Rearrangement of the myeloid-lymphoid leukaemia gene in Hong Kong Chinese children with acute leukaemia

Objective. To determine the frequency of rearrangement of the myeloid-lymphoid leukaemia gene in acute leukaemia in Hong Kong Chinese children.

Design. Immunophenotyping, cytogenetic, and molecular analysis.

Setting. Regional hospital, Hong Kong

Participants. Bone marrow or peripheral blood samples were taken from 27 children aged 16 years or younger with acute leukaemia, from September 1995 through February 1998.

Main outcome measures. Gene rearrangement was analysed by Southern blotting of HindIII digestion products of mononuclear cell DNA, followed by hybridisation with the myeloid-lymphoid leukaemia P/S4 probe. Nested reverse transcription–polymerase chain reaction analysis was performed to detect and characterise duplication of the myeloid-lymphoid leukaemia gene.

Results. Only one of 23 (4%) children whose marrow or peripheral blood samples contained adequate material for genetic study showed rearrangement in the myeloid-lymphoid leukaemia gene. No children were positive for partial tandem duplication of the myeloid-lymphoid leukaemia gene.

Conclusion. Myeloid-lymphoid leukaemia gene rearrangement is rare in Hong Kong Chinese children with acute leukaemia.

Introduction

The myeloid-lymphoid leukaemia (MLL) gene—also known as acute lymphoblastic leukaemia-1, HRX, and HTRX-1 gene—is located at chromosome 11q23,¹ and is unique among leukaemia genes in that it
can fuse with a large number of different partner genes on other chromosomes. At least 20 different partner chromosomes have been involved in 11q23 reciprocal translocations.\(^2\) In addition, the leukaemic cells are characterised by features of mixed lineage differentiation.\(^2\) They may show monocytic, lymphoid, or biphenotypic (myeloid and lymphoid) antigen expression, which implies that a pluripotential progenitor cell may be involved in leukaemogenesis.

The involvement of the \(\text{MLL}\) gene in oncogenesis is not limited to chromosomal translocations that result in chimeric fusion genes. The \(\text{MLL}\) gene has recently also been found to be involved in acute leukemias without visible chromosomal translocations. Partial tandem duplication of the \(\text{MLL}\) gene also occurs in some cases.\(^3\)

Conventional cytogenetic analysis can detect only a subset of acute leukemias that harbour \(\text{MLL}\) gene rearrangement, and it cannot distinguish cases containing germline-derived 11q23 alterations from those with rearranged \(\text{MLL}\) gene. In some cases of acute leukaemia, \(\text{MLL}\) gene rearrangements can occur without cytogenetic evidence of 11q23 rearrangement.\(^4,6\)

More sensitive molecular techniques are widely used to accurately identify patients with \(\text{MLL}\) gene rearrangements because these patients have a poor prognosis and are candidates for bone marrow transplantation or other aggressive therapies.\(^2\) For example, the analysis of reverse transcription–polymerase chain reaction (RT-PCR) amplification products provides an invaluable diagnostic tool to identify partial tandem duplication of the \(\text{MLL}\) gene.\(^8\)

Translocations that involve the \(\text{MLL}\) gene at chromosome band 11q23 occur in approximately 10% of patients with acute lymphoblastic leukaemia (ALL), 5% of those with acute myeloid leukaemia (AML), and 85% of those with secondary leukaemias who are treated with topoisomerase II inhibitors.\(^2\) The \(\text{MLL}\) gene is affected specifically in approximately 80% of infants younger than 1 year with ALL and in 3% of older children with ALL.\(^7\)

Most of the published studies, however, have been performed in western countries. The frequency of \(\text{MLL}\) gene rearrangement in Hong Kong Chinese children with acute leukaemia is still not known. In this study, Hong Kong Chinese children with acute leukaemia were analysed to determine the frequencies of rearrangement and partial tandem duplication of the \(\text{MLL}\) gene.

**Methods**

**Patient samples**

From September 1995 through February 1998, bone marrow or peripheral blood samples were taken from 27 children aged 16 years or younger who had a confirmed diagnosis of ALL or AML. The samples were obtained from the Department of Pathology at the Queen Elizabeth Hospital after obtaining informed consent. Mononuclear cells, including blast cells, were isolated from the samples by density centrifugation using a Ficoll-paque gradient and stored at -70\(^\circ\)C.

The diagnosis of acute leukaemia was made according to the French-American-British classification system using morphological, cytochemical, and immunophenotypic criteria.\(^9,10\) For ALL samples, immunophenotyping was performed by streptavidin-biotin immunocytochemical staining using a comprehensive panel of different lineage-associated markers. Based on their pattern of reactivity, leukemias were classified as early B-cell precursor (TdT+, CD19+, HLA-DR+), common (TdT+, CD19+, CD10+), pre-B cell (TdT+, CD19+, CD10+, Cy Ig+), B cell (TdT-, CD19+, CD10+/-, S Ig+), early T-cell precursor (TdT+, CD7+, CD2-), or T cell (TdT+, CD7+, CD2+, CD3+).

**Cytogenetic studies**

The cytogenetic results of all the samples were used to correlate with the molecular aberrations associated with leukaemia. Conventional cytogenetic studies were performed using overnight fluorodeoxyuridine-synchronised culturing of the blast cells. Metaphase chromosomes were analysed by G-banding and karyotyped according to the International System for Human Cytogenetic Nomenclature 1995.\(^11\)

**RNA and DNA isolation**

Single-step simultaneous isolation of total RNA and DNA from cryopreserved cell samples was performed using TRIzol reagent (GIBCO BRL/Life Technologies, Gaithersburg, USA).\(^12,13\) Total RNA and DNA were isolated according to the manufacturer’s protocol with modifications.\(^14\)

**Southern blot analysis**

Southern blot analysis was performed according to standard protocols.\(^14\) The \(\text{MLL}\) probe that was used (P/S4) spanned from exon 7 to part of intron 8 of the \(\text{MLL}\) gene (Fig 1a).\(^15\) The DNA of a bone marrow sample from a patient with previously confirmed \(\text{MLL}\) gene rearrangement was used as a positive control and that from a healthy donor was used as a negative control. Samples were incubated with \(\text{HindIII}\) restriction enzyme.
enzyme, subjected to electrophoresis and Southern blotting, and hybridised with the P/S4 probe.

**Reverse transcription–polymerase chain reaction**
The RT-PCR method of cDNA production was performed as previously described. The first strand of cDNA was reverse-transcribed with the primer 3.1c (5'-AGG AGA GAG TTT ACC TGC TC-3'). The first round of PCR was performed with primers 3.1c and 5.3 (5'-GGA AGT CAA GCA AGC AGG TC-3'). The second round of PCR was performed with 2% of the first-round PCR product and primers 6.1 (5'-GTC CAG AGC AGA GCA AAC AG-3') and 3.2c (5'-ACA CAG A TG GA T CTG AGA GG-3') [Fig 1b]. The RNA of a bone marrow sample from a patient who had been confirmed to have a tandem duplication in the MLl gene was used as a positive control; RNA from a healthy donor was used as a negative control.

Before conducting any nested RT-PCR investigation, the integrity of each RNA sample was checked by performing RT-PCR with β₂-microglobulin (β₂M) primers because RNA degradation could not be detected spectrophotometrically. Oligonucleotide primers that were used to amplify the β₂M DNA sequence were 5'-GGA CAT ACT ACC CTG AAT GA-3' (residues 1705-1724 sense strand) [β₂M₁] and 5'-CAT GCT GTG CAT CAG TAT CT-3' (residues 1806-1825 antisense strand) [β₂M₂]; the result was a 120-bp product. The experiment was considered valid only when the controls showed the expected results.

**Results**
The results of the cytogenetic and molecular analysis of the 27 patients studied for MLl gene rearrangement are shown in the Table. They were all analysed at the time of diagnosis and none was analysed at relapse. There were 16 males and 11 females, with a median age of 7.8 years (range, 3 days to 16 years). Eighteen (67%) patients had ALL, with 14 (78%) of them being of B-cell lineage. All 27 patients were successfully karyotyped and only 2 showed 11q23 aberrations (patients 5 and 22).

Samples from 23 patients contained adequate materials for further genetic study. The P/S4 probe detected a germline fragment of approximately 14 kb after hybridisation with separated HindIII digestion products. The positions of the germline and rearranged bands are shown in Fig 2. Different sizes of rearranged bands were seen because different partner chromosomes were involved, so the locations of breakpoints were different in various MLl gene rearrangements. For
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/age</th>
<th>Specimen</th>
<th>Diagnosis</th>
<th>Karyotype</th>
<th>Southern blot analysis</th>
<th>Nested RT-PCR analysis</th>
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<tbody>
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<td>1</td>
<td>M/4 y</td>
<td>BM*</td>
<td>C-ALL†</td>
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<tr>
<td>5</td>
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<td>BM</td>
<td>T-ALL</td>
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<td>AML-M2</td>
<td>45,X,Y,t(1;8)<a href="8;21">p22;q11</a>[q22;22][20]</td>
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<td>F/7 y</td>
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<td>AML-M3</td>
<td>46,XX[20]</td>
<td>Germline</td>
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<tr>
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<td>C-ALL</td>
<td>46,XY[1]</td>
<td>Germline</td>
<td>Negative</td>
</tr>
<tr>
<td>22</td>
<td>F/3 mo</td>
<td>BM</td>
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<td>46,XX,t(4;11)[q21;23][15]/46,XX[4]</td>
<td>Rearrangement</td>
<td>Negative</td>
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<tr>
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<td>M/5 y</td>
<td>BM</td>
<td>C-ALL</td>
<td>46,XY[2]</td>
<td>Germline</td>
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<tr>
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<td>46,XY[7]</td>
<td>Germline</td>
<td>Negative</td>
</tr>
<tr>
<td>25</td>
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<td>PB</td>
<td>AML-M3 variant</td>
<td>46,XY,inv9[p13q21k, t[15;17][q24; q21][q21]46,XY,inv9[p13q21k][3]</td>
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<td>Negative</td>
</tr>
</tbody>
</table>

*BM bone marrow
†PB peripheral blood
‡ALL acute lymphoblastic leukaemia
§AML acute myeloid leukaemia

Patients 1, 9, 14, and 27, no germline band was visible even after repeating the analysis. This result was most likely due to there being insufficient DNA (<7 μg). Only one patient (patient 22) showed evidence of MLL gene rearrangement, with a single rearranged band (8.5 kb) in addition to a germline band (14 kb). This finding accounted for a frequency of 4% (1/23) of MLL gene rearrangement in childhood acute leukaemia in this study. In addition, the frequency of MLL gene rearrangement in childhood ALL in this study was 7% (1/15). Patient 5, who had a deletion of 11q23 (Table), did not show concomitant MLL gene rearrangement.
Gene rearrangement in acute leukaemia

The results of the nested RT-PCR analysis to detect fusion \textit{MLL} transcripts are shown in Fig 3. A total of 10 samples (from patients 2, 3, 4, 7, 13, 16, 17, 18, 25, and 26) were suspected to have fusion \textit{MLL} transcripts. Because all the patients in this study were analysed at presentation, only those with both positive results from the Southern blot and nested RT-PCR analyses would be considered to show partial tandem duplication of the \textit{MLL} gene rearrangement. Hence, we could not find any partial tandem duplication of the \textit{MLL} gene associated with childhood acute leukaemia in this study. The cytogenetic findings, together with the positive Southern blot results but negative RT-PCR results for patient 22, suggested the presence of an \textit{MLL}/\textit{AF4} rearrangement—that is, (t;4;11)(q21;q23) — in this patient. This rearrangement is the most common (50%) specific chromosomal translocation found in infant ALL.\textsuperscript{18}

**Discussion**

Genetic changes in leukaemic cells have conventionally been identified by cytogenetic analysis. This technique, however, is labour-intensive and reveals only gross chromosomal alterations. In addition, cytogenetic analysis is often complicated by difficulties in obtaining sufficient marrow cells and the frequent low mitotic index of blast cells in infant leukaemia. Advances in molecular biology have led to the application of other techniques such as Southern blot and RT-PCR analysis, which are more sensitive and specific than conventional cytogenetics in the study of genetic alterations in haematological malignancies. Furthermore, molecular analysis is essential to achieve the sensitivity needed for the detection of minimal residual disease.\textsuperscript{7}

In western countries, the \textit{MLL} gene is involved in approximately 3% to 10% cases of childhood ALL.\textsuperscript{7,19} The results of this study showed a frequency of 4% for \textit{MLL} gene rearrangement in acute leukaemia in Hong Kong Chinese children. In particular, the frequency of \textit{MLL} rearrangement in ALL in Hong Kong Chinese children was 7%, which is similar to the figure reported from western countries. Of the 23 patients who gave adequate materials for genetic study, only patient 22 showed \textit{MLL} gene rearrangement, and this patient was the only infant with ALL with the early B-cell precursor phenotype. The molecular rearrangement of the \textit{MLL} gene in acute leukaemia without cytogenetic evidence of 11q23 aberration cannot be reliably demonstrated in this study because of the small sample size. A recent study has suggested that the poor prognosis for a subgroup of patients with \textit{MLL} partial duplication may mean different treatment needs.\textsuperscript{20} However, the frequency of \textit{MLL} gene partial tandem duplication in acute leukaemia in children is still not known. No patient was shown to have a partial tandem duplication of the \textit{MLL} gene in this study, which indicates that this genetic rearrangement is rare in Hong Kong Chinese children with acute leukaemia.

The diagnosis of partial tandem duplication of the \textit{MLL} gene relies on molecular methods such as Southern blot and RT-PCR analyses. The latter method can also be used to monitor minimal residual disease of...
leukaemia during remission. A recent study, however, has suggested that a positive result from nested RT-PCR analysis as the only molecular test may not be sufficient to diagnose leukaemia associated with MLL partial tandem duplication or to indicate minimal residual disease. By using nested RT-PCR analysis, that study showed that 16% of healthy donors had a unique transcript owing to the fusion of two MLL exons, which was consistent with the partial tandem duplication of MLL. However, a corresponding genomic rearrangement or a unique genomic fusion of MLL could not be demonstrated by Southern blot analysis. A similar study also showed that MLL duplication transcripts were detectable by nested RT-PCR analysis in the peripheral blood of 84% to 100% of healthy donors. Marked differences, however, were observed between the MLL fusion transcripts that were detected in healthy donors and transcripts that were detected in patients with leukaemia in whom a partial tandem duplication of the MLL gene had been documented at the genomic level. Consequently, the fusion points of an MLL gene that has undergone partial tandem duplication should be studied by DNA sequencing, so that the MLL fusion transcripts of patients with leukaemia can be distinguished from those detected in healthy donors. Further consideration should also be given to the use of the RNA poly(A)+ fraction to increase the specificity of RT-PCR analysis. Finally, nested PCR assays must be rigorously tested to ensure that they can reliably distinguish between patients with leukaemia who have low levels of disease and healthy individuals who carry rare non-malignant cells that possess a specific molecular defect.

To our knowledge, this is the first study to report the frequencies of the rearrangement and partial tandem duplication of the MLL gene in Hong Kong Chinese children with acute leukaemia. The results provide important clinical and epidemiological information on the MLL gene and acute leukaemia in local children.

References