L2 according to the FAB criteria, was established. Chemotherapy following a standard protocol for childhood ALL (EORTC 5881) was started.

Cytogenetics. Cytogenetic analysis was performed on direct cultures of bone marrow cells prior to treatment. Ten R- and G-banded karyotypes were analyzed and classified according to ISCN 1995 (19).
FISH. FISH was performed as previously described (20). Chromosome 12 abnormalities were analyzed using eleven 12p cosmide probes and YAC 958B8 (Fig. 1) labeled with biotin. 958B8 is a 1.2-Mb YAC containing the ETV6 gene, as was previously published by Golub et al. (10). The ETV6 locus was investigated with the following LL12NCO1 cosmide probes (Lawrence Livermore National Laboratories, Livermore, CA) ordered as follows: 5′end-179A6-50F4-2G8-163E7-184C4-148B6-3′end. The different ETV6 exons present in the cosmids are shown in Fig. 1.4 Cosmid probes for D12S158, PRB3, D12S119, and KRAS2 were described before by Chaffanet et al. (21) and for D12S934 by Baens et al. (22).

Further FISH experiments were performed using a chromosome 13q13 specific cocktail probe IGMTA94/013 (Integrated Genetics), a chromosome 13/21 centromere probe (pUC 1.76), a cosmide containing CSFIR mapped to 5q33 (23), cosmide ICRFC102D12118 (21q22.3, ICRF) and a whole chromosome 12 painting probe (WCP 12, Imageneics). Chromosome 12s were identified by cohybridization with the Texas Red-5-dUTP labeled chromosome 12 alphoid probe (pBR12; Ref. 24) and simultaneous G-banding analysis using 4′,6-diamidino-2-phenylindole dihydrochloride counterstaining. The number of analyzed abnormal cells in each experiment is indicated in Table 1. The FISH data were collected on a Leitz DMRB fluorescence microscope equipped with a cooled black-and-white charge-coupled device camera (Photometrics) run by SmartCapture software (Vysis, Stuttgart, Germany).

Molecular Analysis. Total RNA was isolated from bone marrow cells using the Trizol reagent (BRL). First strand cDNA was reverse transcribed from 1 μg RNA with MuMLV-reverse transcriptase (BRL) according to standard procedures with pd(N6) random primers. The following primers derived from ETV6 and AML1 sequences were used to amplify the ETV6-AML1 fusion RNA: ETV6F (5′-TCCCCGCCTGAAGAGCACGCC) and AML1R (5′-AGCGGCAACGCGCCTCGCTCAT). For the amplification of the AML1-ETV6 fusion RNA, the following primers were used: AML1F (5′-GGAGGAAGCGATGGCTTCAGACAGC) and ETV6R (5′-CCACAGTCGAGCCAGCTCCTGCG).

DNA was isolated from a bone marrow sample and used for Southern analysis by standard methods. A probe for CDKN1B was obtained by PCR with the following primers derived from the CDKN1B cDNA (F: GGACTTGGAGAACGC ACTGC; R: GGAGCTGTAGTAGAGACTCGG), amplifying a genomic fragment of 1.2 kb.

RESULTS

Cytogenetics and FISH. Cytogenetic analysis of the reported case performed at the time of diagnosis revealed clonal chromosome abnormalities in 50% (5 of 10) of analyzed bone marrow cells described as 44, X, −X, del(2)(q14q22), del(5)(p13p14), inv(12)(p12q24), der(12)t(12;13)(p11q12), −13. A representative R-banded karyotype is shown in Fig. 2. To confirm the inv(12) detected by cytogenetics, FISH was performed with a chromosome 12 painting probe (WCP 12) and YAC 958B8 containing ETV6. The uniform painting of the inv(12) and a hybridization signal from the YAC on the long arm of this abnormal chromosome were in agreement with the cytogenetic data. However, a second hybridization signal from this
Table 1 Results of FISH analysis

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<th>inv(12) 12p</th>
<th>inv(12) 12q</th>
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PBR-12 was additionally used as a marker for chromosome 12 in the majority of FISH experiments.

12p13/ETV6 specific 958B8 YAC was unexpectedly detected on a small acrocentric, identified as a chromosome 21 (Fig. 3A). This observation suggested a cryptic t(12;21) translocation masked by a t(12;13) and prompted us to investigate this case with other 12p-specific DNA probes. D12S158, a 12p13.3 specific cosmid, and the ETV6 probes 179A6, 50F4, 2G8, 163E7, and 184C4 were present on chromosome 21 (e.g., 50F4; Fig. 3B), whereas 148B6 was absent (Fig. 3C). These results confirmed the translocation of the distal part of chromosome 12p to chromosome 21, with a 12p breakpoint within ETV6 between 184C4 and 148B6. All these ETV6-specific probes, together with probes for CDKN1B, D12S119, and KRAS2, were absent from the der(12)t(12;13) (e.g., CDKN1B; Fig. 3D) and D12S934 (12p11) was present, demonstrating an interstitial deletion of 12p sequences ranging from exon 8 of ETV6 (148B6) distal to D12S934 [del(12)(p11p13)].

The complex rearrangements involving chromosomes 12, 13, and 21 were further analyzed by FISH with a chromosome 13q13 cocktail probe (IGMataka94/013), chromosome 12 (pBR12), 13/21 (pUC 1.76) centromeric probes, and a cosmid assigned to 21q22.3. The 13q13 probe hybridized to the normal chromosome 13 and the der(12)t(12;13) (e.g., CDKN1B; Fig. 3D) and D12S934 were found on the short arm of chromosome 5, which was further confirmed by cohybridization with 5q33 specific CSF1R probe (Fig. 3F). Interestingly, FISH analysis with the same 12p cosmid probes performed on the inv(12) chromosome showed splitting of the signal of the CDKN1B probe demonstrating that the 12p breakpoint occurred within or near the CDKN1B gene. Consequently, hybridization signals from other probes centromeric to CDKN1B, namely, D12S119, KRAS2, and D12S934, were found on the short arm of the inv(12) chromosome. These experiments unexpectedly also revealed an interstitial microdeletion that involved the 5' end of ETV6. Briefly, hybridization signals from 179A6, 50F4, 2G8, 163E7, and 184C4 probes were lost (e.g., 50F4; Fig. 3B), but the 3' end ETV6 probe 148B6 hybridized to the inv(12) chromosome (Fig. 3C). Taken together with the normal signal observed with the YAC 958B8 described above, this indicates the presence of a microdeletion possibly limited to 5'ETV6.

All FISH results are summarized in Table 1 and schematically illustrated in Fig. 4. The FISH findings led to a correction of the description of the original karyotype as follows: 44, X, −X, der(12)t(12;13) (Fig. 3E). Taken together, these observations indicated the dicentric nature of this derivative chromosome with a 12p breakpoint telomeric to D12S934. The cosmid specific for 21q22.3 hybridized to a normal chromosome 21 and, unexpectedly, to a short arm of chromosome 5, which was further confirmed by cohybridization with 5q33 specific CSF1R probe (Fig. 3F).

![Fig. 2. R-banded karyotype of the reported case. Arrows, chromosomes involved in complex rearrangements described in "Results."](image-url)
Fig. 3. Examples of FISH results with YAC 958B8 (A); cosmids 50F4 (B), 146B6 (C), and CDKN1B (D); centromeric probes for chromosome 12 and 13/21 (E); and 21q22.3 probe (F). Big arrows, arrowheads, and small arrows, inv(12), der(12)t(12;13), and chromosome 21s, respectively. *, a normal chromosome 13 (E) and der(5) (F). E, inset, the der(5) chromosome identified by a cohybridization of 21q22.3- and 5q33-specific cosmid probes.

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Molecular Investigations. The expression of ETV6, AML1, and chimeric ETV6-AML1 mRNAs was investigated by RT-PCR (Fig. 5). A signal for the ETV6-AML1 transcript was present in the bone marrow sample of the patient and absent in a control EBV-transformed lymphocyte sample, confirming the translocation of S'ETV6 to chromosome 21. The size of the PCR products also demonstrates that fusion occurred between the same ETV6 and AML1 exons as described by Romana et al. (9) and Golub et al. (8). No products were obtained with primers designed to amplify an AML1-ETV6 chimeric mRNA. This is in agreement with the translocation of 21q sequences to chromosome 5. A control PCR amplifying a ETV6 fragment (bp 991-1164 of the ETV6 cDNA; Ref. 8) showed a clear signal for the EBV-transformed sample and a weak signal with the patient’s bone marrow RNA (data not shown). Because FISH data show that this part of ETV6 is completely deleted in the leukemic cells, the signal must result from normal cells present in the bone marrow sample, a finding confirmed by the chromosome data.

FISH with cosmid 123C12, which carries the CDKN1B gene, resulted in a split signal. To investigate this, DNA isolated from the bone marrow sample was digested with EcoRI, BamHI, and HindIII, and a Southern hybridization was performed with a CDKN1B probe obtained by PCR. With EcoRI, the tumor sample showed an aberrant fragment of 8 kb, whereas the control yielded a band at 13 kb. To exclude a polymorphism, the same probe was hybridized to a Southern blot with 20 normal control DNAs, and in each case, a band at 13 kb was detected. With BamHI and HindIII, genomic fragments of approximately 6.5 kb were detected in both the tumor sample and the control sample (Fig. 6).
DISCUSSION

Recurrent involvement of the short arm of chromosome 12 in karyotypic abnormalities observed in hematological malignancies suggests a significant role of this chromosome region in leukemogenesis. The ETV6 gene, recently identified and assigned to 12p13, was only found in this region, and its overexpression is associated with acute leukemia. However, the role of this gene in the pathogenesis of leukemia is still not fully understood.

The ETV6 gene, recently identified and assigned to 12p13, was found to be rearranged in several cases of ALL. One of these recurrent rearrangements, t(12;21)(p13;q22), not apparent at the cytogenetic level, appeared as the most common abnormality in childhood B-cell ALL. Using FISH with 12p-specific DNA probes, we not only identified another cryptic 12:21 translocation in a pediatric patient with B-cell ALL, but also discovered additional biallelic rearrangements of CDKN1B. This translocation resulted in an ETV6-AML1 fusion similar to the one described by Romana et al. (9) and Golub et al. (8). FISH revealed that the breakpoint occurred proximally to cosmid 184C4, which contains two exons coding for bp 353-1033 of ETV6, and the presence of ETV6-AML1 fusion mRNA in leukemic cells was demonstrated by RT-PCR, which detected a fragment of the same size as expected based on previous observations. In addition, FISH results showed that the 3' end ETV6 domain (148B6) was deleted from the dic(12;13) chromosome together with CDKN1B, D12S119, and KRA52, and that the 21q22.3 sequences were translocated to the short arm of chromosome 5. Therefore, the reciprocal chimeric transcript containing the 5' end AML1 and the 3' end ETV6 domain is absent in the malignant cells, as confirmed by the RT-PCR experiments. In contrast, Romana et al. (9) and Golub et al. (8) found that both derivative chromosomes of a t(12;21) are transcriptionally active, although the level of the 5'-AML1-ETV6-3' mRNA expression in two cases analyzed by the latter group was low. The results presented here, together with the recent data reported by Shurtleff et al. (17) and Romana et al. (18), clearly indicate that the 5'-ETV6-AML1-3' chimeric protein is sufficient for the development of leukemia.

In the previously published ETV6-AML1 documented ALLs, the wild-type ETV6 allele was frequently deleted, as demonstrated using Southern blot and FISH approach (8, 9, 17, 18, 25). FISH analysis of the inv(12) of the case reported here revealed that a microdeletion occurred within the region covered by YAC 958B8 that affected only the 5' end of ETV6 encoding the helix-loop-helix domain (probes 179A6, 50F4, 2G8, 163E7, and 184C4) but not the 3' end of ETV6 (148B6). The part of ETV6 that is deleted spans approximately 270 kb, and together with the observation of normal FISH signals with the 958B8 probe, this indicates that the deletion is limited to the ETV6 gene. This finding strongly suggests that ETV6 is the actual target of 12p13 deletions associated with t(12;21). The biallelic alteration of ETV6 caused by the t(12;21) plus a partial deletion, leading to its functional inactivation in leukemic cells, fulfills a criterion for its being a tumor suppressor gene. However, recent reports indicate that del(12)(p) is a secondary event in ALL with t(12;21) (25) and does not occur in all cases with ETV6-AML1 fusion (17, 18, 25), so the alternative possibility remains that the t(12;21) fusion proteins act in a recessive fashion and thus requires a deletion of the normal ETV6 allele.

Another intriguing FISH finding in our case is a putative rearrangement of CDKN1B as a result of the inv(12)(p13q24). Southern blot analysis with a genomic probe containing the two coding exons of CDKN1B detected an abnormal fragment after digestion with EcoRI. This indicates that the breakpoint occurred within a 8-kb fragment containing the CDKN1B gene, and it is tempting to speculate that this rearrangement inactivated the CDKN1B gene. We also showed that the CDKN1B allele on the dic(12;13) was deleted; hence, the tumor cells might present a homozygous inactivation of CDKN1B. The presence of normal cells in the tumor sample prevents an analysis of this by RT-PCR, and further molecular studies are necessary to prove this point.

The CDKN1B gene, which is situated at most 2.9 Mb centromeric to ETV6 (5), belongs to a family of genes coding cyclin-dependent kinase inhibitors including p15INK4B, p16INK4A, p18, and p21WAF1, which may act as tumor suppressors (26–30). p21WAF1, the product of CDKN1B, has 42% homology with p16INK4A protein in its NH2-terminal region and is also involved in G1 cell cycle arrest. However, in contrast to p16INK4A, in which biallelic deletions have been frequently found in hematological malignancies (31, 32), except for two cases with a homozygous CDKN1B deletion found by Morosetti et al. (33) in a series of 119 analyzed non-Hodgkin’s lymphomas and ALLs, no other biallelic inactivation of CDKN1B could be demonstrated in a series of human leukemias and solid tumors (34–36). Thus, our case is exceptional in this respect, and it is tempting to speculate that biallelic alterations of both ETV6 and CDKN1B might have a synergistic effect in the development of some acute leukemias. More extensive molecular studies of CDKN1B gene in ALL should be performed to investigate this possibility.

In summary, important observations were made in the present study. First, a t(12;21), which is not evident on a cytogenetic level, can occur as a consequence of several possibly complex chromosomal translocations, and a 12p13/ETV6 specific breakpoint can be masked by an interstitial deletion in the same chromosome 12. In addition, we showed that the 5'-ETV6-AML1-3' chimeric gene, rather than the reciprocal product of a t(12;21), plays a critical role in leukemogenesis.

Moreover, the intragenic microdeletion of the 5' part of the ETV6 allele unaffected by the translocations strongly indicates that the gene is the target of 12p deletions associated with a t(12;21) in childhood ALL. Finally, the inactivation of the wild-type function of ETV6 and the putative homozygous inactivation of CDKN1B in the leukemic cells suggest a synergistic involvement of these two candidate tumor suppressor genes in pathogenesis of the reported ALL. More cases need to be analyzed in detail to answer this question.

5 P. Mynen, unpublished observation.
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REFERENCES


2661
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