LCX, Leukemia-associated Protein with a CXXC Domain, Is Fused to MLL in Acute Myeloid Leukemia with Trilineage Dysplasia

Having t(10;11)(q22;q23)

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ABSTRACT

There are a limited number of reports of acute myeloid leukemia (AML) with t(10;11)(q22;q23). We showed that the MLL gene on 11q23 was fused to the LCX (leukemia-associated protein with a CXXC domain) gene on 10q22 in a de novo adult AML-M2 with trilineage dysplasia having t(10;11)(q22;q23). LCX consisted of at least 12 exons and was predicted to encode a 2136-amino-acid protein with an estimated molecular mass of 235.3 kDa. The LCX protein had a zinc-binding CXXC domain that MLL also contains within a methyltransferase domain, three nuclear localization signals, an α-helical coiled-coil region, and two homologous regions to CG2083 proteins of Drosophila melanogaster. We found approximately 12-, 9.5-, and 7.5-kb transcripts of LCX. Expression of the 7.5-kb transcript was detected in fetal heart, lung, and brain, and in adult skeletal muscle, thymus, and ovary. Expression of the 9.5-kb transcript was detected in fetal lung and brain and in adult ovary. Expression of the 12-kb transcript was detected in fetal heart and brain and in adult thymus and ovary. LCX was expressed in 8 of 22 leukemic cell lines, but not in EBV-induced normal B-cell lines. The MLL-LCX fusion protein lacked a CXXC domain of LCX, but retained an α-helical coiled-coil region at the COOH terminus, similar to MLL-SEPTING, MLL-CD-CRELI, MLL-AF1p/Eps15, and MLL-AF6, which suggests that these fusion proteins are involved in the pathogenesis of 11q23-associated leukemia through similar mechanisms.

INTRODUCTION

A variety of chromosomal aberrations have been reported in hematological malignancies (1). Recent studies have demonstrated that several chromosomal rearrangements and molecular abnormalities are strongly associated with distinct clinical subgroups and are predictive of clinical features and therapeutic responses (1–3). The 11q23 translocation is a frequent cytogenetic abnormality found in hematological malignancies, occurring in 5–6% of patients with AML, 7–10% of patients with ALL, 60–70% of infants with acute leukemias, and in most patients with therapy-related leukemias induced by inhibitors of topoisomerase II (1). At least 40 chromosomal regions for partners of 11q23 have been observed. The MLL gene (Ref. 4; also called ALL-1 and HRX) has been identified in 11q23 translocations (5, 6), and at least 28 partner genes for MLL have been cloned from leukemias with various 11q23 translocations that formed fusion transcripts with MLL. The functions of some genes have been clarified, including those for a Ras-binding protein (AF6; Ref. 7), an RNA polymerase II elongation factor (ELL/MEN; Ref. 8), transcriptional coactivator/histone acetyltransferase (CBP and p300; Refs. 9–11), and ABL and eps8-binding protein (ABI-1; Ref. 12). Recently, it was shown that both the “knock-in” of AF9 into MLL and the HRX-ENL (MLL-ENL) fusion caused myeloid malignancies in mice (13, 14).

There are a limited number of reports of AML with t(10;11)(q22;q23) (15–17), but no partner gene on 10q22 has been identified yet. In this study, we analyzed a de novo AML-M2 with trilineage dysplasia having t(10;11)(q22;q23), and identified LCX (leukemia-associated protein with a CXXC domain) as a novel fusion partner of the MLL gene.

MATERIALS AND METHODS

Patient. A 67-year-old male presented with pancytopenia. He was diagnosed as having AML-M2 with trilineage dysplasia that expressed CD7, CD13, CD19, CD33 and HLA-DR and was cytogenetically characterized as 46, XY, t(10;11)(q22;q23) in 20 BM cells. He achieved complete remission with intensive chemotherapy but relapsed 24 months later and died of infection 26 months after the diagnosis.

Cell Lines. B-precursor ALL (REH and NALM26), B-ALL (BALM14, BALM9, BJAB, and A4/FUK), T-ALL (L-SMY and MOLT-14), acute monocytic leukemia (THP-1), AML (HEL, KG-1, ML-1, Kasumi-3, and SN-1), acute megakaryoblastic leukemia (CMS and CMY), chronic myelogenous leukemia (K562, MOLM7, TS9:22 and SS9:22) cell lines and EBV-induced normal B-cell lines (EB1 and EB2) were analyzed by Northern blot analysis. MOLT-14 was also used to generate a PCR product at the 5′ region of LCX.

Southern Blot Analysis. High molecular weight DNA was extracted from BM at diagnosis and 10 μg of DNA was analyzed with a 0.9-kb BamHI fragment (designated probe x (18)) and a 523-bp LCX cDNA probe spanning exons 7–10 (nucleotides 4700–5222) as reported previously (19).

cDNA Panhandle PCR. Total RNA was extracted from BM cells of the patient using Isogen LS (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and analyzed by modified cDNA panhandle PCR method as follows (20–22).

In brief, first-strand cDNAs were synthesized with MLL-random hexamer oligonucleotides, MLL-N. After primer 1 extension with MLL-1, and extension in stem-loop templates, sample was amplified by first PCR with MML1 and MLL2. Then, one-twenty-fifth of the products were used for nested PCR with MLL-3 and MLL-4. Four μl of the products were electrophoresed in a 3% agarose gel. The MLL-random hexamer oligonucleotides and primers used were as follows: MLL-N, 5′-TCAGAGAAGAGTTAGAAAGGAATGTC-CTC-TNNNNN-3′; MLL-1, 5′-TGAAGAAGTTGGACACT-3′; MLL-2, 5′-TACTAAGACCGGCAAAGA-3′; MLL-3, 5′-GTCAAGAAACTTACCATC-3′; and MLL-4, 5′-AGCCAGAAGAACAGACCT-3′.

RT-PCR and Genomic PCR. Four μg of total RNA was reverse transcribed to cDNA in a total volume of 33 μl with random hexamers using the Ready-To-Go You-Prime First-Strand beads.

Received 12/17/01; accepted 5/10/02.

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1 Supported by Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan, a Grant-in-Aid for Scientific Research on Priority Areas and Grant-in-Aid for Scientific Research (B) and (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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3 The abbreviations used are: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; BM, bone marrow; RT, reverse transcription; FISH, fluorescence in situ hybridization; ORF, open reading frame; MTDM, DNA methyltransferase; CGBP, human C-terminal binding protein.
amplified under the same condition as RT-PCR. The primers used were as follows: ALL-6GS3, 5'-TGGACATGTTGCAGGTAGTCG-3'; UN10GA1, 5'-CAGACACCTTGTCCTCCAC-3'; ALL-6GA1, 5'-GGATTACAGACCC-CCACTACA-3'; and UN10GS3, 5'-CATGCAACACCCATCATGCC-3'.

**Nucleotide Sequencing.** Nucleotide sequences of phage clones, PCR products, and, if necessary, subcloned PCR products were analyzed as described previously (19).

**Screening of the cDNA Libraries.** The unknown 189-bp novel cDNA probe derived from the MLL-LCX chimeric products was used for screening cDNA libraries derived from the BALM14 cell line (19) and human fetal lung 5'-STRETCH cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA).

**FISH Analysis.** Chromosomal mapping of the BAC clone RP11-119F7 was performed by the FISH method (23). FISH analysis of the patient's leukemic cells using a YAC clone specific to 11q23 and RP11-119F7 was carried out as described previously (23).

**Northern Blot Analysis.** Each 1-μg aliquot of mRNA derived from REH, BALM14, BALM9, BJAB, A4/FUK, NALM26, and SN-1, each 20-μg aliquot of total RNA derived from the others; and multiple human tissue Northern blots (CLONTECH Laboratories, Inc.) were analyzed with the same LCX cDNA probe (32P-labeled) as used for screening a cDNA library (19).

**RESULTS**

**Cloning and Identification of the MLL Fusion cDNA in t(10;11)(q22;q23).** Southern blot analysis of DNA prepared from the leukemic cells of the patient using probe x showed a chromosomal breakpoint within the breakpoint cluster region of the MLL gene at 11q23 (data not shown). To clone the chimeric transcripts, we performed cDNA panhandle PCR analysis of total RNA from BM mononuclear cells of the patient at diagnosis. Three products were obtained, and one of them was found to represent a fusion transcript of MLL (Fig. 1A). This product, 248 bp in size, contained a fusion product of a 36-bp sequence of exon 5 [nomenclature is updated (24) but used according to the commonly used nomenclature (25)] in the MLL gene at the 5' region to an unknown 102-bp sequence, but the reading frame terminated shortly after the fusion point [Fig. 1C(a)]. To verify that this fusion product was indeed expressed in the leukemic cells, we performed the seminested PCR analysis using primers MLL-2 and UN10A for the first-step PCR analysis and using primers MLL-4 and UN10A for the seminested PCR analysis (Fig. 1, B and C(b)). Three products were obtained, and one of them, 299 bp in size, contained a fusion product of a 110-bp sequence of exons 5–6 in the MLL gene at the 5' region to a 189-bp sequence at the 3' region [Fig. 1C(b)]. The 189-bp sequence contained an unknown 90-bp sequence and a 99-bp sequence derived from panhandle PCR product. The ORF was preserved in this 299-bp fusion transcript. The other 225-bp product contained a fusion product of a 36-bp sequence of exon 5 in the MLL gene at the 5' region to the unknown 189-bp sequence found in the 299-bp fusion transcript at the 3' region, but the reading frame terminated shortly after the fusion point. The same fusion transcript that was detected by panhandle PCR was also detected by seminested PCR, but this product was very minor. These different fusion products may have arisen through alternative splicing.

**Isolation and Characterization of the LCX Gene.** The unknown 189-bp sequence identified in the chimeric transcript was used as a probe to screen cDNA libraries derived from the BALM14 cell line and human fetal lung. We isolated four overlapping clones by several rounds of screening (Fig. 2A). An RT-PCR analysis of the MOLT-14 cell line using primers ES2S and L16RA1 yielded a product of 559 bp (ES1) that confirmed the 5' end of the gene and completed the ORF. These clones were assembled into one contiguous sequence, resulting in a total size of 8497 nucleotides (Figs. 2A and 5; GenBank...
PSORT II indicated three bipartite nuclear localization sites and an estimated molecular mass of 235.3 kDa. The LCX gene (RP11-119F7) was assigned to band 10q22 (arrows). B, the same metaphase spread stained with 4’,6-diamidino-2-phenylindole (DAPI). DAPI images were inverted and enhanced in terms of band image contrast. C, arrowheads indicate the fusion signal of the 13HH4 (MLL) and RP11-119F7 (LCX).

Accession no. AF430147. This sequence was compared with known genomic sequences in public databases and was found to contain at least 12 exons. Clone B14-4, lacking exons 5–6, was shown to be alternatively spliced. Computer analysis indicated a 6408-bp ORF encoding a 2136-amino-acid protein with an estimated molecular mass of 235.3 kDa.

The prediction of protein localization sites in cells using the PSORT II indicated three bipartite nuclear localization sites and an α-helical coiled-coil region (Fig. 2B). One of three nuclear localization sites was fused to MLL, similar to other MLL partners such as AF3p21, AF4, AF9, AF15q14, ENL, and ELL (6, 26–29). Motif analysis using the Pfam database showed a cysteine-rich domain. This domain exhibits a high degree of homology to a zinc-binding CXXC domain, which is conserved in DNA methyltransferase (MTDM), MLL, and others (Figs. 2B and 6). Sequence comparisons using the FASTA file showed two homologous regions shared by Drosophila CG2083 (Fig. 2B). Therefore, we designated this gene LCX (leukemia-associated protein with a CXXC domain).

**Molecular Cloning and Sequence Analysis of Chromosomal Breakpoints.** Restriction mapping by Southern blot analysis using the LCX probe, as well as probe x (data not shown), allowed us to locate both breakpoints of MLL and LCX (Fig. 2C). We cloned each genomic junction of the breakpoints by genomic PCR using two sets of primers (ALL-6GS3 and UN10GA1, and ALL-6GA1 and UN10GS3) followed by sequencing (Fig. 2D). In the der(10), an 8-bp sequence of the LCX intron 8 at the 5’ region of the breakpoint was deleted.

**Chromosomal Assignment of the LCX Gene.** To assign the chromosomal location of the LCX gene, we found a BAC clone (RP11-119F7) after comparing the LCX gene with known genomic sequences in public databases. The BAC clone showed specific signals at 10q22 in all of the metaphase cells tested (Fig. 3, A and B). We next performed a FISH analysis of leukemic metaphase using a YAC clone specific to 11q23 (13HH4) and RP11-119F7, and confirmed fusion signals in both der(10) and der(11) (Fig. 3C).

**Expression of the LCX Gene.** To examine expression of the LCX gene, we performed Northern blot analysis of polyadenylated RNA from various human tissues using the LCX probe spanning exons 7–10, and detected three types of transcripts of approximately 7.5-, 9.5-, and 12-kb (Fig. 4, A and B). Expression of the 7.5-kb transcript was detected in fetal heart, lung and brain and in adult skeletal muscle, thymus, and ovary. Expression of the 9.5-kb transcript was detected in fetal lung and brain and in adult ovary. Expression of the 12-kb transcript was detected in fetal heart and brain and in adult thymus and ovary. Although we repeated additional rounds of library screenings, no clones differed from the sequences found here. We next performed Northern blot analysis using various fragments of LCX. A fragment within exon 4 of LCX detected all three bands; however, a fragment within exon 2 of LCX detected only a 9.5-kb band (data not shown), which suggested that the 8459 bp of the identified LCX sequence was compatible with the 9.5-kb transcript. Expression of these transcripts was found in one of two T-ALL cell lines, two of four B-ALL cell lines, one of two B-precursor ALL cell lines, two of five AML cell lines, none of two acute megakaryoblastic leukemia cell lines, one of one acute monocytic leukemia cell lines, one of four chronic myelogenous leukemia cell lines, and none of two EBV-induced normal B-cell lines (Fig. 4C).

**DISCUSSION**

In this study, we isolated a novel fusion partner of the MLL gene, LCX, in a de novo AML-M2 with trilineage dysplasia having t(10;11)(q22;q23). The t(10;11)(q22;q23) is a rare chromosomal translocation reported in only four patients, three of whom were characterized as having complex karyotypes involving t(10;11)(q22;q23) (15–17). All of the patients were adult and received diagnoses of AML-M4 or -M5, and the above three patients died within 11 months after diagnosis. Although MLL gene rearrangement was detected in all four of the patients reported, the fusion partners of the MLL in these patients were not analyzed molecularly. To clarify the clinical characteristics of AML with t(10;11)(q22;q23), more cases with the fusion transcripts are needed.

In the current patient’s leukemic cells, three different MLL-LCX fusion transcripts were detected. By sequencing analysis, we found an
in-frame fusion of exon 6 of MLL and exon 9 of LCX. Although two additional out-of-frame fusion transcripts that may have produced truncated MLL proteins were expressed in the patient’s leukemic cells, these fusion transcripts were not thought to be crucial to leukemogenesis, as reported previously (13). On the other hand, we could not detect LCX-MLL fusion transcripts by RT-PCR (data not shown), which suggested that the 5'-MLL-LCX-3' in-frame fusion transcript is crucial to leukemogenesis, as described previously (14).

Fig. 4. Northern blot analysis of RNAs from fetal (A) and adult (B) human tissues, and from leukemic and EBV-induced normal B-cell lines (C). 28S, 28S ribosomal RNA.

Fig. 5. Sequencing of LCX. Arrowhead, the breakpoint. Shaded box, a CXXC domain. Boxed areas, nuclear localization signal sites. Underlined residues, a potential α-helical coiled-coil region.
The LCX protein has a zinc-binding CXXC domain, which is one of some zinc-finger domains but different from the plant homeodomain finger encoded by various genes including other partners, AF10 and AF17 (30, 31). The CXXC domain contains eight conserved cysteine residues that bind zinc, and belongs to a small group of proteins, including IDAX, a human homologue of Idax for the inhibition of the Dvl and Axin complex (32), Drosophila CG9973, MTDMD (33), methyl-CpG binding domain protein 1 (MBD1/PCMI; Refs. 34, 35), CGBP (36), and MLL (Fig. 6; Refs. 37, 38). In some proteins such as MTDMD and CGBP, it was suggested that this domain was associated with the distinction between unmethylated and methylated DNA (36, 39). On the other hand, the same region of MLL is part of a domain with transcriptional repressor activity in vivo (40). Because this domain is not included in the fusion protein, the replacement of this domain of LCX by the one in MLL might be involved in leukemogenesis.

The LCX and Drosophila CG2083 proteins have two homologous regions. Drosophila CG2083 belongs to the Drosophila melanogaster genomic scaffold section, but its function has not been clarified. Although the functions of the two regions remain unknown, these homologous regions may include potential common functional domains of LCX and Drosophila CG2083. In particular, the region at the COOH terminus was retained in MLL-LCX; therefore, this region may be involved in leukemogenesis.

Expression of the LCX gene was found in fetal heart, lung, and brain, but not in adult heart, lung, or brain, which suggests that LCX may play a key role in the fetal development of these tissues. Three different bands that were considered to be derived from three isoforms of LCX were detected by Northern blot analysis. The 8459 bp of the identified LCX sequence was considered to be compatible with the 9.5-kb transcript, but we could not determine the precise structure of the other two transcripts. It is suggested that these two transcripts shared a common 3′ region of exon 4; however, these transcripts may lack exon 2 or have a different exon 2, similar to AF4 (6). The LCX gene was expressed in 8 of 22 leukemic cell lines but not in EBV-induced normal B-cell lines. Further analysis is needed to clarify the role of LCX in normal development and leukemogenesis.

The function of the MLL fusion protein remains unknown, although a few interesting reports have been published (13, 14, 41). In the MLL-ENL fusion protein, a transcriptional activation domain in ENL was found to be important for leukemogenesis (14). On the other hand, it was shown that the artificial MLL-lacZ fusion gene was sufficient to cause leukemia with long latency in mice, and it seemed to be important that lacZ could contribute to leukemogenesis through oligomerization of the Mll-lacZ fusion protein (41). Similarly, the α-helical coiled-coil region at the retained COOH terminus of fusion proteins, which was found in LCX as well as in SEPTIN6, CDCREL1, AF1p/Epsl15, and AF6 (22, 42–45), might contribute to leukemogenesis. Functional analyses of the LCX and MLL-LCX fusion proteins may provide new insights into leukemogenesis involving 11q23 translocations.

ACKNOWLEDGMENTS

We express our appreciation to Shoko Sohma, Hisae Soga and Yumiko Takatani for technical assistance.

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