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

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Citation: Cerveira N, Micci F, Santos J, Pinheiro M, Correia C, Lisboa S, Bizarro S, Norton L, Glomstein A, Åsberg AE, Heim S, and Teixeira MR. Molecular characterization of the MLL-SEPT6 fusion gene in acute myeloid leukemia: identification of novel fusion transcripts and cloning of genomic breakpoint junctions. Haematologica 2008; doi: 10.3324/haematol.12594

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-I6-01-0	*MLL* intron 6	5'-CAAAGCAAAACACTGTCTCCAAAA-3'	419_442
MLL-I6-01-In	*MLL* intron 6	5'-AAAATTTAGGCTTGGCAAGGC-3'	443_463
MLL-I6-02-0	*MLL* intron 6	5'-GTTTCTTCCTTGTTGCTTTTCCC-3'	1079_1101
MLL-I6-02-In	*MLL* intron 6	5'-TGGCCCCACATGTTCTAGC-3'	1109_1127
MLL-I8-01-0	*MLL* intron 8	5'-AGAAATAAATACATGTTGGGTGGCA-3'	438_462
MLL-I8-01-In	*MLL* intron 8	5'-GAGGTGAAGGGAGGGTGTCTG-3'	467_487
MLL-I8-02-0	*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-I8-03-0	*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-I8-04-0	*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

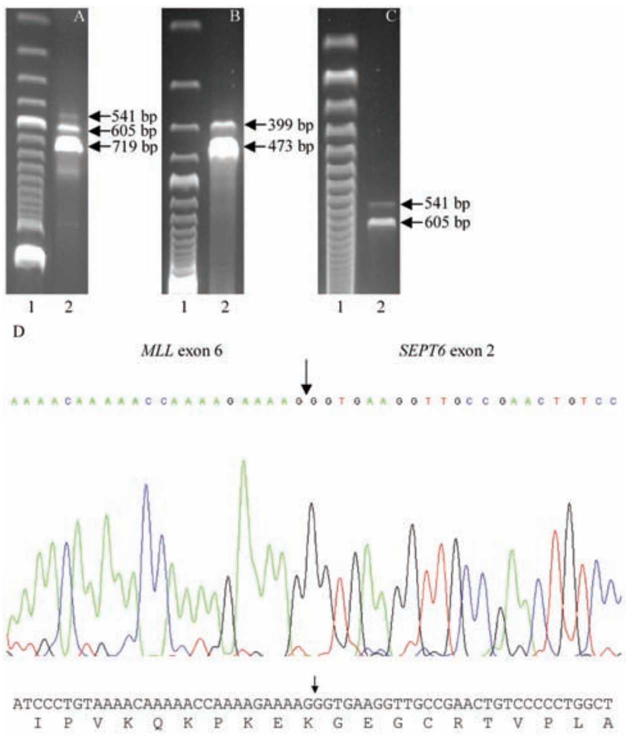
High molecular weight DNA and RNA were extracted from the bone marrow sample (patient 1) or from fixed cell suspension remaining after completion of the cytogenetic analysis (patients 2 and 3; cells from peripheral blood) using 1 mL of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA)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

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

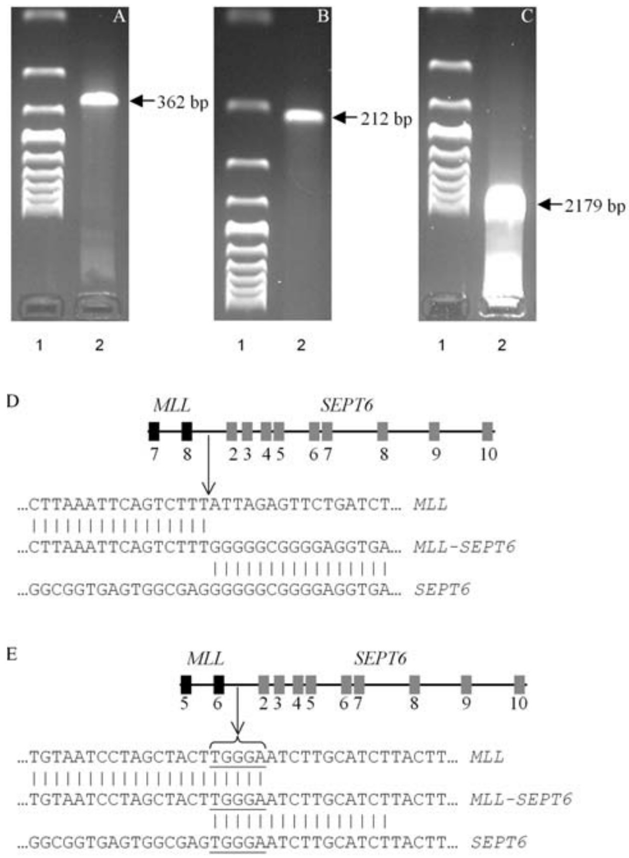
RT-PCR for the detection of *MLL-SEPT6* fusion transcripts was performed as follows: for cDNA synthesis, 1 μg of RNA was subjected to reverse transcription with random hexamers using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA) according to the manufacturer's 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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