

## ***MLL-SEPTIN6* fusion recurs in novel translocation of chromosomes 3, X, and 11 in infant acute myelomonocytic leukaemia and in t(X;11) in infant acute myeloid leukaemia, and *MLL* genomic breakpoint in complex *MLL-SEPTIN6* rearrangement is a DNA topoisomerase II cleavage site**

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We examined the *MLL* translocation in two cases of infant AML with X chromosome disruption. The G-banded karyotype in the first case suggested t(X;3)(q22;p21)ins(X;11)(q22;q13q25). Southern blot analysis showed one *MLL* rearrangement. Panhandle PCR approaches were used to identify the *MLL* fusion transcript and *MLL* genomic breakpoint junction. *SEPTIN6* from chromosome band Xq24 was the partner gene of *MLL*. *MLL* exon 7 was joined in-frame to *SEPTIN6* exon 2 in the fusion transcript. The *MLL* genomic breakpoint was in intron 7; the *SEPTIN6* genomic breakpoint was in intron 1. Spectral karyotyping revealed a complex rearrangement disrupting band 11q23. FISH with a probe for *MLL* confirmed *MLL* involvement and showed that the *MLL-SEPTIN6* junction was on the der(X). The *MLL* genomic breakpoint was a functional DNA topoisomerase II cleavage site in an *in vitro* assay. In the second case, the karyotype revealed t(X;11)(q22;q23). Southern blot analysis showed two *MLL* rearrangements. cDNA panhandle PCR detected a transcript fusing *MLL* exon 8 in-frame to *SEPTIN6* exon 2. *MLL* and *SEPTIN6* are vulnerable to damage to form recurrent translocations in infant AML. Identification of *SEPTIN6* and the *SEPTIN* family members *hCDCrel* and *MSF* as partner genes of *MLL* suggests a common pathway to leukaemogenesis.

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**Keywords:** *MLL*; *SEPTIN6*; complex translocation; infant leukaemia; spectral karyotype; DNA topoisomerase II

### Introduction

Translocations of the *MLL* gene at chromosome band 11q23 are the most common molecular abnormalities in leukaemia in infants (reviewed in Felix, 2000). *MLL* translocations in infant leukaemias are *in utero* events (Ford *et al.*, 1993; Gale *et al.*, 1997; Gill-Super *et al.*, 1994; Mahmoud *et al.*, 1995; Megonigal *et al.*, 1998). *MLL* has many partner genes that encode proteins of several different types (reviewed in Ayton and Cleary, 2001; Felix, 2000; Rowley, 1998). Genomic breakpoint junction sequences or *MLL* chimeric transcripts involving 32 partner genes have been described, but others have not yet been cloned (Huret, 2001). Several *MLL* fusions with nuclear transcription factors (Corral *et al.*, 1996; Lavau *et al.*, 1997) or with proteins central to transcriptional regulation (Lavau *et al.*, 2000a,b) transform hematopoietic progenitors (Lavau *et al.*, 1997, 2000a,b), and/or are leukaemogenic in transgenic mice (Corral *et al.*, 1996) or in mouse models created by retroviral-mediated gene transfer (Lavau *et al.*, 1997, 2000a). Whereas many *MLL* partner proteins have structural motifs of nuclear transcription factors (LAF-4, AF4, AF5α, AF5q31, AF6q21, AF9, AF10, *MLL*, AF17, ENL, AFX) (Borkhardt *et al.*, 1997; Chaplin *et al.*, 1995; Gu *et al.*, 1992; Hillion *et al.*, 1997; Morrissey *et al.*, 1993; Nakamura *et al.*, 1993; Prasad *et al.*, 1994; Schichman *et al.*, 1994; Taki *et al.*, 1996, 1999a; Tkachuk *et al.*, 1992), proteins involved in

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transcriptional regulation (CBP, ELL, p300) (Ida *et al.*, 1997; Sobulo *et al.*, 1997; Taki *et al.*, 1997; Thirman *et al.*, 1994) or, in one case, a nuclear protein of unknown function (AF15q14) (Hayette *et al.*, 2000), other MLL partner proteins are found in the cytoplasm (AF1p, AF1q, AF3p21, GMPS, LPP, GRAF, FBP17, ABI-1, GAS7, EEN) (Bernard *et al.*, 1994; Borkhardt *et al.*, 2000; Daheron *et al.*, 2001; Fuchs *et al.*, 2001; Megonigal *et al.*, 2000a; Pegram *et al.*, 2000; Sano *et al.*, 2000; So *et al.*, 1997; Taki *et al.*, 1998; Tse *et al.*, 1995) or at the cell membrane (AF6, LARG, GPHN) (Eguchi *et al.*, 2001; Kourlas *et al.*, 2000; Prasad *et al.*, 1993).

Septins are cytoplasmic proteins with roles in cell division, cytokinesis, cytoskeletal filament formation and GTPase signalling (Cooper and Kiehart, 1996; Field and Kellogg, 1999). In AML of infant twins, we identified the hCDCrel (human Cell Division Cycle related) gene at chromosome band 22q11.2 as the first SEPTIN gene fused with MLL (Megonigal *et al.*, 1998). hCDCrel involvement has been shown in other cases (Tatsumi *et al.*, 2001), indicating that the MLL-hCDCrel is a recurrent translocation. The MSF (MLL Septin-like fusion) gene at chromosome band 17q25 is a partner gene of MLL in infant and treatment-related leukaemias (Osaka *et al.*, 1999; Taki *et al.*, 1999b). SEPTIN6 is the third SEPTIN family member disrupted by MLL translocations. We identified recurrent involvement of SEPTIN6 in two cases of infant AML. MLL-SEPTIN6 chimeric transcripts were recently reported in four cases of infant AML (Borkhardt *et al.*, 2001; Ono *et al.*, 2002), but the genomic breakpoint junctions were not cloned. Together with prior results on the MLL-hCDCrel genomic breakpoint junction in AML of infant twins (Megonigal *et al.*, 1998), results described herein on the genomic sequencing, FISH and SKY characterization, and DNA topoisomerase II cleavage assays of a complex translocation suggest that the MLL gene and SEPTIN family genes are vulnerable to damage to form translocations associated with infant AML.

## Results

### Case histories

Patient 62 presented at 20 months of age with hepatosplenomegaly, massive adenopathy, anemia, thrombocytopenia, a WBC of  $397 \times 10^9/L$  and CNS leukaemia. The bone marrow was 91% replaced by French-American-British (FAB) M4 blasts that expressed CD33, CD15, CD11b and HLA DR. The original G-banded karyotype in eight of eight metaphases was 47,X,t(X;3)(q22;p21)ins(X;11)(q22;q13q25),+6,del(11)(q13). The patient died of infectious complications during induction.

Clinicopathologic features of patient 23, who presented at age 10 months with a WBC count of  $13.4 \times 10^9/L$ , 13% circulating blasts and pancytopenia, have been described (Felix *et al.*, 1998). The marrow morphology was FAB M2. The immunophenotype was

CD11+, CD13+, CD15+, CD33+; no lymphoid antigens were expressed. The karyotype in 25 out of 30 metaphases revealed 46,Y,t(X;11)(q22;q23). Four months later, only partial remission had been achieved after treatment according to protocol CCG 2891 (Woods *et al.*, 1996); the karyotype was 45,Y,t(X;11)(q22;q23),-7[1]/46,XY[23]. The patient was removed from protocol and underwent a haploidentical transplant with his mother's marrow. He remains disease free with chronic graft-versus-host disease 7 years from diagnosis.

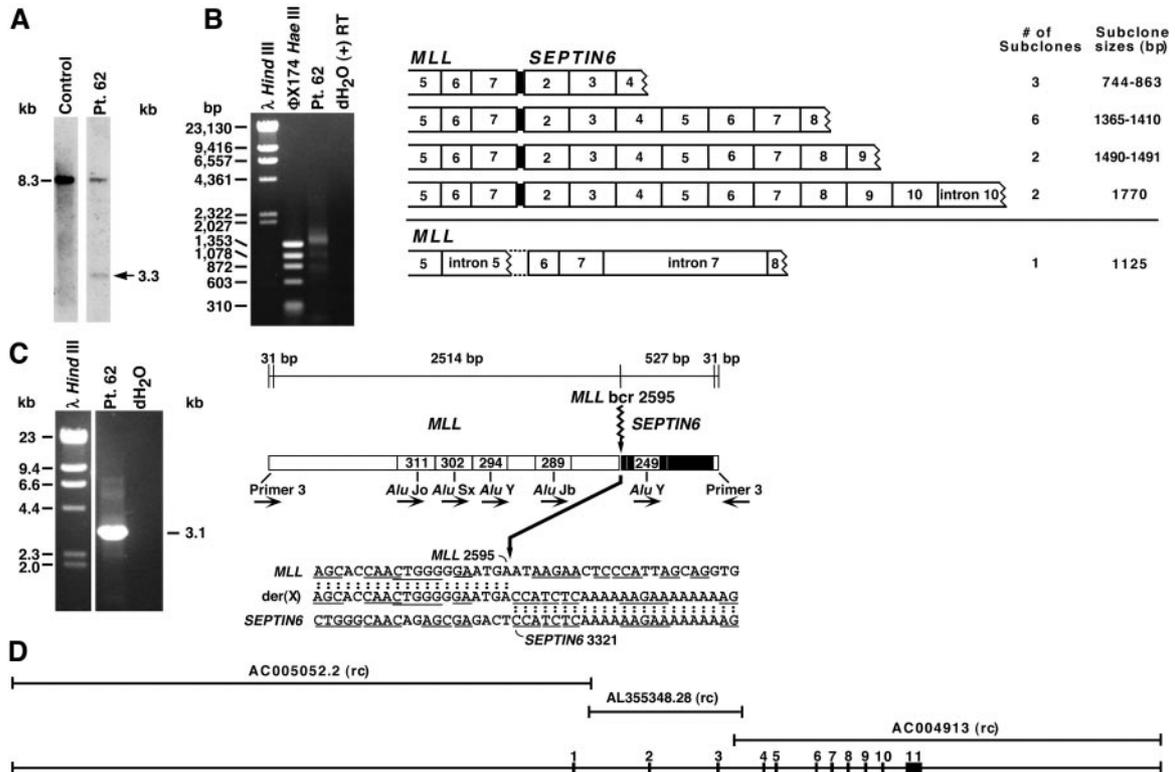
### Molecular and cytogenetic characterization of a complex MLL-SEPTIN6 rearrangement

Although the G-banded karyotype of the AML of patient 62 did not show involvement of band 11q23, Southern blot analysis of the MLL bcr was performed because the morphology was FAB M4. MLL bcr rearrangement suggested that the t(X;3)(q22;p21)ins(X;11)(q22;q13q25) disrupted MLL (Figure 1a). The single rearrangement was consistent with loss of the 3' portion of the MLL bcr during the translocation.

cDNA panhandle PCR identified the partner gene of MLL (Figure 1b). Sequencing of recombination PCR-generated subclones from cDNA panhandle PCR revealed two types of MLL-containing transcripts. The majority of subclones contained an in-frame fusion of MLL exon 7 to exon 2 at position 24 of the 4612 bp SEPTIN6 cDNA from chromosome band Xq24 (GenBank no. D50918); two subclones indicated incomplete processing of this transcript (Figure 1b). The second type of transcript contained MLL sequence only and was also incompletely processed (Figure 1b). Amplification of the same first-strand cDNA with MLL and SEPTIN6-specific primers and sequencing of the 357 bp product confirmed the fusion transcript (not shown). There was no evidence of alternative splicing of the fusion transcript from either cDNA panhandle PCR or PCR with gene-specific primers.

The corresponding MLL-SEPTIN6 genomic breakpoint junction was isolated by panhandle variant PCR. The product size (Figure 1c) was consistent with the ~3.3 kb MLL bcr rearrangement on the Southern blot (Figure 1a). The MLL genomic breakpoint was position 2595 in intron 7; the SEPTIN6 genomic breakpoint was position 3321 of 17407 in intron 1 (GenBank no. AC005052.2) (Figure 1c). The MLL breakpoint was near Alu Y and Alu Jb repeats; the SEPTIN6 breakpoint was near an Alu Y (Figure 1c). Several two- to five-base homologies were present near the breakpoints in both genes (Figure 1c). The sequence of the 564 bp product obtained by PCR with MLL- and SEPTIN6-specific primers confirmed the breakpoint junction (not shown).

Spectral karyotype analysis (SKY) and fluorescence *in situ* hybridization analysis (FISH) allowed visualization of the chromosomal abnormalities and the translocation. SKY analysis of 10 metaphase cells indicated a more complex translocation and disruption of band 11q23. The spectral karyotype was 47,X,



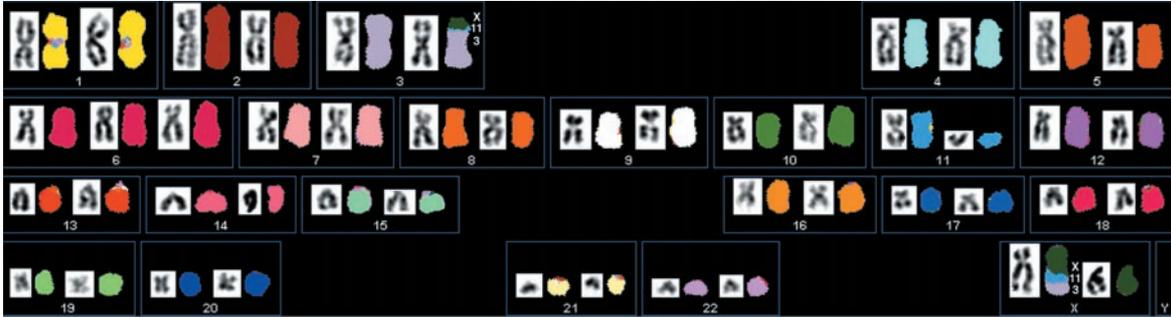
**Figure 1** (a) *MLL* bcr rearrangement in AML of patient 62. *Bam*HI-digested DNA was hybridized with B859 fragment of *ALL-1* cDNA (Gu *et al.*, 1992). 8.3 kb fragment is from unrearranged *MLL* allele; arrow shows rearrangement. (b) cDNA panhandle PCR analysis of total RNA from diagnostic marrow of patient 62. Smear in third lane of gel shows products of various sizes from amplification of 5'-*MLL*-NNNNNN-3'-primed first strand cDNAs with *MLL*-specific primers (left). The products were subcloned by recombination PCR. Thirteen subclones contained an in-frame fusion of *MLL* exon 7 to *SEPTIN6* exon 2. Subclones with *SEPTIN6* intron 10 in sequence are from incompletely processed transcripts (top right). Subclone with *MLL* sequence only contains intronic sequence, indicating an incompletely processed transcript (bottom right). (c) Panhandle variant PCR analysis of genomic DNA from diagnostic marrow of patient 62. Three panhandle variant PCRs gave products consistent with *MLL* bcr rearrangement size on Southern blot (c.f. a); gel (left) shows example. Products of one reaction were subcloned by recombination PCR; one subclone was sequenced in entirety. 3103 bp sequence is summarized (right). 31-base sequence of primer 3 from *MLL* exon 5 used in final round of panhandle variant PCR and complement are at 5' and 3' ends. 2514 additional bases of 5' sequence are from *MLL* bcr. Corkscrew arrow indicates *MLL* breakpoint at position 2595 in intron 7. 527 bases of 3' sequence are from *SEPTIN6* intron 1. Alu repeats are shown (middle right). Underlines indicate short homologies between *MLL* and *SEPTIN6* (bottom right). Breakpoint junction was confirmed in another subclone and in products of independent PCR performed with gene-specific primers. (d) Genomic sequence entries in GenBank comprising human *SEPTIN6*. Each GenBank entry appears in reverse complement (rc) from orientation of transcription

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) (Figure 2).

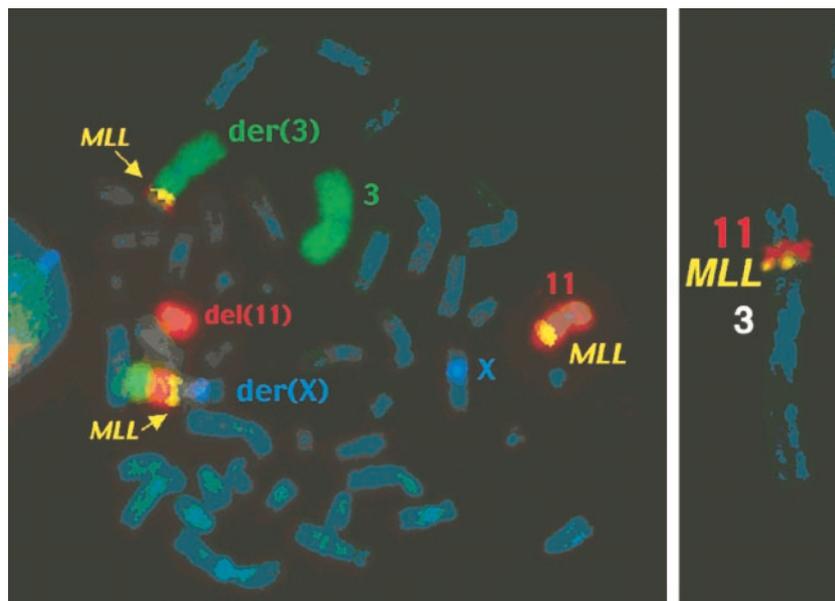
FISH analysis of 12 of 14 metaphase cells with the *MLL* probe (Ventana) detected one *MLL* signal on the normal chromosome 11 and signals on the der(X) and the der(3), confirming *MLL* disruption. The location of the signal on the der(3) at the interface between material from chromosome 11 and chromosome 3 suggested that the *MLL-SEPTIN6* fusion was created on the der(X) and that, cytogenetically, no reciprocal fusion could be created through this aberration (Figure 3). Since the single *MLL* bcr rearrangement on Southern blot analysis was consistent with deletion of the 3' bcr, detection of split *MLL* signals on the der(X) and der(3) chromosomes by FISH suggested that the *MLL* sequences on the der(3) chromosome were distal to the bcr.

*MLL* genomic breakpoint in complex rearrangement is a DNA topoisomerase II cleavage site

A DNA topoisomerase II *in vitro* cleavage assay was performed to determine the feasibility of DNA topoisomerase II cleavage at the *MLL* bcr translocation breakpoint in the AML of patient 62. *MLL* position 2595, which was the translocation breakpoint, was the 5' side or -1 position of a naturally-occurring, enzyme-only cleavage site. The DNA topoisomerase II inhibitor etoposide enhanced cleavage at this site 1.2-fold over cleavage without drug (Figure 4). Although stronger cleavage was discerned at several sites in the substrate both with and without drug and position 2595 did not appear to be a highly preferred cleavage site, detection of cleavage after heating to 65°C indicates stability of the cleavage complexes formed at this position (Figure 4). The assay was repeated with reproducible results.



**Figure 2** SKY analysis of exemplary metaphase cell from marrow of patient 62 at AML diagnosis. The chromosomes are arranged in karyotype fashion. Inverted DAPI-image (left) and the respective SKY-classification (right) are shown for each chromosome. SKY analysis of ten metaphases was interpreted as 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)



**Figure 3** Metaphase FISH analysis of AML of patient 62. FISH analysis of a metaphase cell with painting probes for chromosome 3 (green), chromosome 11 (red), a centromere probe for the X chromosome (blue), and a probe for *MLL* (yellow) (left) confirmed the complex translocation seen by SKY. The simultaneous hybridization with a probe for *MLL* (yellow) showed *MLL* signals on the normal chromosome 11, the der(X) and the der(3). The image at right shows the der(3) chromosome from a metaphase chromosome with better resolution. The signals for *MLL* (yellow) are located at the interface between material from chromosome 3 (green) and chromosome 11 (red). These results indicate that the 5'-*MLL-SEPTIN6*-3' junction identified by panhandle variant PCR was on the der(X)

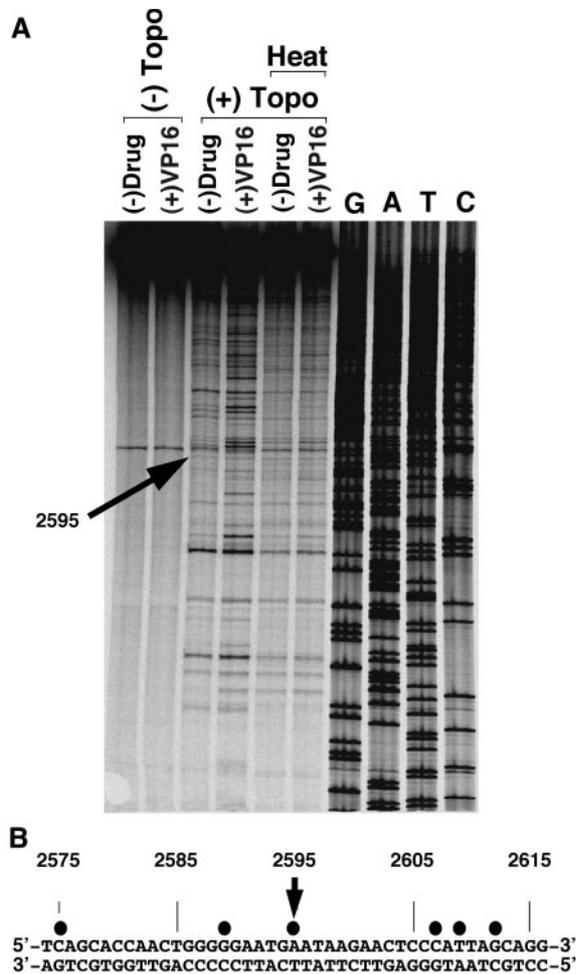
#### Detection of *MLL-SEPTIN6* fusion transcript in infant AML with *t(X;11)(q22;q23)*

In the FAB M2 AML of patient 23, the *t(X;11)(q22;q23)* translocation was the only clonal abnormality detected by the karyotype (Felix *et al.*, 1998). Southern blot analysis of the *MLL* bcr showed two rearrangements consistent with an *MLL* translocation (Figure 5a) (Felix *et al.*, 1998). cDNA panhandle PCR revealed the fusion transcript with *MLL* exon 8 fused in-frame to *SEPTIN6* exon 2. The point of fusion at position 24 of the 4612 bp *SEPTIN6* cDNA (GenBank no. D50918) was the same as in the fusion transcript in the AML of patient 62 (Figure 5b). The presence of *SEPTIN6* intron 3 sequence in the majority

of subclones was consistent with a related, incompletely processed transcript (Figure 5b). Additional subclones contained *MLL* exon 5 and 6 sequence only (Figure 5b). Amplification of the same first-strand cDNA with *MLL*- and *SEPTIN6*-specific primers and sequencing of the 471 bp product confirmed the fusion transcript (not shown). There was no evidence of alternative splicing of the fusion transcript.

#### Discussion

Cytogenetic detection of chromosome bands Xq22 and Xq24 as novel chromosomal partners of band 11q23 in



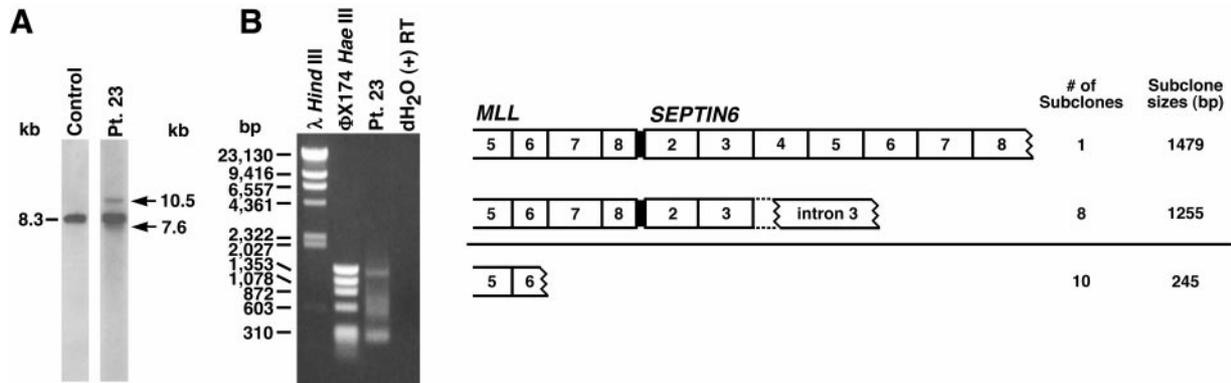
**Figure 4** (a) DNA topoisomerase II cleavage assay of *MLL* intron 7/exon 8 coordinates 2490 to 3077 containing normal homologue of *MLL* genomic breakpoint in AML of patient 62. Autoradiograph of cleavage products after 10 min incubation of 25 ng (30 000 c.p.m.) singly 5' end-labeled DNA with 147 nM human DNA topoisomerase II $\alpha$ , 1 mM ATP and, where indicated, 20  $\mu$ M etoposide (VP16) is shown (top). Heat indicates reactions incubated for 10 min at 65°C before trapping of covalent complexes. The indicated nucleotide (*MLL* position 2595), which was the translocation breakpoint, was the 5' side or -1 position of a cleavage site (bold arrow); the cleaved phosphodiester bond is 3' to this position. The DNA topoisomerase II inhibitor etoposide enhanced cleavage at this site 1.2-fold. Detection of cleavage after heating to 65°C indicates stability of the cleavage complex formed at this position. (b) Summary of DNA topoisomerase II *in vitro* cleavage sites proximal to *MLL* genomic breakpoint in AML of patient 62. Dots indicate bases at 5' side (-1 position) of cleavage sites identified. Numbers are relative nucleotide positions in normal genomic sequence. Arrow indicates correspondence of normal homologue of the translocation breakpoint to DNA topoisomerase II cleavage site

AML of infants and young children by several groups in recent years (Felix *et al.*, 1998; Harrison *et al.*, 1998; Mitelman *et al.*, 2001; Nakata *et al.*, 1999) and the identification of *MLL* involvement by Southern blot analysis in two of these cases (Felix *et al.*, 1998; Nakata *et al.*, 1999) predicted that one or more partner genes of *MLL* would be identified in these regions of chromosome Xq. Here we identified *SEPTIN6* as the

partner gene in a cryptic *MLL* rearrangement in a case of FAB M4 infant AML in which the original G-banded karyotype suggested involvement of band Xq22 but not band 11q23, and in a second case of FAB M2 infant AML with t(X;11)(q22;q23) (Felix *et al.*, 1998). Resultant in-frame transcripts contained a 5'-*MLL* exon 7-*SEPTIN6* exon 2-3' fusion in one case and a 5'-*MLL* exon 8-*SEPTIN6* exon 2-3' fusion in the other.

In another recently reported case of infant AML of FAB M2 morphology with cryptic rearrangement of band 11q23, FISH identified ins(X;11)(q24;q23q23), also associated with a 5'-*MLL* exon 8-*SEPTIN6* exon 2-3' fusion transcript (Borkhardt *et al.*, 2001). cDNA panhandle PCR characterization of three additional cases of FAB M1 or FAB M2 infant AML with in-frame 5'-*MLL* exon 7-*SEPTIN6* exon 2-3', 5'-*MLL* exon 8-*SEPTIN6* exon 2-3', or alternatively spliced 5'-*MLL* exon 8-*SEPTIN6* exon 2-3' and 5'-*MLL* exon 7-*SEPTIN6* exon 2-3' fusion transcripts, has just been described (Ono *et al.*, 2002). The karyotype suggested t(5;11)(q13;q23) and add(X)(q22) in one case (Ono *et al.*, 2002). In the second case the karyotype was normal but FISH unmasked the abnormality ins(X;11)(q22-24;q23) (Ono *et al.*, 2002). The cytogenetic abnormality in the third case was described as add(X)(q2?),del(11q?) (Ono *et al.*, 2002). These results further demonstrate the utility of cDNA panhandle PCR (Megonigal *et al.*, 2000a,b) for partner gene identification in complex rearrangements. Recently, *SEPTIN6* was annotated at chromosome band Xq24 in the human genome project (<http://genome.ucsc.edu>). Molecular detection of *MLL-SEPTIN6* transcripts in the two leukaemias with cytogenetic Xq22 breakpoints in the present study and in the cases with cytogenetic Xq24 breakpoints described by others (Borkhardt *et al.*, 2001; Ono *et al.*, 2002) indicate the difficulty in precise cytogenetic breakpoint definition in leukaemias with *MLL-SEPTIN6* rearrangements, as well as the recurrent nature of this translocation. Six other cases with simple or complex cytogenetic rearrangements of chromosome bands 11q23 and Xq22 or Xq24 have been reported (Mitelman *et al.*, 2001), which also potentially could be found to involve *MLL* and *SEPTIN6*.

Unlike most *MLL* translocations in which the 5'-*MLL-PARTNER GENE-3'* genomic breakpoint junction is created on the der(11) chromosome, combined SKY and FISH analyses of the AML of patient 62 showed that the 5'-*MLL-SEPTIN6-3'* genomic breakpoint junction was not on the der(11) chromosome, but on the der(X), where the partner gene resides. Similarly, in two of the recently reported cases of infant AML described above, FISH suggested that the 5' portion of *MLL* had been inserted into the X chromosome and that the genomic breakpoint junction from which the *MLL-SEPTIN6* transcript was produced was on the der(X) (Borkhardt *et al.*, 2001; Ono *et al.*, 2002). Noteworthy also is that in the AML of patient 62 SKY indicated disruption of chromosome band 3p21. The *AF3p21* gene at band 3p21 encoding a SH3 protein has been identified as a partner gene of *MLL* in treatment-related AML (Sano *et al.*, 2000).



**Figure 5** (a) *MLL* bcr rearrangement in AML of patient 23. *Bam*HI-digested DNA from marrow at AML diagnosis was hybridized with B859 fragment of *ALL-1* cDNA (Gu *et al.*, 1992). 8.3 kb fragment is from unrearranged *MLL* allele; arrows show two rearrangements. (b) cDNA panhandle PCR analysis of total RNA from diagnostic marrow of patient 23. Smear in third lane of gel shows products of various sizes from amplification of 5'-*MLL*-NNNNNN-3'-primed first strand cDNAs with *MLL*-specific primers (left). The products were subcloned by recombination PCR. Nine subclones contained an in-frame fusion of *MLL* exon 8 to *SEPTIN6* exon 2. Subclones with *SEPTIN6* intron 3 in sequence are from incompletely processed transcripts (top right). Other subclones contained only *MLL* (bottom right)

However, because the single *MLL* bcr rearrangement in the AML of patient 62 involved *SEPTIN6*, the detection of split *MLL* signals on the der(X) and the der(3) chromosomes by FISH suggested that the *MLL* sequences on the der(3) chromosome were distal to the bcr.

In the leukaemia of patient 62, the complex translocation suggested damage to the genome. Therefore, the corresponding *MLL-SEPTIN6* genomic breakpoint junction was studied in detail. DNA topoisomerase II has been implicated in the DNA damage leading to *MLL* translocations because of epidemiological associations of chemotherapeutic and dietary DNA topoisomerase II inhibitors, respectively, with treatment-related and infant acute leukaemias (Ross, 1998; Ross *et al.*, 1996; Smith *et al.*, 1999). An *in vitro* DNA topoisomerase II cleavage assay was undertaken to investigate the potential relationship between functional DNA topoisomerase II cleavage sites and the *MLL* genomic breakpoint. DNA topoisomerase II catalyzes transient and reversible cleavage and religation of both strands of the double helix (Fortune and Osheroff, 2000). Etoposide decreases the religation rate and is often used in these assays to enhance the detection of the cleavage complexes, which otherwise are transient and more difficult to discern (Fortune and Osheroff, 2000). The detection of heat-stable cleavage complexes at position 2595 even without drug enhancement indicates that the translocation breakpoint sequence is a naturally-occurring site of DNA topoisomerase II cleavage that is resistant to religation. Although position 2595 was not a highly preferred cleavage site, stability of the broken DNA may have been more relevant to the translocation. One model for *MLL* translocations involves DNA topoisomerase II mediated chromosomal breakage and formation of the translocations when the breakage is repaired (Felix, 2000). The correspon-

dence of the translocation breakpoint and the DNA topoisomerase II cleavage site is consistent with this model. The microhomologies between *MLL* and *SEPTIN6* near the genomic breakpoint junction observed in the sequence, like those at other *MLL* genomic breakpoint junctions (Felix *et al.*, 1997, 1999; Gillert *et al.*, 1999; Megonigal *et al.*, 1998, 2000a; Super *et al.*, 1997), may suggest that nonhomologous end-joining was involved in the repair (Gillert *et al.*, 1999; Lovett *et al.*, 2001). DNA topoisomerase II cleavage assays of the involved genomic regions of *SEPTIN6* and other *SEPTIN* family members would be highly relevant to further testing of this model.

Nine mammalian *SEPTIN* family members have been identified so far (Kinoshita *et al.*, 2000). Human analogues for most of the nine can be found in GenBank. The *SEPTIN* genes comprise a gene family in which several different members can fuse with *MLL*. The characterization of identical, non-constitutional 5'-*MLL*-hCDCrel-3' genomic breakpoint junction sequences in infant twins established that *MLL* translocations in infant AML are *in utero* events (Megonigal *et al.*, 1998). The *MLL*-hCDCrel genomic breakpoint junction sequence in the AMLs of the infant twins contained evidence of DNA damage and repair (Megonigal *et al.*, 1998) similar to that in the *MLL-SEPTIN6* genomic breakpoint junction sequence in the AML of patient 62. That hCDCrel, *MSF(AF-17q25)* and *SEPTIN6* are all disrupted by *MLL* translocations (Borkhardt *et al.*, 2001; Megonigal *et al.*, 1998; Ono *et al.*, 2002; Taki *et al.*, 1999b; Tatsumi *et al.*, 2001) suggests that *SEPTIN* family members are particularly vulnerable to damage and recombination to form *MLL* translocations associated with infant AML. Besides the *SEPTIN* family, *LAF-4* is the only other gene family with three members (*LAF-4*, *AF4*, *AF5q31*) that fuse with *MLL* (Ayton and Cleary, 2001; Huret, 2001; Nilson *et al.*, 1997; Taki *et al.*, 1999a).

First identified in budding yeast and later in *Drosophila*, Septin proteins are believed to be important in septation, cell division, cytokinesis, vesicle trafficking and exocytosis (Beites *et al.*, 2001; Kartmann and Roth, 2001; Kinoshita *et al.*, 2000). Although their roles in mammalian cells are incompletely understood, the *SEPTIN* genes all encode GTP binding proteins with a central, conserved GTPase domain, a variable N-terminal extension domain and a C-terminal coiled coil; the various Septin proteins may function in heteropolymeric complexes with each other in the cytoskeleton (Cooper and Kiehart, 1996; Field and Kellogg, 1999; Kinoshita *et al.*, 2000). Murine Septin6 expression is detected in synaptic vesicles in specific regions of the brain (Kinoshita *et al.*, 2000). In the human, several alternatively spliced *SEPTIN6* transcripts are differentially expressed in adult and fetal tissues (Ono *et al.*, 2002).

This study brings to six the cases of infant leukaemia in which molecular rearrangement of *MLL* with *SEPTIN6* has been reported. The complex rearrangement of chromosomes 3, X and 11 is a novel translocation. The fusion transcripts in both leukaemias that we studied and in those reported (Borkhardt *et al.*, 2001; Ono *et al.*, 2002) joined 5' *MLL* sequences in-frame with *SEPTIN6* exon 2, 5' in the coding sequence of this gene. Predicted fusion proteins from the *MLL-SEPTIN6* translocations would contain the N-terminal AT-hook, DNA methyltransferase, and repression domains of *MLL* and all three domains of Septin6. Investigation of the cellular localization of the resultant fusion proteins and their role in leukaemogenesis is warranted. The identification of three *SEPTIN* family members as partner genes of *MLL* suggests an important common pathway to leukaemogenesis in AML with these translocations. The high WBC count, organomegaly and myelomonocytic morphology in the AML of patient 62 are archetypal features of leukaemias with *MLL* translocations, but the FAB M2 morphology of the AML of patient 23 and FAB M1 and FAB M2 morphologies in other cases (Borkhardt *et al.*, 2001; Ono *et al.*, 2002) indicate heterogeneity in presenting features. One patient in this study and two of the four reported patients (Borkhardt *et al.*, 2001; Ono *et al.*, 2002) have survived, suggesting heterogeneity in outcomes also. The role of *SEPTIN* family aberrations may extend to yet other cancers, since *MSF* loss of heterozygosity is frequently observed in cancers of the ovary and breast (Russell *et al.*, 2000).

## Materials and methods

The IRB at the Children's Hospital of Philadelphia approved this research.

### Southern blot analysis

The *MLL* breakpoint cluster region (bcr) was examined in *Bam*HI-digested DNA using the B859 fragment of *ALL-1* cDNA (Gu *et al.*, 1992).

### cDNA panhandle PCR analysis of *MLL* fusion transcripts

First-strand cDNAs were synthesized from 0.5–1 µg of total RNA using oligonucleotides containing *MLL* exon 5 sequence at the 5' ends and random hexamers at the 3' ends (Megonigal *et al.*, 2000a,b). Second-strand cDNA synthesis, formation of stem-loop templates, and PCR with *MLL*-specific primers were as described (Megonigal *et al.*, 2000a,c). Products were subcloned by recombination PCR; the subclones were screened by PCR and sequenced (Megonigal *et al.*, 2000a,b).

*MLL* fusion transcripts were confirmed by amplifying 2 µl of the same first-strand cDNAs with the *MLL* exon 5 sense primer 5'-AGTGAGCCCAAGAAAAAGCA-3' corresponding to positions 3973 to 3992 in the HUMHRX cDNA (GenBank no. L04284) and the *SEPTIN6* exon 2 antisense primer 5'-GCACAGGATGTTGAAGCAGA-3' corresponding to positions 134 to 115 in the KIAA0128 cDNA (GenBank no. D50918). The products were gel-purified and sequenced.

### Panhandle variant PCR

Genomic DNA from the leukaemia of patient 62 was studied by panhandle variant PCR. Reactions were performed and the products were subcloned by recombination PCR as described (Megonigal *et al.*, 1998). The breakpoint junction was confirmed by PCR with primers 5'-TCTGTTGCAA ATGTGAAGGC-3' corresponding to positions 2288–2307 in *MLL* intron 6 (GenBank no. U04737) and 5'-TTTTTGA-GACGGATTCCCAC-3' corresponding to positions 3557 to 3576 in *SEPTIN6* intron 1 (GenBank nos. AL355348.28; AC005052.2).

### Spectral karyotype analysis (SKY)

Metaphases for SKY were prepared from 10<sup>7</sup> viably frozen bone marrow cells that were thawed and cultured for 24, 48 and 72 h in RPMI 1640 medium (Gibco–BRL, Gaithersburg, MD, USA) supplemented with 20% Fetal Bovine Serum, 10% Giant-Cell-Tumor-Conditioned Medium (Origen<sup>TM</sup>, Gaithersburg, MD, USA), 10 ng/ml IL-3 (Boehringer Mannheim, Indianapolis, IN, USA), 50 ng/ml SCF (Boehringer Mannheim) and 50 ng/ml Flt-3 (R&D Systems, Minneapolis, MN, USA). Chromosomes were harvested and metaphase spreads were prepared following standard procedures (Roulston and Le Beau, 1997). Twenty-four differentially labelled chromosome-specific painting probes were simultaneously hybridized onto metaphase chromosomes as described (Schröck *et al.*, 1996). Probe preparation, slide pretreatment, hybridization and detection were per established protocols (Macville *et al.*, 1997). Ten metaphases were imaged using the SpectraCube<sup>TM</sup>SD200 system (Applied Spectral Imaging, Carlsbad, CA, USA) connected to an epifluorescence microscope (DMRXA, Leica Microsystems, Wetzlar, Germany) and analysed together with the corresponding inverted DAPI images using SkyView<sup>TM</sup> v.1.2.04 software (Applied Spectral Imaging). The karyotype was interpreted according to the guidelines for cytogenetic nomenclature of the ISCN 1995 (Mitelman, 1995).

### Fluorescence in situ hybridization analysis (FISH)

FISH was performed according to standard procedures using chromosome painting probes for chromosomes 3 and 11, a centromere probe for X (Spectrum Acqua, Vysis, Downers

Grove, IL, USA) and a DNA probe for *MLL* (Ventana Medical Systems, Tucson, AZ, USA). Images were acquired using Leica Q-FISH software (Leica Imaging Systems, Cambridge, UK).

#### DNA topoisomerase II in vitro cleavage assay

A DNA fragment spanning *MLL* intron 7/exon 8 positions 2490 to 3077 and containing the normal homologue of the *MLL* genomic breakpoint in the AML of patient 62, was subcloned into pBluescript II SK (Stratagene; La Jolla, CA, USA). The singly 5' end-labelled, double-stranded DNA substrate was prepared from the plasmid as described (Lovett *et al.*, 2001). Twenty-five ng of substrate DNA were incubated with human DNA topoisomerase II $\alpha$ , ATP and MgCl<sub>2</sub> either in absence of drug or in the presence of 20  $\mu$ M etoposide per the same reaction conditions used for other cleavage assays (Lovett *et al.*, 2001). Covalent complexes then were irreversibly trapped by adding SDS, without or following incubation for 10 min at 65°C, the latter to evaluate heat stability (Lovett *et al.*, 2001). The cleavage products were deproteinized and electrophoresed in a denaturing polyacrylamide gel in parallel with a dideoxy

sequencing ladder to map the sites of cleavage (Lovett *et al.*, 2001).

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#### Accession numbers

The sequences reported in this study were deposited in the GenBank database (accession nos. AF512942, AF512943, AF512944, AF512945, AF512946).

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