AF4p12, a Human Homologue to the furry Gene of Drosophila, as a Novel MLL Fusion Partner

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Abstract

More than 35 different partner genes with the mixed lineage leukemia (MLL) gene have been cloned from leukemia cells with translocations involving chromosome 11 band q23. In this study, we report on a novel fusion partner of the MLL gene, AF4p12, which we have identified as the human homologue to the furry gene of Drosophila. AF4p12, highly conserved in evolution, encodes a large protein of 3,105 amino acids. The expression of AF4p12 has been preferentially detected in colon, placenta, and brain tissues and in tumor cells of lymphoid origin. We show that the t(4;11)(p12;q23) translocation results in the creation of a chimeric RNA encoding a putative fusion protein containing 1,362 amino acids from the NH2-terminal part of MLL and 712 amino acids from the COOH-terminal part of AF4p12. FLT3 and HOXA9 genes are overexpressed in this leukemia. We found that the COOH-terminal part of AF4p12 fused to MLL contains a leucine zipper motif and exhibits transcriptional activation properties when fused to Gal4 DNA-binding domains in transient transfection assays. The AF4p12 fragment fused to MLL may contribute to the oncogenic activation of MLL, possibly through specific recruitment of the transcriptional machinery. (Cancer Res 2005; 65(15); 6521-5)

Introduction

11q23 abnormalities involving the mixed lineage leukemia (MLL) gene are found in a variety of hematologic malignancies including acute lymphoblastic leukemia (ALL), acute myeloid leukemia, and cases of myelodysplastic syndromes (1). Recently, we have shown that the involvement of the MLL gene is also recurrent in T-cell ALL, with an incidence of more than 8% (2). This rearrangement is also found in patients with therapy-related leukemia induced by topoisomerase-inhibiting epipodophyllotoxins. MLL, a histone methyltransferase protein, has been reported to assemble a supercomplex of several chromatin-modifying components. This transcriptional regulator is required for normal hematopoiesis and implicated in the maintenance of Hox gene expression (3). The most remarkable feature of MLL in leukemias is the diversity of its fusion partners. More than 35 MLL partner genes have been cloned from leukemia cells with various types of 11q23 translocations (1). These translocations result in the creation of fusion genes that encode chimeric proteins harboring the NH2-terminal amino acids of MLL and the COOH-terminal amino acids of its partner protein. The chimeric proteins localize in the nucleus and exhibit transforming activity. The partner proteins, which share no distinct common sequence motif and are functionally diverse, may arise from either nuclear or cytoplasmic origin (4). It has been shown that the acquisition of heterologous transcriptional effector or homeologization domains may be the mechanism that enables the oncogenic conversion of MLL, which supports the major role of fusion partners (3, 5). Furthermore, MLL translocations specify a distinct gene expression profile in leukemias (6). Although the dysregulation of Hox gene family members is the dominant mechanism in leukemic transformation induced by chimeric proteins, some oncogenic targets (such as the FLT3 gene) could be specific to particular lineages (7).

In the present study, we have identified the AF4p12 gene, a human homologue to the furry gene of Drosophila, as a novel fusion partner of the MLL gene in a patient with treatment-related ALL with t(4;11)(p12;q23) translocation.

Materials and Methods

Patient. A 67-year-old female was diagnosed in 2001 as having therapy-related ALL. This patient had been diagnosed in 1996 with a breast adenocarcinoma treated with 5-fluorouracil, mitoxantrone, cyclophosphamide, and radiotherapy, and in 2000 with an endometrial carcinoma treated with radiotherapy. At diagnosis, her WBC count was 262 109/L with 97% lymphoblasts. Leukemic cells expressed CD19, CD20, CD79a, CD13, CD38, and HLADR but not CD34. Cyto genetic analysis was done at the time of diagnosis. The karyotype of bone marrow cells showed 46, XX, t(4:11)(p12q23) [53]/46,XX [2]. The patient died 1 month after ALL diagnosis without responding to chemotherapy.

Culture conditions. The nonhematopoietic cell lines used in this study were cultured in DMEM containing 10% FCS, 0.03% l-glutamine, 100 µg/mL penicillin, and 100 µg/mL streptomycin sulfate. Hematopoietic cell lines were cultured in RPMI 1640 supplemented with FCS, l-glutamine, and antibiotics.

DNA and RNA analysis. High molecular weight DNA and RNA were extracted from the bone marrow leukemic cells following standard procedures. Southern blot analyses were hybridized with the MLLa cDNA probe (Fig. 1B) which encompasses the MLL breakpoint cluster region. Northern blot and human RNA Master Blot (Clontech Laboratories, Palo Alto, CA) were assayed for hybridization to an AF4p12 probe obtained by PCR amplification of human AF4p12 cDNA fragment (forward primer in exon 44: GTGCAGTCCACTACTGAGCC and reverse primer in exon 49: GGCCTTTTCCAATCTAGGG) and reverse primer in exon 49: GGCCTTTTCCAATCTAGGG. Panhandle PCR. Panhandle PCR amplification of MLL genomic breakpoint was done as previously described (8) apart from using the BgIII enzyme instead of BamHI to digest the DNA containing known MLL.

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sequence juxtaposed to unknown partner. Panhandle PCR products from the der(11) BgIII-rearranged allele (2.8 kbp) were subcloned into the TA cloning vector (Invitrogen, Paisley, United Kingdom) and individual clones were sequenced. To confirm the genomic breakpoint and the presence of MLL/AF4p12 fusion mRNA, exonic sequences from MLL upstream of the junction and from AF4p12 downstream of the junction were used to design primers. Forward primer from MLL exon 6 was GCAAGAGAAAAAGTGGGCTCCCCG and reverse primer from AF4p12 exon 49 was GGCCCT-TTCCAACATACGG. Reverse transcription was done as previously described. Fifty nanograms of genomic DNA and 2 μL of cDNA from leukemic cells and normal controls were amplified by long-distance PCR (Expand Long Template PCR System, Roche Diagnostics, Meylan, France). Thermocycling conditions were 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 7 minutes (or for 30 seconds for cDNA), and a final extension step at 68°C for 10 minutes.

**Transient transfections and reporter assays.** The 2.1 kbp AF4p12 fragment fused to MLL, obtained by reverse transcription-PCR (RT-PCR; sense primer: 5′-ACTAGTACTCGAGAAGACACATATGTTGGAGAAAAGAAA-3′; antisense primer which contained the AF4p12 termination codon: 5′-ACTAGTACTCGAGATCTGAGTTTGCACTCAT-3′; accession no. XM_093839.10), was subcloned into the XhoI site of the pSG5-Gal4-DBD expression vector, in frame with the yeast Gal4 DNA-binding domain coding sequence which contains Gal4 elements responsible for DNA binding, homodimerization, and nuclear localization. The pSG5-Gal4-DBD-AF4p12 construct was sequence checked. For transient transfections, HeLa cells were seeded (8 × 10⁴ cells per well) in 24-well plates and transfected 24 hours later using Exgen 500 (Euromedex, Mundolsheim, France) with the reporter constructs (pSG5-Gal4-luciferase, 200 ng), various amounts of the expression vector (pSG5-Gal4-DBD-AF4p12), and pRI-TK internal control vector (25 ng) under the control of the thymidine kinase promoter (Promega, Madison, WI). When increasing amounts of Gal4-AF4p12 expression vectors were transfected, the amount of transfected SV40 promoter was kept constant (250 ng) by the addition of an empty vector (pSG5-Gal4-DBD wild-type vector) to the transfection mixture. Forty-eight hours after transfection, the luciferase activity was quantified using equivalent amounts of each lysate analyzed with the Dual Luciferase Reporter Assay (Promega). In all experiments, done in triplicate, firefly luciferase activity was normalized by reference to the Renilla luciferase activity expressed by the pRL-TK vector.

**Results and Discussion.**

The t(4;11)(p12;q23) translocation involves the MLL and AF4p12 genes in treatment-related acute lymphoblastic leukemia. Fluorescence in situ hybridization (FISH) analysis on tumor metaphases, done with a commercial probe covering the entire MLL gene (Oncor), gave a split signal between the der(4) and der(11) chromosomes, confirming the involvement of MLL in this case (data not shown). Southern blot analysis provided strong evidence that the t(4;11) translocation cytogenetically detected in treatment-related ALL disrupts the MLL gene. Using the MLLa probe which encompasses the breakpoint cluster region, we detected rearranged fragments in addition to the germ line bands in BgIII- and HindIII-cleaved DNA (Fig. 1A). Southern blot analysis done with probes surrounding the MLL breakpoint probe allowed us to identify der(11) and der(4) rearranged bands (data not shown) and was used for restriction mapping (Fig. 1B).

To characterize the genomic sequences fused to MLL, we carried out a panhandle PCR amplification of MLL genomic breakpoints. In BgIII-cleaved tumor DNA, the MLLa probe detected a 2.8 kbp rearranged band derived from der(11). As expected, panhandle PCR identified a 2.8 kbp product (Fig. 1C). Blast search analysis showed that the nucleotide sequence of this panhandle product contained the MLLa probe (accession no. NT_086650) surrounded by human cDNA sequence (KIAA0826, accession no. XM_093839.10). In line with the nomenclature for MLL fusion partner genes, we named this gene AF4p12 (ALL-1 fused gene from chromosome 4p12). A comparison of human cDNA (XM_093839.10) and genomic DNA chromosome 4 sequences (NT_086650) revealed that the AF4p12 gene extends over 127 kbp and contains at least 61 exons.

To confirm the specificity of this 2.8 kbp fragment, the sequences of the panhandle PCR products were used to design primers encompassing the breakpoint (forward primer from MLL exon 6 and reverse primer from AF4p12 exon 49). As expected, with tumor DNA, we obtained a 3.8 kbp PCR product (data not shown). The sequence analysis of this fragment...
confirmed the sequence obtained by panhandle PCR. The breakpoint occurs 1,434 nucleotides downstream from exon 6 of the MLL gene and 2,429 nucleotides upstream from exon 49 of the AF4p12 gene (Fig. 1D).

FISH analysis on leukemic metaphases, done with a BAC clone (RP11-19132) covering the entire KIAA0826 cDNA, gave three signals, one on the normal chromosome 4, one on the derivative chromosome 4, and one on the derivative chromosome 11, confirming the involvement of AF4p12 in this translocation (data not shown).

Next, to identify the presence of the MLL/AF4p12 fusion mRNA, we did an RT-PCR analysis (forward primer from MLL exon 6 and reverse primer from AF4p12 exon 49) on tumor cells, which successfully yielded products of predicted size (206 bp; Fig. 2A). Sequence analysis of the fusion point revealed an in-frame fusion between MLL exon 6 and AF4p12 exon 49 (Fig. 2B). The putative MLL-AF4p12 fusion protein of 2,074 amino acids contained 1,362 amino acids from the NH2-terminal part of MLL and 712 amino acids from the COOH-terminal part of AF4p12.

Expression analysis of AF4p12 transcripts. The expression of AF4p12, quantified in a variety of human tissues, was expressed in a wide spectrum of normal tissues. The highest steady-state levels were observed in the colon, the placenta, and in different parts of adult brain (substantia nigra, putamen, and thalamus). A weaker expression was observed in the bladder, the kidney, and mammary glands (Fig. 2C). Northern blots of total RNA extracted from several cell lines showed the expression of a single species of AF4p12 mRNA of ~11 kb. A preferential expression was seen in cells of lymphoid origin (Fig. 2D).

AF4p12 is the human orthologue to the furry gene of Drosophila. The human KIAA0826 cDNA sequence identical to AF4p12 cDNA has 11,423 bp with a large open reading frame of 9,318 bp capable of encoding 3,105 amino acids. Comparison of AF4p12 cDNA sequence with those in the human data bank revealed the presence of a protein (accession no. CAB42442) highly homologous to AF4p12. These two proteins showed 60% identity at the amino acid level (74% with conservative substitution). Data bank analysis showed that two paralogues are found in human, rat, and chicken tissues, but only one orthologue gene is found for Drosophila, C. elegans, and Arabidopsis (Fig. 3). AF4p12 is highly conserved in evolution, a feature that indicates a large selective pressure to conserve the specific structural and functional characteristics of the protein. Sequences homologous to AF4p12 can be detected in lower species such as Drosophila, C. elegans, and Arabidopsis (Fig. 3). There is little information about the biological function of the homologues, as almost all were found in genome or EST sequencing projects. Furry, the Drosophila orthologue of AF4p12, is a large and complicated gene that encodes two proteins with no known functional domain. One contains 3,479 amino acids and the second is a truncated version of this large protein. Furry is important for maintaining the integrity of cellular extensions during morphogenesis (9) and for patterning the dendritic field of Drosophila sensory neurons (10). It has been shown that the fission yeast Furry-like protein, Mor2, is essential for cell morphogenesis and is required for the establishment of growth polarity (11). The furry gene and its homologues in yeast and C. elegans have been implicated as being essential for the function of the Ndr family of AGC kinases, essential regulators of the cell cycle and morphogenesis (12, 13).

The fusion domain of AF4p12 to the chimeric protein MLL-AF4p12 displays transcriptional activation potential. Protein motif analysis using the PROSITE database indicated that AF4p12 protein contains two potential leucine zipper regions (amino acids 1,229-1,250 and 2,923-2,944). The second leucine zipper motif is
very similar (77-100%) to the equivalent region in other species (Fig. 4A). Thus, there has been selective pressure to maintain the leucine zipper, suggesting that it has an important functional role. As it has been shown for AF10 (14), the leucine zipper motif is maintained in the MLL-AF4p12 fusion protein (Fig. 4B). Several partners of MLL (AF10, ENL, and ELL) display an ability to activate transcription under experimental conditions. Structure/function studies suggest that the gain of transcriptional effector properties contributes to the transformation of myeloid progenitors by the fusion protein (14–16).

To study the transcriptional properties of AF4p12, we did transient transfection assays in HeLa cells with the 2.1 kbp fragment of AF4p12 fused to MLL cloned into the pSG5-Gal4-DBD expression vector, in frame with the yeast Gal4 DNA-binding domain coding sequence. This 2.1 kbp fragment encodes the COOH-terminal part of the AF4p12 protein (amino acids 2,394-3,105). Transcriptional activity was measured as a function of luciferase protein production. As shown in Fig. 4C, the transfection of HeLa cells with the pSG5-Gal4-luciferase reporter, together with increasing amounts of the construct expressing Gal4-AF4p12, activated transcription in a dose-dependent way. These results indicate that the COOH-terminal part of the AF4p12 protein exhibited transcriptional activation properties when fused to Gal4 DNA-binding domains. The leucine zipper domain found in the COOH-terminal part of AF4p12 fused to MLL is the sole motif found by sequence analysis in this region and could be implicated in the oncogenic activation of MLL. Further experiments are required to explore the consequences of the deletion of the leucine zipper motif on the transcriptional activation properties of AF4p12.

In the present study, we have isolated a novel fusion partner of the MLL gene, AF4p12, localized on chromosome 4 band p12, in a treatment-related ALL with chromosomal abnormalities involving 11q23 and 4p12. The t(4;11)(p12q23) translocation was found as
the sole anomaly in all metaphases analyzed and thus might contribute to leukemogenesis. The AF4p12 fused domain might convert MLL into an oncoprotein by the acquisition of a transcriptional effector domain. Moreover, as previously described in leukemias with MLL fusion proteins (6, 17, 18), quantitative real-time PCR experiments showed that \textit{FLT3} and \textit{HOXA9} were significantly overexpressed in our patient (data not shown). Although the clinical effect of these infrequent fusions may be limited, their characterization will certainly promote additional mechanistic insights into MLL-mediated leukemogenesis.

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