Molecular characterization of the *MLL-SEPT6* fusion gene in acute myeloid leukemia: identification of novel fusion transcripts and cloning of genomic breakpoint junctions

Nuno Cerveira,[1] Francesca Micci,[2] Joana Santos,[1] Manuela Pinheiro,[1] Cecília Correia,[1] Susana Lisboa,[1] Susana Bizarro,[1] Lucília Norton,[3] Anders Glomstein,[4] Ann E. Åsberg,[5] Sverre Heim,[2,6] and Manuel R. Teixeira[1,7]

[1]Department of Genetics, Portuguese Oncology Institute, Porto, Portugal; [2]Section for Cancer Cytogenetics, Department of Medical Genetics, Radiumhospitalet-Rikshospitalet Medical Center, Oslo, Norway; [3]Department of Pediatrics, Portuguese Oncology Institute, Porto, Portugal; [4]Department of Pediatrics, Radiumhospitalet-Rikshospitalet Medical Center, Oslo, Norway; [5]Department of Pediatrics, St Olav University Hospital, Trondheim, Norway; [6]Faculty Division, The Norwegian Radium Hospital, Medical Faculty, University of Oslo, Norway and [7]Biomedical Sciences Institute (ICBAS), University of Porto, Porto, Portugal

ABSTRACT

One of the *MLL* fusion partners in leukemia is the *SEPT6* gene, which belongs to the evolutionarily conserved family of genes of septins. In this work we aimed to characterize at both the RNA and DNA levels three acute myeloid leukemias with cytogenetic evidence of a rearrangement between 11q23 and Xq24. Molecular analysis led to the identification of several *MLL-SEPT6* fusion transcripts in all cases, including a novel *MLL-SEPT6* rearrangement (*MLL* exon 6 fused with *SEPT6* exon 2). Genomic DNA breakpoints were found inside or near Alu or LINE repeats in the MLL breakpoint cluster region, whereas the breakpoint junctions in the *SEPT6* intron 1 mapped to the vicinity of GC-rich low-complexity repeats, Alu repeats, and a topoisomerase II consensus cleavage site. These data suggest that a non-homologous end-joining repair mechanism may be involved in the generation of *MLL-SEPT6* rearrangements in acute myeloid leukemia.

Key words: *MLL-SEPT6*, fusion oncogene, fusion transcript, genomic breakpoint, acute myeloid leukemia.

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Introduction

Abnormalities of 11q23, resulting in fusion of the mixed lineage leukemia (*MLL*) gene with numerous translocation partners, are found in primary acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), as well as in secondary, topoisomerase II inhibitor–related leukemia.[1] The MLL gene codes for a multi-domain protein that is a major regulator of class I homeobox (*HOX*) gene expression.[2] *HOX* genes play a key role in the regulation of hematopoietic development and altered patterns of MLL activity might cause abnormal *HOX* gene expression in hematopoietic stem cells, resulting in blockage of hematopoietic maturation and, eventually, leukemia.[3]

To date, more than 50 *MLL* fusion partners have been cloned.[4] Five of these, *SEPT2*, *SEPT5*, *SEPT6*, *SEPT9*, and *SEPT11*, code for septins[4,5] and belong to an evolutionarily conserved family of genes with 13 members identified so far.[6] As a consequence, the septins are the protein family most frequently involved in rearrangements with *MLL*, suggesting that their involvement in *MLL*-related leukemia is anything but a chance event.[5,6] Septins are conserved GTP-binding proteins that assemble into homo- and hetero-oligomers and filaments with key roles in cell division cytoskeletal dynamics and secretion.[6]

To our knowledge, the fusion between *MLL* and *SEPT6* has so far only been described in 10 AML patients.[7-13] However, the genomic breakpoint junction was only characterized in 2 patients.[9,10] We present a detailed RNA and DNA analysis in 3 new AML patients with the *MLL-SEPT6* rearrangement, one of them showing a novel in-frame fusion transcript.

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Correspondence: Manuel R. Teixeira, Department of Genetics, Portuguese Oncology Institute, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. E-mail: mteixeir@ipoporto.min-saude.pt
The online version of this article contains a supplementary appendix.

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Design and Methods

Patients

The study comprised three cases of AML in which an Xq24-11q23 rearrangement was detected by karyotyping of the leukemic cells. A summary of the clinical, cytogenetic, and molecular genetic data is provided in *Online Supplementary Table S1*.

Patient 1

A 17-month-old girl was admitted to the Portuguese Oncology Institute (Porto, Portugal) in September 2006 because of mucosal and cutaneous pallor and right leg pain, with refusal to walk. Peripheral blood analysis revealed leukocytosis, anemia, and thrombocytopenia, with 28% circulating blasts. The bone marrow was hypercellular containing 82% blasts mainly positive for CD4, CD11b, CD11c, CD15, CD33, CD64, CD65, HLA-DR, and LIS, and negative for CD2, cCD3, CD13, CD14, CD16, CD34, CD36, CD56, CD79a, CD117, and TdT. A diagnosis of acute myeloblastic leukemia with maturation was established. Cytogenetic analysis showed evidence of a rearrangement involving the long arm of chromosome 11 (11q23), with additional material of unknown origin in 11q23 and Xp11. She was treated according to the ELAM 02 protocol (aracytine, mitoxantrone and methotrexate) and entered complete remission after induction chemotherapy. In April 2007, she was submitted to allogeneic bone marrow transplantation and has no evidence of disease at the time of writing.

Patient 2

A previously healthy, 12-month-old boy was admitted to the Rikshospitalet Department of Paediatrics (Oslo, Norway) in March 1997 after a short history of skin bleedings. Peripheral blood analysis revealed leukocytosis (90% myeloblasts), anemia and thrombocytopenia. The bone marrow was hypercellular with more than 90% blasts that were positive for CD45, CD33, CD15, CD13 and HLA-DR and a diagnosis of acute myeloid leukemia was made. Cytogenetic analysis of bone marrow cells revealed a clonal t(X;11)(q24;q23). The spinal fluid contained 14×10^9/L leukocytes, morphologically described as reactive; immunocytochemistry was unsuccessful. The patient was treated according to the Nordic protocol NOPHO-AML 93,[1] and went into complete remission after the first course containing intrathecal methotrexate and intravenous 6-thioguanine, cytarabine, etoposide and doxorubicin. This treatment was discontinued in August 1997 after a total of 6 courses without major complications. In April 2000, he was readmitted with a bone marrow relapse after a few weeks with infections and falling blood values. The leukemic blasts showed the same markers as in 1997.

He was reinduced with the same NOPHO protocol and went into second complete remission after the first course. In September 2000, after two more courses, he underwent bone marrow transplantation from his older, HLA-identical sister donor. There were no procedural complications and at last follow-up, in October 2007, he was still in second complete remission. The boy is active and with no sequelae.

Patient 3

This patient was a newborn boy, the third child of healthy parents, delivered at term in October 1997 after an uncomplicated pregnancy. The boy was transferred to the Department of Paediatrics, St Olav University Hospital (Trondheim, Norway) on suspicion of congenital leukemia. Initial physical examination revealed widespread, firm, bluish cutaneous nodules, petecchiae and hepatosplenomegaly. The peripheral blood values were Hb 16.2 g/dL, platelets 100×10^9/L and WBC 340×10^9/L (90% with monoblastic morphology). The cerebrospinal fluid contained 101×10^9/L cells that by flow cytometric analysis were shown to be monoblasts. Immunophenotyping of peripheral blood confirmed a 90% population of cells expressing CD33, CD13, CD15, CD14 and partly CD24 and HLA-DR. Because of severe tumor lysis syn-

Supplementary Table S1. Sequence of the primers used for the RNA and DNA analyses.

Primer	*Target*	*Sequence*	*Position*
MLL-E5S	*MLL* exon 5	5'-GAGGATCCTGCCCCAAAGAAAAG-3'	3771_3793
MLL-E6S	*MLL* exon 6	5'-GCAAACAGAAAAAAGTGGCTCCCCG-3'	4048_4072
MLL-I6S-In	*MLL* exon6/intron 6	5'-AAACCAAAAGAAAAGGTGAGGAGA-3'	4095_4109/1_9
MLL-E7S-01	*MLL* exon 7	5'-CCTCCGGTCAATAAGCAGGAGAATG-3'	4119_4143
MLL-E7S-02	*MLL* exon 7	5'-TCAGCACTCTCTCCAATGG-3'	4162_4180
MLL-E8S	*MLL* exon 8	5'-GCAGAAAATGTGTGGGAGATGGGAG-3'	4254_4278
MLL-E8S-In	*MLL* exon 8	5'-TTCCTATAACACCCAGGGTGGT-3'	4300_4321
MLL-I6-01-O	*MLL* intron 6	5'-CAAAGCAAAACACTGTCTCCAAAA-3'	419_442
MLL-I6-01-In	*MLL* intron 6	5'-AAAATTTAGGCTTGGCAAGGC-3'	443_463
MLL-I6-02-O	*MLL* intron 6	5'-GTTTCTTCCTTGTTGCTTTTCCC-3'	1079_1101
MLL-I6-02-In	*MLL* intron 6	5'-TGGCCCCACATGTTCTAGC-3'	1109_1127
MLL-I8-01-O	*MLL* intron 8	5'-AGAAATAAATACATGTTGGGTGGCA-3'	438_462
MLL-I8-01-In	*MLL* intron 8	5'-GAGGTGAAGGGAGGGTGTCTG-3'	467_487
MLL-I8-02-O	*MLL* intron 8	5'-CAGGCGGATCACAAGGTCA-3'	878_896
MLL-I8-02-In1	*MLL* intron 8	5'-CACAGTGAAACCCCGTCTCTATT-3'	921_943
MLL-I8-02-In2	*MLL* intron 8	5'-TCTGGAAGGATTCACACCAAAA-3'	1331_1352
MLL-I8-03-O	*MLL* intron 8	5'-TGTTGAGCAGTCAGTGAGACACAA-3'	1970_1993
MLL-I8-03-In1	*MLL* intron 8	5'-CCCTGCCCACTTGCCAT-3'	2012_2028
MLL-I8-03-In2	*MLL* intron 8	5'-TGCCTGCACTGCACTCCTAA-3'	2394_2413
MLL-I8-04-O	*MLL* intron 8	5'-GAGAATCGCTTGAACCCAGG-3'	3113_3132
MLL-I8-04-In	*MLL* intron 8	5'-GATCGCACCACTGCACCC-3'	3156_3173
SEPT6-E2AS-01	*SEPT6* exon 2	5'-CCTGGCTGACGGACTTATTCACC-3'	361_383
SEPT6-E2AS-02	*SEPT6* exon 2	5'-GCACAGGATGTTGAAGCAGA-3'	387_406
SEPT6-E2AS-03	*SEPT6* exon 2	5'-TTGCCCAAACCTGTCTCTCC-3'	410_429
SEP06-I2LDAS-01	*SEPT6* intron 1	5'-CAGCTATACCATCTCTGAAATGCAGGT-3'	1657_1683
SEP06-I2LDAS-02	*SEPT6* intron 1	5'-GGCCGATCAGTGCCCAGTGAATATGTG-3'	4987_5013
SEP06-I2LDAS-03	*SEPT6* intron 1	5'-ATAGATCGACCTTCCCTACGACTCTCTCCC-3'	7718_7747
SEP06-I2LDAS-04	*SEPT6* intron 1	5'-GCAAAGGTAGGAAGGACAGAAGGACAC-3'	11924_11950
SEP06-I2LDAS-05	*SEPT6* intron 1	5'-CCGTCAGCTTGGAAATCACAGATTCTT-3'	17222_17248
SEP06-I2AS-01	*SEPT6* intron 1	5'-ATACACACACAGACGCAGTCACAT-3'	528_551
SEP06-I2AS-02	*SEPT6* intron 1	5'-CACACCACAGAGGTGAGCACA-3'	660_680
SEP06-I2AS-03	*SEPT6* intron 1	5'-CACCTACAGGCCAGCCAACT-3'	751_770
SEP06-I2AS-04	*SEPT6* intron 1	5'-GCATCATCACAGAGAATGTCCC-3'	1531_1552
SEP06-I2AS-05	*SEPT6* intron 1	5'-GGAGAATCGCTTGAACCTGG-3'	2427_2446
SEP06-I2AS-06	*SEPT6* intron 1	5'-CACCATGTTGGCCAGGCT-3'	3132_3149
SEP06-I2AS-07	*SEPT6* intron 1	5'-GGCTTGCCCTGTGCCTT-3'	3767_3783
SEP06-I2AS-08	*SEPT6* intron 1	5'-AGTTTGGGAATACCTTTTTCCAGAG-3'	5377_5401
SEP06-I2AS-09	*SEPT6* intron 1	5'-TCGTATCACCCACTGACCAGC-3'	6034_6054
SEP06-I2AS-10	*SEPT6* intron 1	5'-TGGCTTGATGCTGGTCAGG-3'	7332_7350
SEP06-I2AS-11	*SEPT6* intron 1	5'-GGCAATATCTGAAGGGTTGTTTCT-3'	7782_7805
SEP06-I2AS-12	*SEPT6* intron 1	5'-GAGAATCGCTTGAACGCAGG-3'	9896_9915
SEP06-I2AS-13	*SEPT6* intron 1	5'-TGGGAACTGAGGGTGCATCT-3'	10267_10286
SEP06-I2AS-14	*SEPT6* intron 1	5'-GAGTAGTCGGTATGCTTCCCTATTG-3'	10812_10836
SEP06-I2AS-15	*SEPT6* intron 1	5'-TCAGTCCGCATTGTTCAGAGTT-3'	11957_11978
SEP06-I2AS-16	*SEPT6* intron 1	5'-CCACGCCCAGGTAATTTTTG-3'	12695_12714
SEP06-I2AS-17	*SEPT6* intron 1	5'-CTAGGAGCAGGAAGACATAGGAGG-3'	13639_13662
SEP06-I2AS-18	*SEPT6* intron 1	5'-AACAAAGTAAGATGCAAGATTCCCA-3'	14411_14435
SEP06-I2AS-19	*SEPT6* intron 1	5'-TGTGGTGAGCATTCAATCAGC-3'	16021_16041
SEP06-I2AS-20	*SEPT6* intron 1	5'-CCTTCCACATCTGCCATCTGA-3'	17018_17038

E: exon; I: intron; S: sense; AS: antisense; O: outer; In: inner.

drome and respiratory insufficiency, initially no bone marrow sample was taken, but cytogenetic analysis of cells in the peripheral blood revealed an insertion ins(X;11)(q24;q13q23). A diagnosis of acute myeloid leukemia was made and treatment was started according to the NOPHO-AML 93 protocol with a prophase of low-dose cytarabine and intrathecal methotrexate.[1] Remission was achieved after the first A1 block. Because of prolonged pancytopenia during the treatment period, chemotherapy dosages had to be reduced. CNS-directed therapy was continued for one year. One year after systemic chemotherapy had been stopped, the boy experienced a local bone marrow relapse, confirmed by immunophenotyping. He went into a second complete remission after one course of cytarabine, etoposide, thioguanine and intrathecal methotrexate, and continued treatment according to NOPHO-AML 93 until he received a bone marrow transplantation five months later. Only minor graft-versus-host disease was subsequently observed and he is now, eight years later, doing well but is being evaluated for secondary short stature.

Chromosome banding and molecular cytogenetics

The diagnostic bone marrow samples (from patient 3, the diagnostic culture was of peripheral blood blasts) were cultured for 24 hours in RPMI 1640 medium with GlutaMAX-I (Invitrogen, London, UK) supplemented with 20% fetal bovine serum (Invitrogen, London, UK). Chromosome preparations were made by standard methods and banded by trypsin-Leishman. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[2]

FISH analysis for possible *MLL* rearrangement was performed using the LSI MLL Dual-Colour, Break-Apart Probe (Vysis, Downers Grove, USA) according to the manufacturer's instructions. In 2 cases (patients 2 and 3), initial characterization of the chromosomal breakpoints in the long arm of the X chromosome was performed using bacterial artificial chromosome (BAC) clones RP11-379J1 (maps to the *SEPT6* gene) and CTD-2334F19 (maps to the 5' region of the *SEPT6* gene). The clones were retrieved from the RP11 Human BAC library and Cal Tech Human BAC library D (*P. de Jong libraries http://bacpac.chori.org/home.htm*.

They were cultured in selected media and DNA was isolated following a standard protocol consisting of alkaline lysis, neutralization, and ethanol precipitation.

RNA and DNA extraction

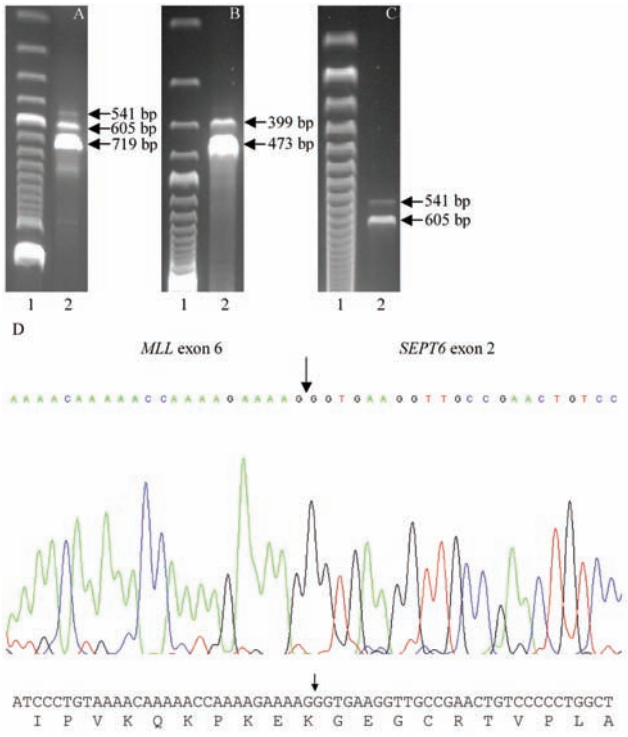
High molecular weight DNA and RNA were extracted from the bone marrow sample (patient 1) or from fixed cell suspension remaining after completion of the cytogenetic analysis (patients 2 and 3; cells from peripheral blood) using 1 mL of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA) according to the manufacturer's instructions.

Reverse-transcription polymerase chain-reaction (RT-PCR)

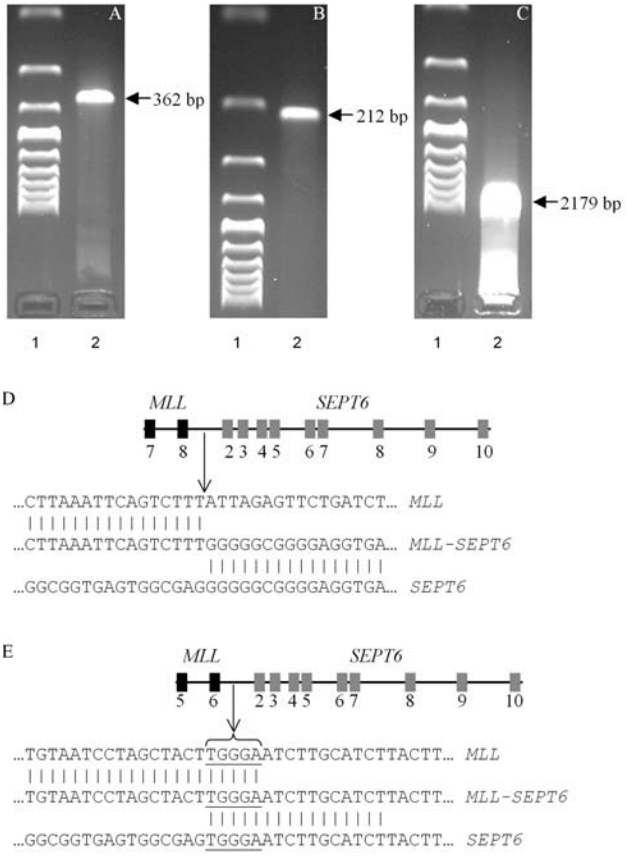
RT-PCR for the detection of *MLL-SEPT6* fusion transcripts was performed as follows: for cDNA synthesis, 1 μg of RNA was subjected to reverse transcription with random hexamers using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Forward primers (*MLL*-E5S, *MLL*-E6S, *MLL*-E7S-01, *MLL*-E7S-02, and *MLL*-E8S) for *MLL* exons 5, 6, 7, and 8 (GenBank accession no. NM_005933) have been previously described (*Online Supplementary Table S1*).[3,4] Reverse primers (*SEPT6*-E2AS-01, *SEPT6*-E2AS-02, and *SEPT6*-E2AS-03) for *SEPT6* exon 2 were derived from the published sequence of *SEPT6* mRNA with GenBank accession n. NM_145799 (*Online Supplementary Table S1*).

PCR reactions were performed in a 50 μL reaction volume containing 2 μL of synthesized cDNA, 5 μL of 10× GeneAmp PCR buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl) (Applied Biosystems, Foster City, USA), 5 μL of 25 mM MgCl2, 0.4 μL dNTP mix (25 mM each dNTP) (Applied Biosystems, Foster City, USA), 0.4 mM of each primer (Metabion, Martinsried, Germany), and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, USA).

Reaction tubes were kept on ice at all times to prevent non-specific amplification and incubated for 5 mins. at 94° C, followed by 35 cycles of 30 secs. at 95° C, 1 min. at 63° C, and 1.5 mins. at 72°C, followed by a final elongation of 10 mins. at 72°C on a GeneAmp PCR System 9700 (Applied BioSystems, Foster City, USA). Amplified products were analyzed on a 2% agarose gel (SeaKem LE Agarose, Rockland, USA) and the results were visualized in an image analyzer ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK).



Supplementary Figure S1. *MLL-SEPT6* fusion transcript analysis. (A) In case 1, three RT-PCR fragments were detected: a major fragment with 719 bp (*MLL* exon 8 fused with *SEPT6* exon 2), a minor fragment with 605 bp (*MLL* exon 7 fused with *SEPT6* exon 2), and a faint band with 541 bp corresponding to out-of-frame fusion between *MLL* exon 7 and *SEPT6* exon 2, with splicing of *MLL* exon 6.[1] 100 bp molecular marker. (B) Case 2 RT-PCR analysis showed the presence of one major fragment of 473 bp (*MLL* exon 6 fused with *SEPT6* exon 2) and a minor fragment of 399 bp resulting from an out-of-frame fusion of *MLL* exon 5 to *SEPT6* exon 2.[1] 100 bp molecular marker. (C) In case 3, two RT-PCR fragments of 605 bp and 541 bp were detected. Sequencing analysis revealed in both cases a fusion between *MLL* exon 7 and *SEPT6* exon 2, with the 541 bp fragment showing an out-of-frame splicing of *MLL* exon 6.[1] 50 bp molecular marker. (D) Partial sequence of the junction of the novel *MLL-SEPT6* chimeric mRNA (type IV) detected in case 2, showing the nucleotide sequence of the fusion transcript. The arrow shows the in-frame fusion between *MLL* exon 6 and *SEPT6* exon 2.



Supplementary Figure S2. *MLL-SEPT6* genomic breakpoint analysis. (A) and (B) Detection by HN-PCR of the genomic breakpoints in patients 1 (362 bp fragment) and 2 (212 bp fragment) respectively. (C) LD-PCR detection of the genomic breakpoint in case 3 (2179 bp fragment). In all cases, a 100 bp molecular marker was used.[1] (D) Schematic representation of the genomic breakpoint (arrow), nucleotide sequence, and corresponding sequences of normal *MLL* and *SEPT6* genes in cases 1 and 3. (E) Schematic representation of the genomic breakpoint, nucleotide sequence (arrow), and corresponding normal *MLL* and *SEPT6* genes (arrow) in case 2. In this case, because of micro-homology at the genomic junction (underlined), we were not able to determine the origin of these 5 nucleotides.

Long-range polymerase chain reaction (LR-PCR)

To characterize the genomic *MLL-SEPT6* fusions, we used the *MLL* exons 5, 6, 7, and 8 primers in combination with five additional primers (*SEP06*-I2LDAS-01, *SEP06*-I2LDAS-02, *SEP06*-I2LDAS-03, *SEP06*-I2LDAS-04, and *SEP06*-I2LDAS-05) located in the large (over 17 Kb) *SEPT6* intron 1 (*Online Supplementary Table S1*). LR-PCR, using the TripleMaster PCR System (Eppendorf, Hamburg, Germany), was performed in a 50 μL reaction volume containing 100 ng DNA, 5 μL of 10x Tuning Buffer, 2.5 μL dNTP mix (10 mM each dNTP) (GE Healthcare, Little Chalfont, UK), 0.4 mM of each primer (Metabion, Martinsried, Germany), and 2 units of TripleMaster Polymerase Mix. Reaction tubes were kept on ice at all times to prevent non-specific amplification. They were then incubated for 3 mins. at 93° C, followed by 10 cycles of 15 secs. at 93° C, 30 secs. at 65° C, and 10 mins. at 68° C, followed by 27 cycles of 15 secs. at 93°C, 30 secs. at 65°C, and 10 mins. at 68°C with an increment of 20 secs. per cycle, on a GeneAmp PCR System 9700 (Applied BioSystems, Foster City, USA).

Amplified products were analyzed on a 0.8% agarose gel (SeaKem LE Agarose, Rockland, USA) and the results were visualized in an image analyzer ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK).

Hemi-nested polymerase chain reaction (HN-PCR)

Since it was not possible to characterize the genomic breakpoint junctions in patients 1 and 2 by LR-PCR, an HN-PCR approach was developed. First-round PCR was performed using forward outer primers located in *MLL* exon/intron 6 (*MLL*-E6S, *MLL*-I6-01-O, and *MLL*-I6-02-O) (patient 2) or exon/intron 8 (*MLL*-I8-01-O, *MLL*-I8-02-O, *MLL*-I8-03-O, MLL-I8-04-O) (patient 1) and reverse primers located in *SEPT6* intron 1/exon 2 (*SEP06*-I2AS-01, *SEP06*-I2AS-02, *SEP06*-I2AS-03, *SEP06*-I2AS-04, *SEP06*-I2AS-05, *SEP06*-I2AS-06, *SEP06*-I2AS-07, *SEP06*-I2AS-08, *SEP06*-I2AS-09, *SEP06*-I2AS-10, *SEP06*-I2AS-11, SEP06-I2AS-12, *SEP06*-I2AS-13, *SEP06*-I2AS-14, *SEP06*-I2AS-15, *SEP06*-I2AS-16, *SEP06*-I2AS-17, *SEP06*-I2AS-18, *SEP06*-I2AS-19, and *SEP06*-I2AS-20) (*Online Supplementary Table S1*). Second-round PCR was performed with forward inner primers *MLL*-I6S-In, *MLL*-I6-01-In, and *MLL*-I6-02-In (patient 2), or *MLL*-I8-01-In, *MLL*-I8-02-In1, *MLL*-I8-02-In2, MLL-I8-03-In1, *MLL*-I8-03-In2, *MLL*-I8-04-In (patient 1) and the same reverse primers as first-round PCR (*Online Supplementary Table S1*). PCR reactions were performed in a 50 μL reaction volume containing 2 μL of first-round PCR product, 5 μL of 10x GeneAmp PCR buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl) (Applied Biosystems, Foster City, USA), 5 μL of 25 mM MgCl2, 0.4 μL dNTP mix (25 mM each dNTP) (Applied Biosystems, Foster City, USA), 0.4 mM of each primer (Metabion, Martinsried, Germany), and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, USA). Reaction tubes were kept on ice at all times to prevent non-specific reactions and then incubated for 5 mins. at 94°C, followed by 35 cycles of 30 secs. at 95°C, 1 min. at 63° C, and 1.5 mins. at 72°C, followed by a final elongation of 10 mins. at 72°C on a GeneAmp PCR System 9700 (Applied BioSystems, Foster City, USA). Amplified products were analyzed on a 2% agarose gel (SeaKem LE Agarose, Rockland, USA) and the results were visualized in an image analyzer ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK). Strict measures were taken to avoid problems associated with contamination.

Sequencing

Sequence analysis was directly performed on the amplified RT-PCR or PCR product by use of the BigDye Terminator Cycle Sequencing Chemistry (Applied Biosystems, Foster City, USA) on an automated sequencer ABI Prism 310 Genetic Analyser (Applied BioSystems, Foster City, USA) according to the manufacturer's instructions. When multiple bands were observed, gel band extraction and purification was performed with the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), again according to the manufacturer's instructions.

Bioinformatic sequence analysis

The presence of specific recombination-related DNA sequence motifs known to be associated with site-specific recombination, cleavage, and gene rearrangement,[5,6] such as the topoisomerase II consensus cleavage site, VDJ recombination sequence, translin binding sequence, χ-like sequence, and purine/pyrimidine repeat regions, was investigated with SEQ tools and RepeatMasker.[7,8]

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Design and Methods

The study comprised three cases of childhood AML in which Xq24 and 11q23 rearrangements were detected by karyotyping and/or molecular cytogenetic analyses of the leukemic cells. Molecular studies involved RT-PCR, LD-PCR, HN-PCR, sequencing, and bioinformatic analyses. For detailed information on patients, methods and results see *Online Supplementary Appendix*.

Results and Discussion

Karyotyping and molecular cytogenetics

The 3 AML patients showed cytogenetic evidence of a rearrangement involving the long arm of chromosome 11 (11q23), the first with additional material of unknown origin in 11q23 and Xp11 (patient 1), the second as a translocation between 11q23 and Xq24, and the third as an insertion of 11q13q23 into Xq24 (Table 1, Figure 1). FISH analysis on leukemic metaphases was performed, demonstrating in all cases a break in *MLL* (Figure 1) and the presence of *MLL* sequences in Xq (cytogenetically cryptic in case 1). The known localization of *SEPT6* in Xq24 prompted further analysis with BACs mapped to this band in patients 2 and 3. In patient 2, the breakpoint was mapped to clone CTD-2334F19, suggesting a breakpoint in or near the 5' region of the *SEPT6* gene. In patient 3, the breakpoint was mapped to the overlapping region of the two BAC clones RP11-379J1 and CTD-2334F19, which suggested that the break occurred in the 5' region of the *SEPT6* gene. In patient 1, BAC analysis could not be performed due to lack of material. Rearrangements recombining 11q23 and Xq24 resulting in *MLL-SEPT6* fusions are usually complex as a result of the opposite orientation of *MLL* and *SEPT6* on the respective chromosome arms. At least four different types of chromosomal rearrangements have been described that can generate the *MLL-SEPT6* in-frame fusion.[7-10] The combined chromosome banding and molecular cytogenetic investigations of our 3 patients confirm that complex, sometimes cryptic, chromosome rearrangements are common in AML patients with *MLL-SEPT6* rearrangements (Table 1).

Characterization of MLL-SEPT6 fusion transcripts

RT-PCR followed by sequencing analysis led to the identification of *MLL-SEPT6* fusion transcripts in all 3 cases. In patient 1, two major PCR fragments of 719 bp and 605 bp were detected (*Online Supplementary Figure 1A*). Sequencing analysis revealed a fusion of *MLL* exon 7 and *MLL* exon 8 with *SEPT6* exon 2. In addition, a minor band of 541 bp detected in this patient (*Online Supplementary Figure S1A*) was shown by sequencing analysis to correspond to an out-of-frame fusion between *MLL* exon 7 and *SEPT6* exon 2 with splicing of 74 bp corresponding to *MLL* exon 6. RT-PCR analysis of patient 2 revealed one major band of 473 bp and a minor band of 399 bp (*Online Supplementary Figure S1B*). Sequencing analysis revealed the presence of two

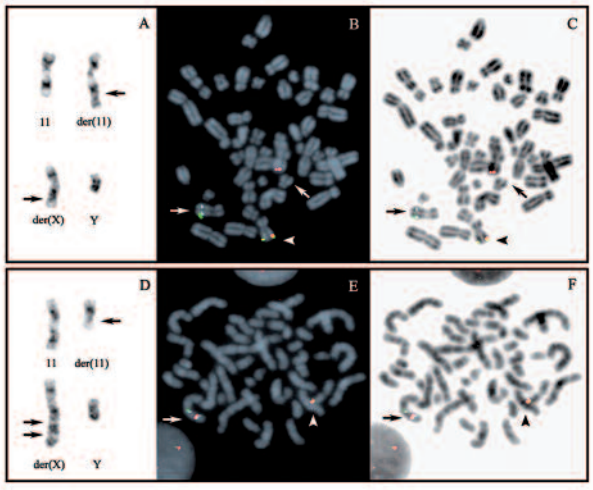

Figure 1. G-banding and FISH analyses of the leukemic cells of patients 2 and 3. (A) Partial karyotype of case 2, with arrows indicating breakpoints of rearranged chromosomes. (B) FISH and (C) inverted DAPI images of metaphase plate from case 2. The hybridization was performed using a locus-specific, break-apart probe for *MLL* (green and red signals) and the BAC clone CTD-2334F19 (blue signal). The blue signal has moved to the derivative chromosome 11, indicating that the breakpoint is in the 5' of the *SEPT6* gene. Arrows indicate derivative chromosomes, arrow heads are pointing to the normal chromosome 11. (D) Partial karyotype of case 3. (E) FISH and (F) inverted DAPI images of a metaphase plate from case 3. Hybridization was performed using the *MLL* probe and the clone CTD-2334F19. The BAC clone (blue colour) splits giving two signals on the rearranged X chromosome, indicating that the breakpoint is inside that clone.

novel *MLL-SEPT6* chimeric transcripts: an in-frame fusion between *MLL* exon 6 and *SEPT6* exon 2 and an out-of-frame fusion of *MLL* exon 5 to *SEPT6* exon 2 (*Online Supplementary Figure S1D*). The novel *MLL-SEPT6* in-frame fusion variant is expected to give rise to a chimeric fusion protein, where the N terminus of *MLL* fused to almost the entire open reading frame of *SEPT6*, except for the first nine amino acids.

In patient 3, RT-PCR analysis showed the presence of two PCR fragments of 605 bp and 541 bp (*Online Supplementary Figure S1C*). Sequencing analysis demonstrated fusions between *MLL* exon 7 and *SEPT6* exon 2, with the smaller fragment showing, as in patient 1, an out-of-frame splicing of *MLL* exon 6. The 10 cases of *MLL-SEPT6* rearrangement described so far showed fusions between *SEPT6* exon 2 and *MLL* exon 7 (3 cases), *MLL* exon 8 (3 cases), both *MLL* exons 7 and 8 (3 cases), or *MLL* exon 9 (one case).[8-13] The novel *MLL-SEPT6* chimeric transcript we here describe between *MLL* exon 6 and *SEPT6* exon 2 may be called type IV, after the three fusion types previously identified (Table 1).

SEPT6 belongs to an evolutionarily conserved family of genes that encode a P loop-based GTP-binding domain flanked by a polybasic domain and, in most cases, a coiled-coil-region.[6] The SEPT6 protein possesses all the three domains and, as previously reported in cases of gene fusion involving *MLL* and other septins (*MLL-SEPT2, MLL-SEPT5, MLL-SEPT9*, and *MLL-*

SEPT11), almost the entire open reading frame of *SEPT6*, containing all the three septin function-defining domains, is fused with the N-terminal moiety of *MLL*. A relevant role of septins in *MLL*-related leukemia, besides activation of the *MLL* protein by dimerization, is therefore a possibility that should not be ruled out.[5,6] Additional support for this hypothesis comes from the observation that all 13 patients reported so far with *MLL-SEPT6* rearrangement were children (age range; 0-29 months) with AML (the FAB-typed included one M1, five M2, four M4 and one M5; Table 1). Since the majority (65%) of pediatric patients with *MLL* rearrangements have ALL,[4] we hypothesize that the SEPT6 domains of the MLL-SEPT6 chimeric protein contribute to myeloblastic leukemogenesis in children. In fact, the *MLL* fusion with the other septins (*SEPT2, SEPT5, SEPT9*, and *SEPT11*) is also preferentially associated with myeoloblastic rather than lymphoblastic leukemogenesis.[4,5,14] In all 3 cases studied we observed the presence of out-of-frame alternative splicing variants, something that has not been previously reported[7-13] and whose biological relevance is not clear.

Characterization of MLL-SEPT6 genomic breakpoints

The genomic breakpoints in all cases occurred in the *MLL* 8.3 kb breakpoint cluster region (BCR) and in *SEPT6* intron 1 (*Online Supplementary Figure 2*). In patient 1, a total of six suggestive HN-PCR fragments were gel extracted and sequenced. Sequencing of the amplification products showed that the breakpoint was located 231 bp downstream of *MLL* exon 8 and 476 bp downstream of *SEPT6* exon 1 (*Online Supplementary Figure 2D*). The HN-PCR study of case 2 revealed a total of 11 suggestive HN-PCR fragments that were gel extracted and sequenced. Sequencing analysis showed that the genomic breakpoints were located 629_634 bp downstream of *MLL* exon 6 and 14410_14415 bp upstream of *SEPT6* exon 2 (*Online Supplementary Figures 2B and 2E*). In this case, the exact position of the genomic breakpoint could not be determined due to the presence of an identical 5-bp microhomology sequence (TGGGA) at the *MLL-SEPT6* genomic junction. In patient 3, an LD-PCR fragment of 2179 bp was detected (*Online Supplementary Figure 2C*). Interestingly, partial direct sequencing of the amplification product revealed the

Table 1. Clinical, karyotyping, FISH and RT-PCR data on all known acute myeloid leukemia-patients with *MLL-SEPT6*, and classification of the fusion variants.

Patient	*Age (mo.)*	*Sex*	*Diagnosis*	*Karyotype*	*FISH*	*RT-PCR (MLL/SEPT6 fusion)*	*Type*	*Reference*
1	17	F	AML-M2	47,X,add(X)(p11),+6,add(11)(q23)[20]	MLL	exon 7/exon 2 exon 8/exon 2 exon 5-7/exon 2	Type II Type I Out-of-Frame	Present study
2	12	M	AML	46,Y,t(X;11)(q24;q23)[11])/46,XY[9]	MLL	exon 6/exon 2 exon 5/exon 2	Type IV Out-of-Frame	Present study
3	0	M	AML	46,Y,ins(X;11)(q24;q13q23)[11]	MLL	exon 7/exon 2 exon 5-7/exon 2	Type II Out-of-Frame	Present study
4	6	F	AML-M2	46,X,ins(X;11)(q24;q23)	MLL	exon 8/exon 2	Type I	8
5	20	F	AML-M4	47,X,der(X)t(X;11)(q22;q23)t(3;11)(p21;q12), der(3)t(3;11)(p21;q23)t(X;11)(q22;q25), +6,der(11)del(11)(q12?qter)	MLL	exon 7/exon 2	Type II	9
6	10	M	AML-M2	46,Y,t(X;11)(q22;q23)[25]/46,XY[5]	Not done	exon 8/exon 2	Type I	9
7	3	F	AML-M2	46,XX,t(5;11)(q13;q23)[6]/46, idem,add(X)(q22)[12]	MLL	exon 7/exon 2 exon 8/exon 2	Type II Type I	7
8	7	M	AML-M2	46,XY[20]	MLL	exon 7/exon 2	Type II	7
9	6	F	AML-M1	46,X,add(X)(q2?),del(11q?)[20]	MLL	exon 7/exon 2 exon 8/exon 2	Type II Type I	7
10	29	M	AML-M5	46,Y,ins(X;11)(q24;q23q13)[13]/46,XY[7]	MLL	exon 7/exon 2	Type II	11
11	8	M	AML-M4	46,XY	Not done	exon 8/exon 2	Type I	10
12	13	M	AML-M4	46,Y,ins(11;X)(q23;q24q22) [14]/46,idem,i(10)(q10)[6]	MLL	exon 9 / exon 2	Type III	12
13	26	F	AML-M4	46,XX,t(11;17)(q23;q?25)[20]	MLL	exon 7/exon 2 exon 8/exon 2	Type II Type I	13

same genomic breakpoint junction as detected in patient 1, with fusion of nucleotide 231 downstream of *MLL* exon 8 with nucleotide 476 downstream of *SEPT6* exon 1 (*Online Supplementary Figure 2D*).

We searched for topoisomerase II consensus cleavage sites in the vicinity of the breakpoint regions in *MLL* introns 6 and 8 and *SEPT6* intron 1. We found one sequence with 100% homology with the topoisomerase II consensus cleavage site located in *SEPT6* intron 1 (GTTTTCCTGTTGTTGTTT), nucleotide position 9533_9550 bp downstream of *SEPT6* exon 1. We also searched the breakpoint junctions (15 bp either side) for repetitive DNA sequence elements and motifs known to be associated with site specific recombination, cleavage, and gene rearrangement, but none could be found. Translocations may or may not involve gain or loss of genetic material at the genomic breakpoint junctions. Patients 1 and 3, as well as the two previously reported cases, showed no nucleotide(s) deletion or duplication at the breakpoint junction.[9,10] In our patient 2, the exact position of the genomic breakpoint could not be determined due to an identical 5-bp microhomology region at the *MLL-SEPT6* genomic junction, so it is unknown whether duplications and/or deletions occurred in this particular patient. The identification of identical microhomologies at genomic junctions suggests that the non-homologous DNA end-joining (NHEJ) pathway may be involved in this rearrangement.[15] In patients 1 and 3, the genomic junction mapped near a 484 bp LINE1 repeat in *MLL* intron 8, whereas the genomic breakpoint in *SEPT6* intron 1 mapped near two GC-rich low complexity repeats. In patient 2, the *MLL* intron 6 genomic breakpoint occurred inside a 298 bp Alu repeat, whereas the breakpoint junction in the *SEPT6* intron 1 mapped near a 300 bp Alu repeat. Although repetitive sequences may occur near or spanning breakpoint junctions by chance, it is plausible that introns with a high density of repetitive sequences, such as *SEPT6* intron 1, are vulnerable to breaking and non-homologous pairing that can lead to gene fusions such as *MLL-SEPT6*. Strikingly, although the breakpoints in the large *SEPT6* intron 1 seem to be distributed all over the intronic region (our patient 2, and cases 6 and 11 in the literature; Table 1), patients 1 and 3 showed exactly the same genomic breakpoint both in *MLL* and *SEPT6*. Since the genomic breakpoint junctions in these 2 patients were cloned by different methods (HN-PCR and LD-PCR), on separate occasions, and taking the strictest anti-contamination measures, these findings can only be explained by the presence of a hot-spot for recombination at the said sites. Supporting this hypothesis, in addition to the above-mentioned high density of repetitive sequences in *SEPT6* intron 1, is the detection of a topoisomerase II consensus site-specific cleavage in the same intron. Identical genomic breakpoints or breakpoint clustering within very narrow regions have been reported before, namely in *MLL-AF4*,[16] *MLL-AF9*,[17] and *TCF3-PBX1*[18] leukemias. Interestingly, the presence of an *MLL* intron 8 genomic breakpoint in patient 3 does not seem to translate into fusion transcripts that include *MLL* exon 8, since the only in-frame fusion transcript detected showed a fusion between *MLL* exon 7 and *SEPT6* exon 2. Splicing of *MLL* exon 8, which includes the first of the four zinc fingers of the first zinc finger domain of the *MLL* gene, has been previously described in acute leukemia with *MLL* rearrangements, both in cases with translocation [(t(4;11), t(9;11), and t(11;19)][19,20] and tandem duplication,[21] changing the structural and possibly the functional features of the first zinc finger region of the MLL protein.

The *MLL* genomic breakpoints in *MLL-SEPT6* AML patients seem to occur preferentially in the telomeric half (between introns 7 and 11) of the *MLL* BCR. This is characteristic of infant AML (the *MLL-SEPT6* cases with genomic breakpoint characterization have an age at diagnosis from 0 to 17 months) and topoisomerase II inhibitor-related secondary leukemia, and a putative association with *in utero* exposure to topoisomerase II inhibitors has been hypothesized.[22] The detection of a topoisomerase II recognition sequence in *SEPT6* intron 1 also supports the hypothesis that exposure to topoisomerase II inhibitors, can result in double-strand DNA breaks that trigger the error-prone non-homologous end-joining pathway, which in turn can lead to formation of the *MLL-SEPT6* fusion oncogene.

Authorship and Disclosures

NC designed and performed the research, analyzed the data and wrote the paper. FM and JS performed the research, analyzed the data and wrote the paper. MP, CC, SL, and SB performed the research and analyzed the data. LN, AG, and AEA clinically assessed the patients. SH and MRT analyzed the data and wrote the paper. The authors reported no potential conflicts of interest.

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