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Validation of a Multiplex RT-PCR Assay for Screening Significant Oncogene Fusion Transcripts in Children with Acute Lymphoblastic Leukaemia

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ABSTRACT

In childhood acute lymphoblastic leukaemia (ALL), cytogenetics play an important role in diagnosis, allocation of treatment and prognosis. Conventional cytogenetic analysis, involving mainly karyotyping in our experience, has not been successful in a large proportion of cases due to inadequate metaphase spreads and poor chromosome morphology. Our aim is to develop a highly sensitive and specific method to screen simultaneously for the four most frequent fusion transcripts resulting from specific chromosomal translocations, namely, both the CML- and ALL-type BCR-ABL transcripts of t(9;22), E2A-PBX1 transcript of t(1;19), the MLL-AF4 transcript of t(4;11) and TEL-AML1 (also termed ETV6-CBFA2) of the cryptic t(12;21). A multiplex reverse-transcription polymerase chain reaction protocol (RT-PCR) was developed and tested out on archival bone marrow samples and leukaemia cell lines. In all samples with a known translocation detected by cytogenetic techniques, the same translocation was identified by the multiplex-PCR assay. Multiplex RT-PCR assay is an effective, sensitive, accurate and cost-effective diagnostic tool which can improve our ability to accurately and rapidly risk-stratify patients with childhood ALL.

Keywords: risk-stratification, multiplex, RT-PCR, acute lymphoblastic leukaemia

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BACKGROUND

Acute lymphoblastic leukaemia (ALL), the most common subtype of childhood cancer is a heterogenous disease which requires a risk-stratified approach to treatment[1]. Leukaemic blast genetic makeup is one of the most important prognostic factors in childhood ALL and detection of specific chromosomal translocations allows the identification of prognostically relevant subgroups[2].

Previously reported translocations that have been shown to carry an adverse prognosis for patients with B-lineage ALL are t(4;11)/MLL-AF4, t(9;22)/BCR-ABL and t(1;19)/E2A-PBX1[3-6]. The most frequent fusion transcript in childhood ALL, t(12;21)/TEL-AML1 which occurs in 25% of patients, in contrast to the previously mentioned cytogenetic abnormalities, has been reported to be associated with a favourable prognosis. ALL patients with the t(12;21) translocation show an excellent response to treatment with very low incidence of relapse following complete remission[7-9].

Cytogenetic changes in acute leukaemia were first identified by routine banding techniques, but the advent of fluorescent in situ hybridisation (FISH) and molecular techniques has allowed more sensitive and specific analysis of genetic abnormalities[10]. Banding techniques, in our experience, yield false-negative or uninterpretable results in a fairly large number of cases because of lack of adequate metaphases and poor chromosome morphology. Molecular-based analyses are not only more sensitive but also can detect cryptic translocations; the most notable example is the submicroscopic t(12;21)/TEL-AML1 fusion transcript[11,12].

Molecular-based assays can be performed successfully on a higher proportion of cases than conventional karyotyping, because they require a smaller amount of tumour cells and do not require mitotic cells. Although FISH techniques are equally capable to detect these fusion transcripts, performing these tests on individual samples is not very efficient. Also, the high cost of FISH probes is often prohibitive in countries with limited resources.

Standard reverse-transcriptase polymerase chain reaction (RT-PCR) assays for the individual detection of the prognostically significant fusion transcripts in ALL is also labour-intensive, material-demanding and not practically feasible. In addition, several primer sets are required for each translocation because of breakpoint diversity in these fusion genes, further increasing the number of reactions that need to be performed.

Pallisgaard et al[13] reported on a successful multiplex RT-PCR method which was able to detect 29 fusion transcripts simultaneously. However, we were looking

Table 1. Primers used in the multiplex PCR protocol.

Multiplex panel	Fusion transcript	1st round primers (5' to 3')	Fusion transcript	2nd round primers (5' to 3')
A	PBX1:459L18	GCCACGCCTTCCGCTAAC	PBX1:436L21	CATGTTGTCCAGCCGCATCAG
	E2A:1075U21	TTCTCGTCCAGCCCTTCTACC	E2A:1173U19	CTACGACGGGGGTCTCCAC
	TEL:871U23	CACTCCGTGGATTTCAAACAGTC	TEL:944U23	CTCATCGGGAAGACCTGGCTTAC
	AML1A:1891L23	AGCCGAGTAGTTTTCATCATTGC	AML1A:1772L21	AGCACGGAGCAGAGGAAGTTG
B	BCR:1698U19	CGCTCTCCCTCGCAGAACT	BCR:1777U19	ACTGCCCGGTTGTCGTGTC
	BCR:3060U23	GAGTCACTGCTGCTGCTTATGTC	BCR:3128U22	CACGTTCCTGATCTCCTCTGAC
	ABL:661L20	TTTTGGTTTGGGCTTCACAC	ABL:642L23	ACACCATTCCCCATTGTGATTAT
C	MLL:3730U20	CCGCCTCAGCCACCTACTAC	MLL:3751U20	GGACCGCCAAGAAAAGAAGT
	MLL:3955U24	AGCACTCTCTCCAATGGCAATAGT	MLL:3996U24	AGCAGATGGAGTCCACAGGATCAG
	AF4:1636L29	GAATTTGAGTGAGTTTTTGAAGATGTATC	AF4:1606L25	GTTTTTGGTTTGGGTTACAGAACT
All (ABC)	E2A:1075U21	TTCTCGTCCAGCCCTTCTACC	E2A:1173U19	CTACGACGGGGGTCTCCAC
	E2A:1883L22	TTTTCCTCTTCTCGCCGTTTCA	E2A:1884L19	AGGTTCCGCTCTCGCACTT

to develop a technically simple and cost-effective method to screen only the proven prognostically-important oncogene fusion transcripts in pre-B ALL, which forms the main bulk of our leukaemia patients. Thus, we have adapted this protocol to develop a multiplex-PCR that allows the simultaneous detection of the rearrangements of t(12;21)/TEL-AML1, t(1;19)/E2A-PBX1, t(4;11)/MLL-AF4 and both major and minor breakpoints of t(9;22)/BCR-ABL in a single assay.

MATERIALS AND METHODS

Patients and Materials

The multiplex assay was performed using the leukaemic blasts of twenty archival bone marrow specimens from patients with precursor B-cell ALL diagnosed at the University of Malaya Medical Centre, Kuala Lumpur. The diagnosis was made via standard morphological and immunophenotyping criteria. Immunophenotyping was performed via flow cytometry using monoclonal antibodies against HLA-DR, CD34, CD19, CD10, sIgM, CD7, CD5, CD3, CD13, CD33, CD41 and CD61. As part of the diagnostic work-up, all specimens underwent standard karyotyping. Written informed consent was obtained from patients' parents or legal guardians. In addition, archival specimens from patients with known translocations diagnosed at the National University of Singapore (Patients 4, 5 and 6) were also used.

Validation of the multiplex assay was performed using leukaemic cell lines REH[14], RS4[15], K562[16], SupB15[17] and 697[18] served as positive controls for t(12;21), t(4;11), CML-type t(9;22), ALL-type t(9;22) and t(1;19) respectively and the leukaemic cell line HL60 was used as a negative control[19].

RNA preparation and cDNA synthesis

Total RNA was extracted from leukaemic cells by Trizol reagent (Life Technologies, Gibco, Gaithersburg, MD) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using 10 units of Omniscript™ reverse transcriptase (Qiagen Inc, Valencia, CA) in RT buffer containing 0.5 mM dNTP mix, 1uM Oligo-dT, 10 U RNAse inhibitor at 37ºC for 60 minutes.

Multiplex PCR analysis

To verify the integrity of the isolated RNA and the correct synthesis of the cDNA, we included an internal positive control in which a 690bp segment of the ubiquitously expressed transcription factor E2AmRNA was co-amplified.

Following cDNA synthesis a multiplex-PCR to detect chimeric transcripts derived from the four translocations was performed. PCR amplification was performed as nested reactions with a Biometra T-gradient Thermoblock thermal cycler (Gottingen, Germany). The first PCR was carried out in a final volume of 25 µl with 10X PCR-Buffer, 200 µM dNTP, 5% DMSO (Sigma), 0.2 µM of forward and reverse primers of E2A, 5 pmol of each primer pair and 0.02 U of HotStar Taq™ DNA polymerase (Qiagen).

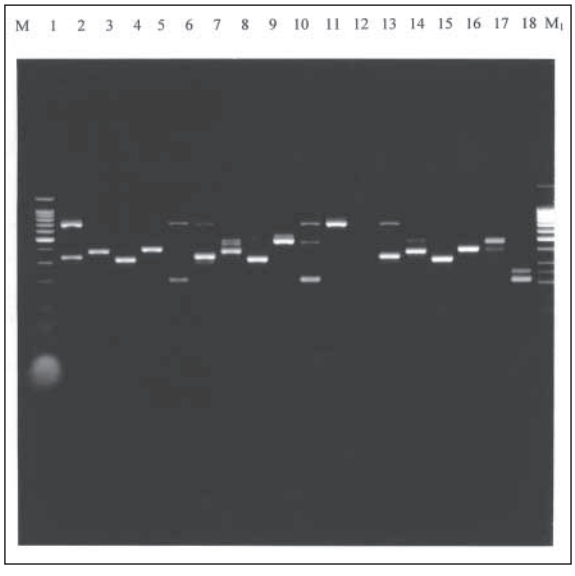
The initial PCR consisted of activation of the polymerase at 95ºC for 15 minutes, followed by 30 cycles of PCR amplification (annealing at 58ºC for 30 seconds, elongation at 72ºC for 60 seconds and denaturation at 95ºC for 30 seconds). After the first PCR, 1 ul aliquots from each of the PCR reactions were transferred to second round mixtures that were identical to the first. The primer sequences are listed in Table I.

Table II. Cytogenetics and multiplex RT-PCR data from 20 ALL patients.

Patient	Karyotype findings at diagnosis	Mutiplex RT-PCR findings
1	No metaphase spreads	t(12;21) / TEL-AML1
2	Insufficient marrow sample	t(12;21) / TEL-AML1
3	No metaphase spreads	t(12;21) / TEL-AML1
4	46XX ins(4;11)q21; q13 q23	t (4;11) / MLL-AF4
5	46 XX, t(1;19)(q23;p13)	t (1;19) / E2A-PBX1
6	47Y del(X) (q21)+der (22) t(9;22)	t (9;22)p190 / BCR-ABL
7*	46 XY, t(9;22)	t (9;22)p210 / BCR-ABL
8	46 XX	Negative
9	46 XY	Negative
10	No metaphase spreads	Negative
11	46 XY	Negative
12	46 XY	Negative
13	46 XY	Negative
14	No metaphase spreads	Negative
15	No metaphase spreads	Negative
16	Insufficient marrow sample	Negative
17	No metaphase spreads	Negative
18	No metaphase spreads	Negative
19	No metaphase spreads	Negative
20	No metaphase spreads	Negative
21	2n = 54; too few metaphase spreads	Negative

* Patient with known chronic myelogenous leukaemia used to test the multiplex RT-PCR assay.

Fig.1 Representative agarose gel analysis of different PCR products by multiplex RT-PCR from patients and control cell lines.



M = 100 bp DNA molecular marker.
Lanes 1-5 = representative patient samples with fusion transcripts.
Lane 6 = positive control cell line REH.
Lane 7 = positive control cell line 697.
Lane 8 = positive control cell line Sup B15
Lane 9 = positive control cell line K562
Lane 10 = positive control cell line RS4
Lane 11 = negative control cell line HL60
Lane 12 = negative control, H20 (no cDNA)
Lane 13-18 = split-out PCR on patient samples found to be positive in multiplex reaction
M1 = 50 bp DNA molecular marker

Fifteen microlitres of the PCR products were electrophoresed in a 2% agarose gel for 60 minutes at 120 V and visualised by ethidium bromide staining. Products from the multiplex PCR were subjected to confirmation of specific transcripts via individual (split out) PCR reactions. These were performed separately using the specific primers for each transcript. Conditions for the latter PCR reactions were identical to both the first round and nested reaction.

RESULTS

Our main aim was to develop a technically-simple and cost-effective method to screen for the prognostically significant fusion transcripts in childhood ALL. To test the performance of this multiplex-PCR assay, we first tested the detection of these four translocations in the cell lines REH (TEL-AML1), RS4 (MLL-AF4), K562 (CML-type BCR-ABL), Sup B15 (ALL-type BCR-ABL) and 697 (E2A-PBX1) which were used as positive controls. All the four translocations were detected (Fig. 1). PCR products were discriminated by their fragment size on an agarose gel .

Once the PCR conditions were established and optimised, 20 selected ALL samples were analysed. We compared previous karyotyping findings in these patients with the new results from the multiplex RT-PCR assay to see if all results were concordant, thus validating the molecular method. The cases selected included those with known cytogenetic abnormalities and those in whom karyotyping was unsuccessful.

Patients 1 – 3 (Table II) were found to have the TEL-AML1 fusion transcript. As the cytogenetic findings were not supportive and we did not have FISH probes to validate this finding, these PCR products were subjected to genetic sequencing using the ABI-Prism Sequencer for confirmation.

Patients 4 – 7 were found to have MLL-AF4, E2A-PBX1 and BCR-ABL fusion transcripts respectively (Fig. 1) and this was concordant with their karyotyping results. The presence of the latter two transcripts were also confirmed by individual (split-out) PCR using specific primers for this translocation.

Patients 8 – 21 included those with either a normal karyotype and those without cytogenetic results at diagnosis. All the cases were negative for any of the four translocations using the multiplex PCR.

DISCUSSION

Identification of specific chromosomal abnormalities is an important tool for diagnosis and risk-stratification in childhood ALL. Current management strategies, using protocols with different intensity according to risk-group has helped to improve survival rates of paediatric ALL to 80% in some centres[20].

Presence of the t(1;19) has been associated with an inferior treatment outcome on several clinical trials as compared to patients without this abnormality[21]. However, the poorer outcome associated with this chromosomal abnormality could be negated with more intensive chemotherapy[22]. Children with Philadelphia chromosome positive ALL have been recognised to have a dismal prognosis with five-year survival rates of only 25 to 30%[23,24]. Consequently, these patients routinely undergo allogeneic stem cell transplant in first remission. This strategy has improved their survival rate to 65%[24]; thus underlying the importance of recognising Philadelphia chromosome positivity in ALL patients.

By cytogenetic analysis, the detection of t(9;22) is usually not difficult as illustrated by both patients used in this study. However, the detection of t(4;11) by routine karyotyping is difficult and often leads to false-negative results. The cryptic t(12;21)/TEL-AML1 transcript is virtually impossible to detect via cytogenetic analysis and for these reasons, the PCR-based method is valuable.

Molecular detection methods based on specific PCR of individual translocations have been used in some laboratories in Malaysia. Although this method is sensitive, it is used routinely only to detect specific translocations such as t(9;22) in chronic myelogenous leukaemia. In addition, this method is laborious as several PCR reactions are required to screen a single patient. To address this situation, we have developed this multiplex-PCR assay to screen simultaneously for the prognostically-important translocations in B-lineage ALL, the commonest type of haematological malignancy in childhood, and validated the methodology with both cell lines and patient samples. Further validation with a larger population sample is required before adopting this protocol into the routine diagnostic work-up of new ALL patients.

Although we often have problems with unsuccessful karyotyping whilst the PCR-based methods are faster and more sensitive, the role of karyotyping cannot be wholly replaced. This is because the PCR-based techniques cannot detect abnormalities in chromosomal number and unknown balanced translocations, all of which may also be prognostically important. Thus both methods are complimentary to each other.

In conclusion, we believe this multiplex-PCR assay is useful to detect the prognostically significant oncogene fusion transcripts in childhood B-lineage ALL namely t(12;21)/TEL-AML1, t(4;11)/MLL-AF4, t(1;19)/E2A-PBX1 and t(9;22)/BCR-ABL. It is fast, accurate and sensitive and unlike other molecular methods like FISH, is relatively affordable in countries with limited economic resources.

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