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

患急性白血病香港華裔兒童中脊椎淋巴白血病基因的重組

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 *Hin*dIII 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.

目的：確定患急性白血病香港華裔兒童中脊椎淋巴白血病基因重組的頻率
設計：免疫學表現，基因及分子生物學分析。
安排：香港地區醫院。
參與者：骨髓或外圍血樣本取自1995年9月到1998年2月間，27名患急性白血病的16歲及以下的兒童。
主要結果測量：基因重組由單核細胞DNA的欣德III消化產品南方涂污方法，接著按與脊椎淋巴白血病P/S4探針雜交化進行分析。進行巢倒轉轉錄脢－聚合鏈反應分析以檢測和特性化複製脊椎淋巴白血病基因。
結果：23名病童含有足夠骨髓或外圍血樣本中，只有1名(4%)可研究展示脊椎淋巴白血病基因重組。沒有病童對脊椎淋巴白血病基因的局部協力複製呈陽性。
結論：脊椎淋巴白血病基因重組在患急性白血病的香港華裔兒童中是罕見的。

Key words:
Child;
Chromosomes;
Gene rearrangement;
Hong Kong;
Leukemia, lymphocytic, acute/genetics;
Leukemia, myeloid/genetics

關鍵詞：
兒童；
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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 *MLL* gene in oncogenesis is not limited to chromosomal translocations that result in chimeric fusion genes. The *MLL* gene has recently also been found to be involved in acute leukaemias without visible chromosomal translocations. Partial tandem duplication of the *MLL* gene also occurs in some cases.[3]

Conventional cytogenetic analysis can detect only a subset of acute leukaemias that harbour *MLL* gene rearrangement, and it cannot distinguish cases containing germline-derived 11q23 alterations from those with rearranged *MLL* gene. In some cases of acute leukaemia, *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 *MLL* gene rearrangements because these patients have a poor prognosis and are candidates for bone marrow transplantation or other aggressive therapies.[7] 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 *MLL* gene.[8]

Translocations that involve the *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 *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 *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 *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°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, leukaemias were classified as early B-cell precursor (TdT+, CD19+, HLADR+), 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 *MLL* probe that was used (P/S4) spanned from exon 7 to part of intron 8 of the *MLL* gene (Fig 1a).[15] The DNA of a bone marrow sample from a patient with previously confirmed *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 *Hin*dIII restriction

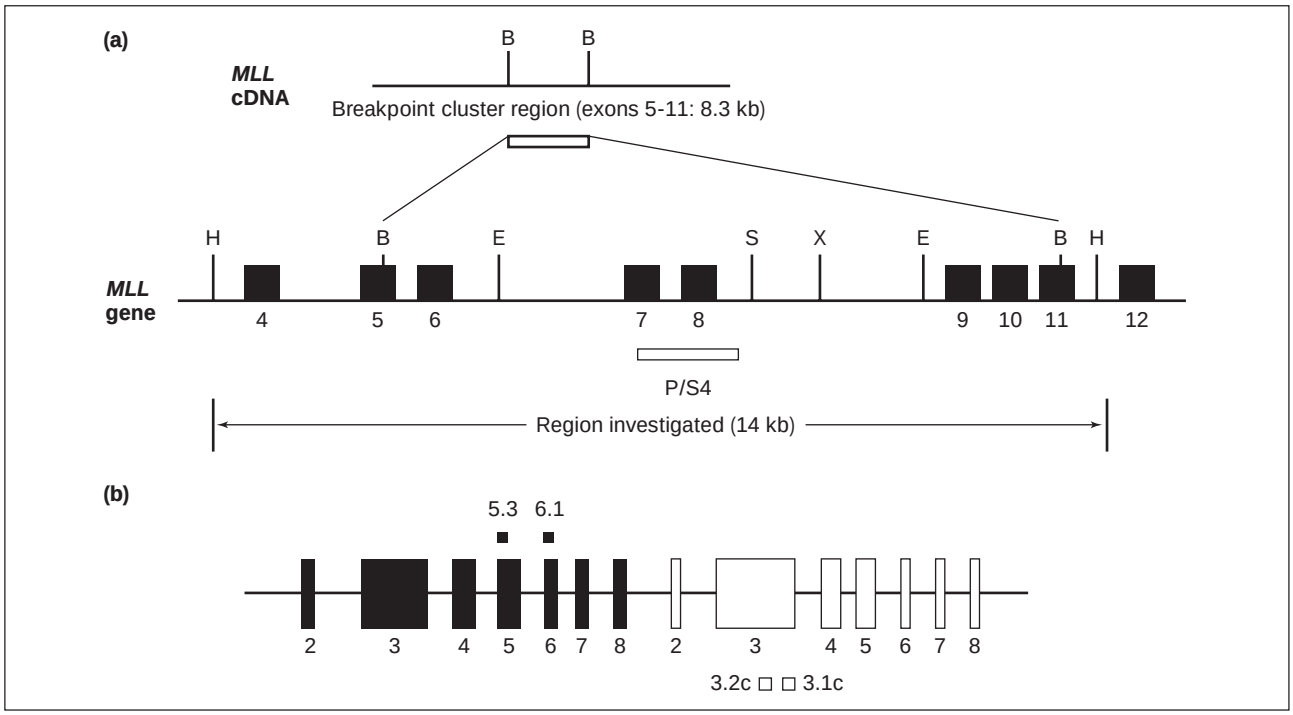


Fig 1 (a). Schematic representation of partial restriction map of the myeloid-lymphoid leukaemia gene
Breakpoints in the *MLL* gene cluster within the two *Hin*dIII sites can be detected by the probe P/S4. Vertical lines represent restriction enzyme digestion sites (H=*Hin*dIII, B=*Bam*HI, E=*Eco*RI, S=*Sac*I, X=*Xba*I); exons are indicated by black boxes
1 (b). Schematic representation of partial tandem duplication of the myeloid-lymphoid leukaemia gene spanning exons 2-8
The location of primers (5.3, 6.1, 3.2c, 3.1c) used for the reverse transcription-polymerase chain reaction amplification of the fusion *MLL* transcript are also shown; exons are indicated by vertical boxes

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.[15] 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 3.2 c (5'-ACA CAG ATG GAT 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 β_2-microglobulin (β_2M) primers because RNA degradation could not be detected spectrophotometrically.[16] Oligonucleotide primers that were used to amplify the β_2M DNA sequence were 5'-GGC CAT ACT ACC CTG AAT GA-3' (residues 1705-1724 sense strand) [β_2M_1] and 5'-CAT GCT GTG CAT CAG TAT CT-3' (residues 1806-1825 antisense strand) [β_2M_2]; the result was a 120-bp product.[17] 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).

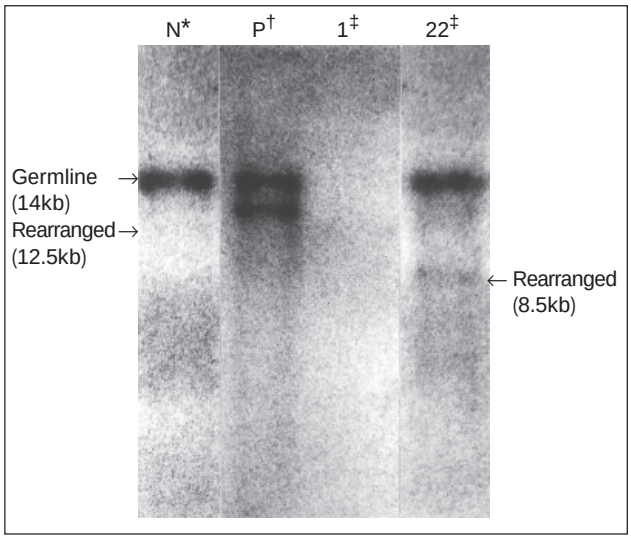
Samples from 23 patients contained adequate materials for further genetic study. The P/S4 probe detected a germline fragment of approximately 14 kb after hybridisation with separated *Hin*dIII digestion products. The positions of the germline and rearranged bands are shown in Fig 2. Different sizes of rearranged bands were seen because different partner chromosomes were involved, so the locations of breakpoints were different in various *MLL* gene rearrangements. For

Table. Cytogenetic and molecular analysis of 27 patients studied for *MLL* gene rearrangement

Patient No.	Sex/age	Specimen	Diagnosis	Karyotype	Southern blot analysis	Nested RT-PCR analysis
1	M/4 y	BM*	C-ALL‡	54,XX,+X,+3,+6,+8,+10,+15,+21, +mar 1,+mar 2x2, +mar 3[3]/46, XY[14]	Indeterminate	Negative
2	M/6 y	BM	AML§-M1	46,XY,t(10;11)(p13;q13)[17]/46,XY[3]	Germline	Indeterminate
3	F/3 y	BM	Pre-B ALL	46,XX[10]	Germline	Indeterminate
4	M/6 y	BM	Early T-precursor ALL	46,XY[18]	Germline	Indeterminate
5	M/9 y	BM	T-ALL	46,XY,t(10;11)(p12,q13),del(11)(q23) [12]/46,XY[15]	Germline	Negative
6	F/16 y	BM	B-ALL	46,XX[2]	Germline	Negative
7	M/11 y	BM	AML-M2	45,X,-Y,t(1;8)(p22;q11) t(8;21) (q22;q22)[20]	Germline	Indeterminate
8	F/7 y	BM	AML-M3	46,XX[20]	Germline	Negative
9	F/5 y	BM	C-ALL	46,XX[9]	Indeterminate	Negative
10	M/5 y	BM	T-ALL	46,XY[6]	Germline	Negative
11	F/11 y	BM	AML-M0	46,XX,der(19)?t(11;19)(p13,q13)[4]/ 46,XX[11]	Germline	-
12	M/16 y	PB†	AML-M2	47,XY,+22[17]/46,XY[2]	Germline	Negative
13	M/6 d	PB	AML-M7	47,XY,+21c[21]	Germline	Indeterminate
14	M/3 d	BM	AML-M7	47,XY,+21c[22]	Indeterminate	Negative
15	F/13 y	BM	Early B-precursor ALL	46,XX,t(1;9)(p10;q10)[4]/46,XX[7]	Germline	Negative
16	F/13 y	BM	AML-M1	46,XX[19]	Germline	Indeterminate
17	F/11 y	BM	C-ALL	47-52,XX,+X,add3(p14;p21),+8,+18, +19,+21,+22[cp14]/46,XX[1]	Germline	Indeterminate
18	M/9 y	BM	C-ALL	59,XY,+4,+5,+6,+7,+8, +10,+13,+17, +18,+18,+20, +21,+22[4]/46,XY[2]	Germline	Indeterminate
19	M/4 y	BM	Pre-B ALL	46,XY[4]	Germline	Negative
20	M/3 y	BM	C-ALL	46,XY[1]	Germline	Negative
21	F/3 y	PB	C-ALL	45,XX,-9,add(9)(p?24)[7]/46,XX[6]	Germline	Negative
22	F/3 mo	BM	Early B-precursor ALL	46,XX,t(4;11)(q21;q23)[15]/46,XX[4]	Rearrangement	Negative
23	M/5 y	BM	C-ALL	46,XY[2]	Germline	Negative
24	M/10 y	BM	Pre-B ALL	46,XY[7]	Germline	Negative
25	M/15 y	PB	AML-M3 variant	46,XY,inv(9)(p13q21)c, t(15;17)(q24; q21)[10]/46, XY,inv(9)(p13q21)c[3]	Germline	Indeterminate
26	M/15 y	BM	T-ALL	46,XY,add(9)(p24)[15]/4,XY[3]	Germline	Indeterminate
27	F/10 y	BM	C-ALL	51-54,XX,+4,+8,+10,+11,+14,+15, +21,+21[cp11]/46,XX[3]	Indeterminate	Negative

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

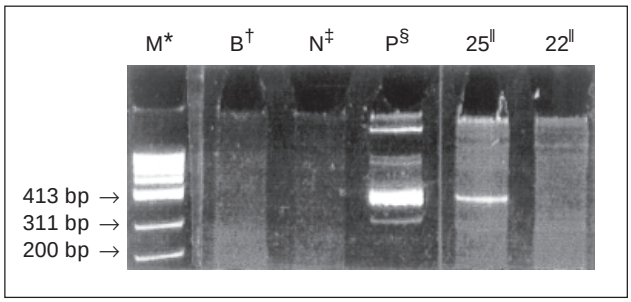
patients 1, 9, 14, and 27, no germline band was visible even after repeating the analysis. This result was most likely due to there being insufficient DNA (<7 μg). Only one patient (patient 22) showed evidence of *MLL* gene rearrangement, with a single rearranged band (8.5 kb) in addition to a germline 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.



*N healthy control
†P positive control
‡ 1 and 22 are patient samples

Fig 2. Southern blot analysis of the myeloid-lymphoid leukaemia gene, after hybridisation with the P/S4 probe
The hybridsation pattern of *Hin*dIII-digested fragments shows a germline band (14 kb) and rearranged bands (12.5 kb. 8.5 kb)

The results of the nested RT-PCR analysis to detect fusion *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 *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 *MLL* gene rearrangement. Hence, we could not find any partial tandem duplication of the *MLL* gene associated with childhood acute leukaemia



*M molecular weight markers
†B reagent blank
‡N healthy control
§P positive control
‖ 22 and 25 are patient samples

Fig 3. Ethidium bromide–stained polyacrylamide gel (6%) of electrophoresed products from the nested reverse transcription–polymerase chain reaction
External primer pair 3.1c and 5.3 were used for the first round of amplification and internal primer pair 3.2c and 6.1 were used for the second round. For the positive control, the 474-bp and 360-bp PCR products corresponded to fusions of exons 8 to 2 and 7 to 2, respectively

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 *MLL/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.[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.[7]

In western countries, the *MLL* gene is involved in approximately 3% to 10% cases of childhood ALL.[7,19] The results of this study showed a frequency of 4% for *MLL* gene rearrangement in acute leukaemia in Hong Kong Chinese children. In particular, the frequency of *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 *MLL* gene rearrangement, and this patient was the only infant with ALL with the early B-cell precursor phenotype. The molecular rearrangement of the *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 *MLL* partial duplication may mean different treatment needs.[20] However, the frequency of *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 *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 *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.[21] 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.[21] 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.[22] 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.[21] 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.

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