RAPID COMMUNICATION

The Chromosome 4q21 Gene (AF-4/FEL) Is Widely Expressed in Normal Tissues and Shows Breakpoint Diversity in t(4;11)(q21;q23) Acute Leukemia

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The chromosomal translocation, t(4;11)(q21;q23), is the most common type of 11q23 chromosomal abnormality, being highly prevalent in infant acute leukemias and associated with a poor prognosis. The t(4;11) results in the fusion of an 11q23 gene (MLL, HRX, Htrx-1, or ALL-1) and a 4q21 gene (AF-4 or FEL). To further evaluate the 4q21 gene and its role in t(4;11) acute leukemia, we have cloned a 38-kb genomic region and mapped exons of the AF-4 gene. The 4q21 breakpoints in 19 cases of t(4;11) acute leukemia were analyzed by Southern analysis and pulsed-field gels. Seventeen of the 19 cases had breakpoints on chromosome 4q21 that were scattered in this 38 kb region. Expression of the AF-4 gene was studied in a total of 28 various nonhematopoietic, hematopoietic, and t(4;11) leukemic cell lines. The AF-4 gene was expressed in all cell lines as a major and a minor transcript. In addition to the normal transcripts, two fusion transcripts from the derivative 11 and derivative 4 chromosomes were identified in all t(4;11) cell lines except B1, which had only the der(11) transcript. These findings suggest that the breakpoints on 4q21 cluster over a broader area than do the breakpoints in the 11q23 gene, and that der(11) encodes the fusion RNA found consistently in leukemia cells.

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N O N R A N D O M aberrations involving chromosome 11 band q23 are frequently seen in human acute lymphoblastic and myeloid leukemias. These abbreviations include deletions and reciprocal translocations with chromosomes 1, 4, 6, 9, 10, 14, 17, and 19. Molecular cloning of the 11q23 region has shown gene fusion events resulting from these reciprocal translocations.1,4 The t(4;11)(q21;q23) acute leukemia is the most common of the 11q23 abnormalities and has distinct clinical and phenotypic features; t(4;11) represents the vast majority of infant (<1 year) acute lymphoblastic leukemia (ALL) and is usually associated with a mixed lymphoblastic/monocytic phenotype, hyperleukocytosis, and a CD10 negative CALLA-negative immunophenotype as well as a poor prognosis with conventional chemotherapy.5,6 The t(4;11) breakpoints have recently been cloned and the translocation fuses an 11q23 gene (named MLL, HRX, Htrx-1, or ALL-1)5,7,8 to a 4q21 gene (AF-4 or FEL).1,3 The 11q23 gene, which is widely expressed in a variety of hematopoietic cell lines as well as epithelial and glial cell-originated tumor cell lines, very likely encodes a transcriptional factor based on its sequence homology to the Drosophila trithorax gene.9,10 The function of the 4q21 gene is still uncertain; however, its predicted protein possesses nuclear localization consensus sequence as well as serine/proline-rich and hydrophilic regions, suggesting that it may be a transcription factor.2 Tight breakpoint clustering on 11q23 has been shown in a number of studies.5,10,11 However, the degree of 4q21 breakpoint clustering is less well defined.1 While fusion transcripts from both der(11) and der(4) have been reported in the t(4;11) leukemic cell lines studied, it has not yet been proven which fusion product is oncogenic.1,12

We have further characterized the 4q21 breakpoint genomic region, shown the location of three exons, and mapped the translocation breakpoints in 17 of 19 acute leukemias and cell lines with the t(4;11)(q21;q23). In addition, we analyzed the expression of the 4q21 gene in a large number of human tissues and hematopoietic and nonhematopoietic cell lines. We also show evidence that the der(11) product is likely to be the important fusion protein in leukemogenesis.

MATERIALS AND METHODS

Fresh leukemic cells and cell lines. Patient samples were selected according to the availability of sufficient material, and included patients enrolled in the Childrens Cancer Group (CCG). Nineteen cases with cytogenetically proven t(4;11)(q21;q23) (15 fresh leukemias and 4 cell lines) were used in this study. Among them, 8 cases (no. 1, 4, 5, 7, 8, 10, 11, and 12) have been used in a previous chromosome 11q23 breakpoint mapping study.5 The RS4;11 cell line, established in our laboratory,13 is available from (American Type Culture Collection (ATCC; Rockville, MD). The AN4;11 cell line was established from an infant with t(4;11) ALL by injecting 3 x 10⁸ patient leukemic cells into the peritoneum of severe combined immunodeficient (SCID) mice. Tumor was harvested 2 months after injection and a cell line was established in culture in RPMI 1640 supplemented with 20% fetal bovine serum (HyClone Laboratories Inc, Logan, UT). MV4;11 and B1 are established t(4;11) cell lines and have been described in detail elsewhere.14,15 Additional cell lines from human tissues used for mRNA extraction include Nalm 6, HPB0, and IE8 (B-cell lines); Ew36 (B-cell lymphoma cell line); HL-60 and K562 (acute lymphoblastic leukemia [ANLL] and myeloid cell lines); THP-1 and U937 (monocytic cell lines); Jurkat, RPMI 8402, CEM, and HSB-2 (T-
cell lines); MT (T/myeloid leukemia cell line)9; NC-37 and FJO (lymphoblastoid cell lines); MG-63 and OHS (human osteosarcoma cell lines); Hs68 and CCD-19Lu (human lung fibroblast cell lines); COLO 201 (human colon adenocarcinoma cell line); SK-MEL-2 (human melanoma cell line); M418 (human neuroblastoma cell line); JEG3 (human choriocarcinoma cell line); and AF-4/FEL (gene in acute leukemia)10,11.

Genomic screening and restriction mapping In our previous studies of the t(4;11) breakpoint in the RS4;11 cell line, a rearranged 3.7-kb EcoRI fragment [a der(4) clone] was isolated from the RS4;11 genomic library. A polymerase chain reaction (PCR)-amplified product, BP7 (Fig 1A), was generated from this EcoRI fragment and confirmed as part of the chromosome 4 region by somatic cell hybrids.3 In this study, a germline restriction map of the 4q21 breakpoint region was generated by obtaining clones from a lung fibroblast W131 genomic DNA library (Strategene, San Diego, CA) by screening with the BP7 probe. Six independent overlapping clones in the 4q21 region were initially identified and used to generate probes for subsequent restriction mapping. Restriction mapping of these clones was performed by probing Southern blots of single and double restriction enzyme digests as well as by probing UV-irradiated and subsequently restriction-digested DNA blots with 32P-labeled T3 and T7 primers. Individual HindIII, EcoRI, Sst I, and BamHI restriction enzyme sites were mapped in this region. Several restriction enzyme-digested fragments from these phage clones were subsequently cloned into the Bluescript vector (Strategene) so that these cloned fragments could be used as probes in 4q21 breakpoint mapping experiments (Fig 1A). Probes used in these studies, including BP7, 4AE1 (a 4.9-kb EcoRI fragment), 4AE2 (a 5.4-kb EcoRI fragment), 4AE3X1 (a 2.4-kb EcoRI-Xba I fragment), 4AE3X2 (a 1.2-kb EcoRI-Xba I fragment), 4AE5X1 (a 3.3-kb Xba I-Sst I fragment), and 4AE6BS (a 2.5-kb BamHI-Sst I fragment). A 38-kb genomic DNA region was identified by these probes. To identify exon/exons in the breakpoint clustering genomic region we mapped, two cDNA clones, pc606 and PL12 (detailed below), were labeled with the Genius nonsotope system (Boehringer Mannheim, Indianapolis, IN) and hybridized to genomic clone blots. A 45-bp exon was further characterized by using PCR to amplify the 4AE1 genomic clone with cDNA primers; the product was then subsequently sequenced (Sequenase Protocol; US Biochemical Corp, Cleveland, OH). To identify possible additional small exons, two oligonucleotides (5' exon: ctaagatgcttctagcttggctggtt; exon 3': gacccattgactgctgctcctgct) 5' and 3' to this 45-bp exon, detailed in Fig 1A, were generated based on the sequence of pc606 and PL12. Both oligonucleotide probes were end-labeled with Digoxigenin-NHS ester (Boehringer Mannheim) and hybridized to genomic clone blots.

DNA preparation, pulsed-field gel electrophoresis (PFGE), and Southern analyses Preparation of DNA in agarose blocks and

![Fig 1.](https://www.bloodjournal.org)
restriction enzyme digestion for PFGE was performed as described. Germline DNA control was isolated from either normal peripheral blood lymphocytes or human placental DNA. One-percentage agarose gels were run in a contour-clamped homogeneous electric field (CHEF) PFGE electrode array as described.

High molecular weight DNA was digested, fractionated by CHEF, and then transferred to a nylon membrane (Nytran; Schleicher and Schuell, Inc, Keene, NH) in 10X saline sodium phosphate EDTA (SSPE). The electrophoresis and blotting conditions have been described previously.

DNA samples were also extracted and prepared for conventional Southern analysis. Germline DNA control was isolated from either normal individual peripheral blood lymphocytes (PBL) or human placental DNA. Southern blot analyses were performed according to standard methods. Membranes were stripped and reexposed to film for an appropriate time before the hybridization with a different probe. To preanneal repetitive sequences recognized by the 4AE2 and 4AE6B probes during Southern analysis, 300 µg/mL of total human placental DNA (Sigma Chemical Co, St Louis, MO) was included in the hybridization solution.

Preparation of polyA+ RNA and hybridization analyses. PolyA+RNA was isolated from cells using the FastTrack mRNA isolation Kit (Invitrogen, San Diego, CA). All adherent cell lines, including Hs68, CCD-114Lu, SK-MEL-2, and JEG3, were grown to 90% confluence before the extraction was performed. Five micrograms of polyA+ RNA was size fractionated in a 0.7% agarose gel containing 2.2 mol/L formaldehyde; the gel was then capillary transferred to a Hybond-N (Amersham, Arlington Heights, IL) nylon membrane and cross-linked by UV light using a Stratalinker (Stratagene). The membranes were prehybridized for 3 to 6 hours and hybridized for 24 hours in a solution containing 5X SSPE, 2% sodium dodecyl sulfate (SDS), 10X Denhardt’s, and 50% formamide. 32P-labeled cDNA probes were made by random priming (US Biochemical), washed out at high stringency (0.1X standard saline citrate = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0, 50°C), and exposed to Kodak X-Ormat film (Eastman Kodak, Rochester, NY) for 2 to 4 days. The membranes were stripped and reprobed according to the manufacturer’s instruction. The multiple tissue Northern (MTN) blot (Clontech, Palo Alto, CA) was used to study the AF-4 gene expression in normal human tissues. The cDNA probes used were pci11i (chromosome 11 origin, MLL gene probe), pc606 (der(11) fusion cDNA of RS4;11), pc754 (chromosome 4 origin, AF-4 gene probe), and PL12 (der(11) fusion cDNA of RS4;11) (Fig 1A). The membranes were stripped and reprobed accordingly to the manufacturer’s instruction. The multiple tissue Northern (MTN) blot (Clontech, Palo Alto, CA) was used to study the AF-4 gene expression in normal human tissues. The cDNA probes used were pci11i (chromosome 11 origin, MLL gene probe), pc606 (der(11) fusion cDNA of RS4;11), pc754 (chromosome 4 origin, AF-4 gene probe), and PL12 (der(11) fusion cDNA of RS4;11).

RESULTS

Physical mapping of 4q21 breakpoints in t(4;11) acute leukemias and characterization of a breakpoint cluster region. Identification of clones both centromeric and telomeric to the breakpoint of the RS4;11 cell line resulted in the generation of a 38-kb genomic restriction map for the 4q21 breakpoint region (Fig 1A). Probes from this region (BP7, 4AE2, 4AE3X1, 4AE3X2, 4AE5X1, and 4AE6BS genomic probes and PL12 CDNA probe) were used to investigate the extent of 4q21 breakpoint clustering. Patient samples and cell lines containing t(4;11)(q21;q23) were analyzed by conventional Southern analysis of HindIII, EcoRI, and BamHI digests or, in some cases, by pulsed-field gel analysis of BssHII and Not I digests. The 4q21 rearrangements of leukemia cases were mapped relative to the germline restriction map. In each case that was mapped, rearranged fragments were identified with at least one of the above probes and rearranged fragments were shown by at least two different enzyme digests (Fig 1B). The results, summarized in Fig 1A, indicate that the majority of cases (17 of 19) of the 4q21 translocation breakpoints could be mapped to a 38-kb genomic region. In the two cases in which Southern analysis failed to show rearrangements in this region, evidence of 4q21 breakpoint heterogeneity was shown by the PFGE (data not shown). Using probes (PL12 and pc754) telomeric or centromeric to this 4q21 breakpoint cluster region, we observed significant variation of the size of the der(4) band in Not I and BssHII digests. This result suggests that there is significant 4q21 breakpoint diversity among t(4;11) acute leukemias.

To identify exons around the breakpoint region, a cDNA clone (PL12) of the AF-4 gene was used in the exon mapping study. The hybridization pattern of PL12 to genomic DNA clones indicated that an exon(s) was mapped to an EcoRI-BamHI fragment telomeric to the RS4;11 breakpoint. Using primers designed from the cDNA sequence and amplified from the 4AE1 genomic clone by PCR, a 45-bp exon and its flanking genomic sequences containing consensus splicing signals were identified (Fig 1A). This 45-bp exon was identified at the cDNA junction of the der(11) fusion transcript of the RS4;11 cell line by analyzing the pc606 cDNA transcript sequence. To identify additional small exons that were mapped within the same region and not readily detectable by using large cDNA probes, two oligonucleotide probes representing the cDNA sequence flanking the 45-bp exon were hybridized to genomic DNA clones. Both oligonucleotides showed signals on the genomic DNA clone blots tested and were placed on the restriction map (Fig 1A). This result suggests that at least 3 exons exist in the region we mapped. Because the breakpoints are between or on either side of these 3 exons, these exons are not crucial to the function of the der(11) fusion gene product.

The AF-4 gene is ubiquitously expressed in normal tissues and hematopoietic and nonhematopoietic cell lines. To elucidate the AF-4 expression pattern, we studied normal tissues, hematopoietic cell lines, and nonhematopoietic cell lines by polyA+ RNA analyses. The AF-4 gene is widely expressed in normal tissues, including placenta, heart, skeletal muscle, kidney, and pancreas (data not shown), and in all 24 cell lines without the t(4;11)(q21;q23). When probed with PL12, two large transcripts, one a major transcript estimated to be 10.5 kb and the other a minor transcript esti-
AF4/FEL GENE IN ACUTE LEUKEMIA 1083

Fig 2. Northern blot analysis of the AF-4/FEL gene transcript in various hematopoietic cell lines and nonhematopoietic cell lines using polyA+ RNA. Cell lines include (1) Nalm 6, (2) HPB-5, (3) EW36, (4) HL-60, (5) K562, (6) U937, (7) THP-1, (8) RPMI 8402, (9) HSB-2, (10) MT, (11) HS68, (12) FJ0, (13) NC-37, (14) COLO 201, (15) SK-MEL-2, (16) M418, (17) JEG3, (18) MG-63, and (19) OHS. Also positive are Jurkat, 1E8, CEM, CCD-19Lu, and FHs 173We (data not shown). The probe used is PL12, an AF-4 cDNA probe. The size of transcripts are indicated on the left of the figure. Differences in hybridization for AF-4 normal transcripts between various lanes generally reflect differences in RNA loading as determined by β-actin hybridization signals. The actin controls are shown only on the bottom of SK-MEL-2, M418, JEG3, MG-63, and OHS lanes for the comparison of RNA loading in MG-63 and OHS.

mated at 12 kb, were seen in all hematopoietic and nonhematopoietic cell lines (Fig 2). The ubiquitous expression of the AF-4 gene suggests that this gene product may play a significant role in cellular function in many tissues. Among all the cell lines, it is of interest that only two, the human osteosarcoma cell lines MG-63 and OHS (lanes 18 and 19), had weak expression of the AF-4 gene. We are unable to determine whether these reduced levels are found in the nonmalignant tissue counterparts or whether these differences are a result of malignant transformation of these cells.

der(4) transcripts are not always present in t(4;11) acute leukemias. We analyzed the expression of the AF-4 gene in t(4;11) acute leukemias. In addition to two normal 10.5- and 12-kb transcripts, three t(4;11) cell lines, RS4;11, MV4;11, and AN4;11, also show two additional fusion transcripts (at 14 kb and 12.5 kb). The latter migrates closely with the 12-kb normal transcript (Fig 3). The 12.5-kb transcript, which was also hybridized by the MLL gene probe, pCl11, is determined to be the der(11) fusion transcript. Therefore, the 14-kb transcript recognized only by the chromosome 4 gene probe, PL12, is the der(4) fusion transcript.

Of interest are results from a t(4;11) cell line, B1, probed with PL12. Only the two normal transcripts and the 12.5-kb der(11) fusion transcript are seen without the 14-kb transcript corresponding to the der(4) fusion transcript (Fig 3). Several Northern analyses using B1 mRNA extracted at different time points were performed and gave consistent results. This lack of a der(4) transcript is supported by the reported cytogenetic analysis of the B1 cell line that also showed the absence of a der(4) chromosome.15

DISCUSSION

In the present study, we have cloned part of the genomic DNA of the t(4;11) 4q21 breakpoint region and identified DNA probes that show molecular rearrangements in t(4;11) acute leukemias. Using these probes, rearrangements of 4q21 clustered in a 38-kb region can be identified by molecular analysis in the majority of cases (17 of 19) shown by cytogenetic analysis to have t(4;11)(q21;q23). A previous study mapped 4q21 breakpoints in 6 cases, including RS4;11, to a more limited region of 7 to 8 kb.1 Our present study with 19 cases indicates that the breakpoints are scat-
tered more widely on 4q21 than previously noted. Furthermore, the PFGE data also suggest that occasional t(4;11) acute leukemias may have their 4q21 breakpoints outside the 38-kb region mapped in our study. These data suggest that, at the genomic level, the 4q21 breakpoints are much less clustered than their 11q23 counterpart. Because of the distribution of these 4q21 breakpoints, no single cDNA probe we tested can detect all rearrangements by Southern blot analyses. The inability to show breakpoint clustering by Southern analysis in some cases is similar to that seen in t(11;19)(q21;p13) acute leukemia; studies of t(11;19) acute leukemia also show greater heterogeneity in the 19p13 breakpoints than in the 11q23 breakpoints. The distribution of 4q21 breakpoints in this study shows differential exon usage of the AF-4 gene in the fusion genes, because exons mapped adjacent to RS4;11 breakpoint may or may not be translocated during the chromosomal exchange. Additional study is needed to elucidate the biologic significance and clinical relevance of different exon usage.

The ubiquitous tissue expression of the AF-4/FEL gene is similar to the expression of the MLL gene. One major and one minor transcript of the AF-4 gene were observed in all the cell lines we tested. Several mechanisms can give rise to various mRNAs from a single gene. These mechanisms include several primary transcripts initiated at alternative promoters, differential termination or 3′ posttranscriptional processing (but not splicing), alternative splicing of the same primary transcript, and a combination of these events. Although the full coding region of the AF-4 cDNA has been cloned and two alternative methionines for translation initiation were identified, it is not certain whether the two normal transcripts are due to the alternative 5′ exon usage. The significance and possible differential regulation of more than one transcript may be subtle and difficult to resolve by Northern blot analysis. The AF-4 gene is a serine/proline-rich protein and shows no significant homology to any previously reported proteins. Interestingly, the 19p13 gene, ENL, involved in the t(11;19)(q23;p13) translocation is also serine/proline-rich. The der(11) fusion products resulting from the t(4;11) and t(11;19) translocations suggest that the amino terminal DNA binding domain of the 11q23 gene is important in the transformation event of these acute leukemias.

The expression of the AF-4/FEL gene and its der(4) and der(11) fusion transcripts in a number of t(4;11) cell lines have been described. Because of the presence of both fusion transcripts in t(4;11) cell lines, it is not certain which fusion product is more important in maintaining the malignant phenotype in these cell lines. Our results of RS4;11, MV4;11, and AN4;11 are consistent with previous reports that both der(4) and der(11) fusion transcripts are present. However, our study shows that whereas der(11) fusion transcripts are present in all four t(4;11) cell lines studied, a der(4) transcript is absent in the B1 cell line, a finding not previously reported. The der(4) chromosome is absent in the B1 cell line by cytogenetic analysis as well. These results argue for the importance of the der(11) fusion transcript in the process of leukemogenesis. In related studies, deletion of chromosome 11 DNA located 3′ (telomeric) to the breakpoint has been observed. In addition, an analysis of complex chromosomal translocations involving 11q23 showed that the der(11) is conserved among all the t(4;11), t(9;11), and t(11;19) cases studied. These studies suggest that the derivative 11 fusion protein is the pathogenetically important fusion product. Further studies using appropriate model systems such as transgenic mice may help to clarify this issue.

The fusion products in acute leukemias with t(4;11) and t(11;19) chromosomal translocations show many similarities and suggest that, in other leukemias in which the MLL/HRX gene is joined to DNA on different chromosomes, similar fusion products will be identified.

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