**MLL Self Fusion Mediated by Alu Repeat Homologous Recombination and Prognosis of AML-M4/M5 Subtypes**


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**ABSTRACT**

Fifty-six patients with *de novo* acute myeloid leukemia M4/M5 subtypes were studied for rearrangements of the mixed lineage leukemia gene, MLL (also called HRX, Htrz-1, or ALL-1). Ten patients (18%) showed rearrangements of the MLL gene, 9 in a major breakpoint cluster region within a centromeric 8.3-kb *Bam*HI fragment, whereas rearrangement in one patient was the result of a direct tandem duplication of exons 2-6 of MLL. Analysis of sequences at the duplication junction revealed that the points of MLL fusion within introns 6 and 1 both lie within Alu elements. This suggests the involvement of Alu repeat mediated homologous recombination in MLL self fusion. For the 10 rearranged samples, cytogenetic analysis revealed a normal karyotype in 3, and 3 had abnormalities other than 11q23. Survival analysis of patients revealed no difference between those with rearrangement of MLL and those showing the germ-line configuration.

**INTRODUCTION**

Translocations involving chromosome band 11q23 are observed in different types of acute leukemias, including acute lymphoid leukemia, AML, chemotherapy-related AML, and myelodysplastic syndrome (1-4). At least 25 different partner chromosomes have been involved in reciprocal translocations (5). The more common translocations include t(9;11)(p22;q23) and t(6;11)(p27;q23) in AML and t(11;19)(q23;p13) and t(4;11)(q21;q23) in infant leukemias (6-11). The gene at chromosome 11q23, which is disrupted in chromosomal translocations, is MLL (also called HRX, Htrz-1, or ALL-1, Refs. 12-16). It encodes a predicted M, 431,000 protein that contains several functional domains including zinc finger and AT-hook DNA binding motifs as well as a DNA methyltransferase homology region. Ten different genes fused to MLL during chromosomal translocation have been cloned (reviewed in Ref. 17). These include genes that encode nuclear proteins, cytoplasmic factors, and putative transcription factors. Of particular interest, MLL rearrangement can also be a result of direct tandem duplication of a portion of MLL. The best documented types of MLL self fusion are direct tandem duplication of exons 2-6 and exons 2-8 of MLL (18-22). Recently, deletion of exon 8 of MLL has been observed in T-ALLs (23).

Although MLL spans a region of approximately 90 kb, the breakpoints in MLL have been reported to cluster within a 8.3-kb *Bam*HI genomic fragment that can be detected by Southern hybridization to DNA probes (e.g., P5q, 98.40, FA4, and B9) (16, 24, 25). Sequence analysis of this breakpoint cluster region revealed the presence of Alu repeats, topoisomerase II recognition site-like sequences, and nonamer and heptamer sequences for VDJ recombinase (26, 27). The presence of these sequences in the breakpoint cluster region leads to different hypotheses for the MLL recombination mechanism (18-21, 25-30). Thus, the mechanism underlying MLL rearrangement in different types of acute leukemia is still unclear.

MLL rearrangements are found more frequently in infant than in adult leukemias, e.g., 70% of infant ALLs and 50% of infant AMLs (11, 31, 32) compared with 2.3 and 4.4% in adults, respectively (33, 34). MLL rearrangements in both infant and adult AMLs are significantly associated with the FAB-M4/M5 subtypes (24, 34, 35) and cytogenetic findings of 11q23 translocations (36, 37). MLL rearrangements in both infant ALLs and AMLs are associated with a poor prognosis compared with infants without the MLL translocation (25, 31, 35). It has, therefore, been suggested that infant patients with MLL rearrangement should be treated with more aggressive therapy (25).

However, there are few studies on the prognostic significance of the MLL rearrangement in adult acute leukemia patients with conflicting conclusions. Bower et al. (34) found that adult AML patients with MLL rearrangements showed no significant prognostic difference from those patients carrying the similar phenotypes without MLL rearrangement, while Coco et al. (24) suggested that adult leukemic patients with MLL gene rearrangements were significantly associated with resistant disease and poor prognosis. Thus, we undertook to analyze the prognostic impact of MLL rearrangement status in a series of 56 *de novo* AML-M4 and M5 subtype patients.

**MATERIALS AND METHODS**

**Patients and Samples.** Fifty-six *de novo* AML patients (28 at Queen Mary Hospital in Hong Kong and 28 at Rui-Jin Hospital in Shanghai, China) studied. According to standard FAB criteria (38), 20 patients were classified as AML-M4 and 36 were AML-M5. The clinical details of patients are shown in Table 1. The patients were treated with a standard protocol comprising an induction regimen of 100 mg/m²/day of Ara C, 50 mg/m²/day (days 1-3) daunorubicin, and 75 mg/m²/day (days 1-7) etoposide, followed by consolidation with two courses of Ara C for 5 days, daunorubicin for 2 days, and etoposide for 5 days with the same dosage as induction. For maintenance, an 8-week course of 100 mg/m²/day of Ara C for 5 days and 100 mg/m²/day of threouamine for 5 days was given.

**Cytogenetic Analysis.** Banding and karyotyping were performed on bone marrow aspirates using standard methods as published previously (39). Details of the karyotype are reported according to ISCN 1991 (40).

**Southern Analysis.** Genomic DNA from leukemia patients or the K562 CML cell line was digested with proteinase K and then extracted with phenol/chloroform and precipitated with ethanol. Ten μg of DNA aliquots were digested with restriction enzymes (EcoRI, BamHI, SacI, and XhoI) according to the manufacturers' instructions, and Southern blots were prepared according to standard protocols. For probes, the blots were hybridized to: (a) 98.40 and (b) PS4, which are centromeric and telomeric to the R54:11 cell line breakpoint, respectively (21); (c) ES11, a 880-bp CDNA probe spanning exons 5-11, which detects a 8.3-kb BamHI germ-line fragment; and (d) SAS1, a 289-bp DNA probe from intron 1 of MLL for the detection of MLL direct tandem duplication (18). In some samples, the blots were hybridized to probe E910, an 180-bp probe spanning exons 9 and 10 made using reverse transcription-PCR with a 3'-specific primer (5'-GTGATACCTGTGTTCCGCC-3') designed from exon 10 and a 5' primer from exon 9 of MLL (5'-GTCATATGCAAGTCTC-3').
Table 2 Cytogenetic and molecular analysis of rearranged cases

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age(yrs)</th>
<th>FAB*a</th>
<th>Karyotype</th>
<th>BamHl</th>
<th>EcoRl</th>
<th>SacI</th>
<th>XbaI</th>
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<tbody>
<tr>
<td>17</td>
<td>F/F24</td>
<td>M5</td>
<td>46,XX</td>
<td>R G G R R</td>
<td>R G G G G</td>
<td>R R G R G</td>
<td>R N</td>
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<tr>
<td>18</td>
<td>F/F62</td>
<td>M5</td>
<td>Multiple abnormalities not including chromosome II</td>
<td>R G G G</td>
<td>R G G G</td>
<td>R R G R G</td>
<td>N N</td>
</tr>
<tr>
<td>19</td>
<td>M/M59</td>
<td>M5</td>
<td>46,XY</td>
<td>R G G R R</td>
<td>R G G G G</td>
<td>G G G G R</td>
<td>R G G</td>
</tr>
<tr>
<td>20</td>
<td>M/M75</td>
<td>M5</td>
<td>46,XY</td>
<td>G R R R G</td>
<td>G G R G G</td>
<td>R R G N N</td>
<td>N N</td>
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<tr>
<td>21</td>
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<td>G G G R G</td>
<td>G G R G G</td>
<td>R G G</td>
</tr>
<tr>
<td>47</td>
<td>F/F13</td>
<td>M5</td>
<td>46,XX</td>
<td>R G G N N</td>
<td>R G G N R</td>
<td>R G N N N</td>
<td>N N</td>
</tr>
<tr>
<td>48</td>
<td>M/M6</td>
<td>ND</td>
<td></td>
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<td>R N G N R</td>
<td>N N N N N</td>
<td>N N</td>
</tr>
<tr>
<td>49</td>
<td>M/M1.3</td>
<td>M5</td>
<td>ND</td>
<td>R G G N N</td>
<td>R N G N R</td>
<td>N N N N N</td>
<td>N N</td>
</tr>
<tr>
<td>50</td>
<td>F/F1.8</td>
<td>M5</td>
<td>47-51,XX,+6,+8,+11, +13[8][46,XX]*[1]</td>
<td>R G G N N</td>
<td>R G G N R</td>
<td>R G N N N</td>
<td>N N</td>
</tr>
<tr>
<td>3</td>
<td>M/M71</td>
<td>ND</td>
<td></td>
<td>R G G R N</td>
<td>G G G G N</td>
<td>N N G N N</td>
<td>N N</td>
</tr>
</tbody>
</table>

a FAB, FAB subtype; G, germline configuration; R, rearrangement; ND, not determined; P/S, P/S4; 98, 98.40; E5, E510; SA, SASI; E9, E910; M, male; F, female.

3'). The PCR product was cloned into pKS+II vector (Stratagene) identified by restriction mapping and sequenced using a T7 DNA sequencing kit (Pharmacia). The positions of the probes are shown in Fig. 1A. Only patients who showed rearranged bands with two or more enzyme digests were scored as rearranged.

Amplification and Sequencing of MLL Genomic Duplication Junction in the M5 Patient. One hundred ng of genomic DNA from the patient and K562 cell line DNA were taken for PCR amplification using MLL exon 6 forward primer 5'-ACGCTGGTAATCCCAACACT-3' and exon 2 reverse primer 5'-GGACUCGCACTCTGACTFC-3'. The purified PCR product was analyzed and purified from agarose gel before it was cloned into a pGEM-T vector (Promega Corp.) by TA-cloning. The insert of the vector was then sequenced from both ends by a 373A automatic sequencer.

Sequencing of MLL Intron 1 from a P1 Clone. The P1 human genomic library constructed with SacBII vector (41) was screened by PCR with MLL primers on exon 3, sense primer 5'-GTACAAATTGTACGCGGAG-3' and antisense primer 5'-AACTGGCTTAAATTCCTGCAGGAG-3'. A positive clone, PAC-107-19B, with an insert of about 75 kb, was found to contain the 5' sequence of MLL by fluorescence in situ hybridization to cell lines with known translocations, t(4;11), t(6;11), t(9;11), and t(11;19) (data not shown). The PAC-107-19B clone was partially sequenced with MLL intron 1 primers (MLL-Int1.2R, 5'-CCTTGTACCACTGCTCA-3' and MLL-Int2R 5'-CATACTAGGCAACTGAGGA-3') designed from the sequence of the genomic duplication junction of the patient.

Prognosis and Statistical Analysis. Patients were classified into two groups based on their MLL rearrangement status. The prognostic difference between the two groups was analyzed using the Kaplan-Meier procedure (42). Differences in disease-free survival as well as survival rates between these two groups were analyzed using the log rank test (43, 44). Eleven patients who defaulted from the study were removed from the analysis.

RESULTS

Southern hybridization analysis revealed rearrangement of MLL in 10 of 56 patients (18%). These included of 1 of 20 AML-M4 patients (5%) and 9 of 36 AML-M5 patients (25%). Nine of 10 cases showed rearrangement with P/S4 and E511 within the 8.3-kb vector (Promega Corp.) by TA-cloning. The insert of the vector was then sequenced from both ends by a 373A automatic sequencer.

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Fig. 1. MLL genomic breakpoint analysis of AML-M4/M5 with MLL rearrangement detected by Southern hybridization. Vertical lines represent restriction enzyme sites (S, SacBII; X, XbaI; E, EcoRI; B, BamHI). Exons are indicated by vertical boxes. The locations of probes are also indicated. Horizontal lines above the map show positions of MLL rearrangements in AML-M4/M5 cases, with case numbers (see Table 1) indicated above each bar. The duplicated region of the MLL locus of patient 20 is bracketed by arrows. Alu repeats are indicated as black arrows on the solid line, along with the exon (25).

Table 1 Clinical details of M4/M5 patients

<table>
<thead>
<tr>
<th>No. of patients (M4:M5)</th>
<th>56 (20:36)</th>
<th>10 (1:9)</th>
<th>46 (19:27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>34:22</td>
<td>6:4</td>
<td>28:18</td>
</tr>
<tr>
<td>No. of patients (&gt;18 years)</td>
<td>48</td>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td>Mean age/year (range)</td>
<td>45 (21-85)</td>
<td>57 (24-75)</td>
<td>43 (21-85)</td>
</tr>
<tr>
<td>No. of patients (&lt;12 years)</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean age/year (range)</td>
<td>5.6 (1.3-12)</td>
<td>2.2 (1.3-6)</td>
<td>8.9 (2-12)</td>
</tr>
<tr>
<td>WBC count/×10^9</td>
<td>55 (1.4-354)</td>
<td>88 (1.4-354)</td>
<td>45 (1.7-305)</td>
</tr>
</tbody>
</table>

Clinical outcome

Remission 27 (48.2%) 6 (54.5%) 21 (46.7%)
Non-remission 10 (17.9%) 1 (9.1%) 9 (20%)
Early deaths 8 (14.3%) 2 (18.2%) 6 (13%)
Unaccessible—defaulted treatment 11 (19.6%) 1 (18.2%) 10 (20%)
BamHI fragment, which encompasses the major breakpoint cluster (Fig. 1A). The breakpoint of patient 20 was detected by both 98.40 and ES11 probes in BamHI digests (Table 2 and Fig. 2). Because 98.40 and ES11 hybridize to different regions of MLL, the rearrangement of MLL in this patient could be explained if tandem duplication had occurred. This was confirmed when the SAS1 probe revealed rearranged bands in EcoRI, BamHI, and SacI digests (Fig. 2).

Previous studies (18—22) have shown MLL duplications mainly involve exons 2—6 or exons 2—8. Because only germ-line bands were observed in patient 20 with the P/S4 probe, which spans intron 7 and exon 8, rearrangement in this patient was unlikely to result from a partial duplication of MLL exon 2—8. The rearranged bands in the BamHI and SacI digest (but not in the EcoRI digest) with SAS1 comigrated with the rearranged bands detected by 98.40 (Fig. 2). This suggested that the rearrangement in patient 20 could be a duplication of exons 2—6 of the MLL gene (Fig. 2). The mechanism that mediated this gene rearrangement was studied by PCR using MLL exon 6 forward and exon 2 reverse primers; this amplified a unique 2-kb fragment from patient 20 but not from the K562 cell line (Fig. 3A). Sequence analysis showed that there were some mismatches between the genomic junction fragment and the published MLL intron 6 sequence (Ref. 26, Fig. 3B). To define precisely the fusion points of the patient, we partially sequenced the PAC-107-19B clone, which contains the 5' genomic sequence of MLL. Analysis of the sequences revealed that the points of fusion within introns 6 and 1 both lie within Alu elements belonging to the same Alu subfamily Ss (Fig. 3B). These two Alu elements in different introns showed 83% identity over 300 bp. Our results suggested that portions of the two different Alu elements were fused together to reconstruct a chimeric Alu sequence (Fig. 3C). Thus, Alu repeat-mediated homologous recombination is involved in the molecular mechanism of MLL self fusion. Because 26 bp at the center of the fusion junction are identical in both Alu elements, it was not possible to define the exact fusion point in the patient.

**Cytogenetic Evaluation of Patients with MLL Rearrangements.**

Cytogenetic studies of six rearranged cases are summarized in Table 2. Three cases show a normal karyotype, and three patients had chromosomal abnormalities other than chromosome 11q23. Patient 20
The clinical features of the patients at presentation are shown in Table 1. The DNA sequence of the patient (MLLdup, accession no. U66259) is aligned with the MU intron 6 sequence (intron 6, accession no. U04737 ref 26) and MLL intron 1 sequence (intron 1, accession no. U66259). DNA sequence is numbered according to the KS62 control cell line. B, DNA sequence across the genomic junction of the MU tandem duplication. Duplication junction sequence from the patient (MLL4up, accession U04737 ref 26).

Fig. 3. Genomic junction analysis of the MLL duplication case. In A, a unique 2-kb band was amplified using MLL Ex2 forward and Ex6 reverse primers from the patient but not from the K562 control cell line. B, DNA sequence across the genomic junction of the MLL tandem duplication. Duplication junction sequence from the patient (MLLdup, accession no. U66258) is aligned with the MLL intron 6 sequence (intron 6, U04737 ref 26) and MLL intron 1 sequence (intron 1, accession no. U66259). DNA sequence is numbered according to the breakpoint cluster sequence by Gu et al. (26). The boxed region indicates where recombination has occurred. C, schematic representation of the reconstruction of the chimeric Alu element in MLL tandem duplication by Alu repeat-mediated homologous recombination. Rectangles, exons. Arrows, Alu sequences with the vertex denoting the 3' end of the element.

with MLL direct tandem duplication was not trisomic for chromosome 11.

Statistical Analysis of Prognostic Outcome in the Two Groups. The clinical features of the patients at presentation are shown in Table 1. Kaplan-Meier survival analysis was used to assess the prognostic implications of MLL rearrangement. No significant difference was found in either disease-free survival or survival rate between these two groups of patients using log rank test, i.e., 7 and 13.6 months for group A versus 6 and 13.2 months for group B (Fig. 4a).

To eliminate any discrepancy due to the relative wide age range of the patients, the patients were divided into two groups, age ≥ 18 years (42 germ-line and 6 rearranged cases) and age < 18 years (4 germ-line and 4 rearranged cases), for the calculation of Ps according to standard procedure (44). The Ps obtained from both disease-free survival and survival curves were greater than 0.05, confirming that no difference could be found between these age groups (Fig. 4B). The data suggest that clinical outcome between these two groups could not be due to the native prognostic response difference between the childhood and adult patients.

In summary, our results show that MLL rearrangement in our patients with M4/M5 subtypes, in contrast to infants, does not confer a prognostic significance from patients that do not show rearrangement.

DISCUSSION

Chromosomal translocations involving band 11q23 are specifically associated with FAB-M4 and FAB-M5 subtypes in both infant and adult AML as well as therapy-related-AML (12, 37, 45, 46). Our findings are consistent with the results of infant studies that MLL rearrangements are strongly correlated with AML-M4 and -M5 subtypes (25). Rearrangements of MLL were detected in 10 of 56 patients (18%) with M4/M5 phenotypes, a rate similar to that reported by Bowet et al. (34) and Cimino et al. (47). The strong association with myelomonocytic leukemia (M4) and monocyctic leukemia (M5) subtypes suggests that MLL may play a regulatory role in the differentiation and/or development of monocytic progenitor cells.

Partial duplications of MLL have been reported in a few AML patients with different subtypes (18—22, 48, 49). A recent study by Yu et al. (49) suggested a poor prognosis for a subgroup of patients with MLL partial duplication that may need different treatment. Because the reported breakpoints of all MLL abnormalities, including partial duplication, reciprocal translocation, and internal deletion have MLL breakpoints clustering within an 8.3-kb BamHI genomic fragment, these can allow detection of MLL gene rearrangements by Southern analysis using a centromeric genomic probe (17, 21, 23) that spans this region. Partial duplication of MLL, however, can be distinguished from the rest only by a 5' MLL probe (e.g., SAS1) in which a rearrangement would comigrate with rearranged bands detected by a 3' probe (e.g., 98.40).

Sequence analysis of an AML patient with MLL exons 2—8 tandem duplication (19) revealed the involvement of Alu repeats in both MLL intron 1 and intron 8 fusion junctions. Here we provide additional evidence that Alu repeat-mediated homologous recombination is a molecular mechanism for MLL duplication. In the MLL exons 2—8 tandem duplication patient (19) and the MLL exons 2—6 tandem duplication patient reported here, the same intron 1 Alu element mediated the recombination, although the exact cross-over sites were different (120—160 bp apart). These two fusions would both lead to reconstruction of a chimeric, full-length Alu element. Recombination mediated by Alu repeats had been reported in many human genetic diseases (50—52). Thus, it is possible that Alu elements clustered in

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M4/MS patients showed no significant prognostic difference from those with MLL germ-line configuration.

ALU-MEDIATED MLL SELF FUSION AND PROGNOSIS STUDIES IN AML

MLL may play a critical role in mediating the illegitimate recombination in MLL partial duplication.

Cytogenetic data were available for 6 of 10 MLL rearranged cases, but none showed chromosomal 11q23 abnormalities. Trisomy 11 in AML has been suggested as a marker for MLL gene self fusion (21, 22); however, a normal karyotype was found in patient 20. Cytogenetic studies are often complicated by low mitotic index, and the sensitivity of karyotype analysis may be limited by submicroscopic abnormalities of band 11q23. In this study, molecular analysis has revealed MLL rearrangements in 10 M4/M5 patients, including 6 showing abnormality of band 11q23 abnormalities by conventional cytogenetics. Our results support the use of molecular techniques for the routine diagnosis of MLL rearrangement in adult AML-M4 and -M5 patients (24, 34).

Disease-free survival and survival curves of group A (MLL rearranged) and group B (MLL germ-line) patients showed no significant difference, even after discounting the age factor. Our data also suggest that there is a lack of prognostic significance of MLL rearrangement in adult AML-M4 and -M5 patients. However, this needs to be confirmed in a larger study because of the small number of childhood patients in our cohort. Our results agree with that of Bower et al. (34) in which MLL rearrangement in adult AML-M4/M5 patients showed no significant prognostic difference from those with MLL germ-line configuration.

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