RESEARCH NOTE

RT-PCR and real-time PCR analysis of *E2A-PBX1*, *TEL-AML1*, *mBCR-ABL* and *MLL-AF4* fusion gene transcripts in *de novo* B-lineage acute lymphoblastic leukaemia patients in south India

NATARAJAN SUDHAKAR[1,3], KAMALALAYAM RAGHAVAN RAJALEKSHMY[2], THANGARAJAN RAJKUMAR[1] and KARUNAKARAN NIRMALA NANCY[1]*

[1]*Department of Molecular Oncology, Cancer Institute (WIA) 38, Sardar Patel Road, Chennai 600 036, India*
[2]*Department of Hemato Immunology, Cancer Institute (WIA) 38, Sardar Patel Road, Chennai 600 036, India*
[3]*Department of Biotechnology, Dr M. G. R. Educational and Research Institute, Dr M. G. R. University, Maduravoyal, Chennai 600 095, India*

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Introduction

Chromosomal translocation is the hallmark of leukaemias and acute lymphoblastic leukaemia (ALL) in particular. DNA damage arises from endogenous and exogenous genotoxic factors and lack of complete fidelity in DNA-repair leads to chromosomal translocations (Greaves and Wiemels 2003; Zhang *et al*. 2010). Chromosomal breakpoints tend to occur at transcriptionally active DNA sites and the fusion-gene transcripts generally involve genes which frequently encode cell cycle regulators, transcription factors, signal transduction molecules, receptors or immunoglobulin and TCR molecules (Rabbits 1994; Ferrando and Look 2000). The commonly detected chromosomal translocations and fusion genes in B-lineage ALL (B-ALL) are *mBCR-ABL, E2A-PBX1, TEL-AML1* and *MLL-AF4*. Due to the chromosomal translocations and subsequent expression of fusion gene transcripts, the normal functions of the genes are altered.

Although cytogenetic analysis is a standard procedure to detect chromosomal translocations it has revealed a substantial number of false negative results and an inability to detect some translocations like TEL-AML1 and MLL-AF4 (Shurtleff *et al*. 1995). Reverse transcriptase-PCR (RT-PCR) and real-time PCR detection of fusion gene transcripts is not only sensitive and specific but also requires smaller number of tumour cells (Van Dongen *et al*. 1999; Liang *et al*. 2010). For the detection of chromosomal translocations, the fusion gene is transcribed into fusion mRNA, which is reverse transcribed to cDNA to serve as a PCR target (Foroni *et al*. 1999; Pakakasama *et al*. 2008).

The fusion gene transcripts *E2A-PBX1, TEL-AML1, MLL-AF4* and *mBCR-ABL* were detected by RT-PCR analysis, confirmed by real-time PCR analysis and sequenced to estimate the frequency/prevalence of these translocations in south Indian ALL.

Materials and methods

Patient samples

Peripheral blood (PB) and bone marrow (BM) samples were obtained at diagnosis from 64 B-ALL patients in the age group of 1–25 years (50 pediatric and 14 young adults). Diagnosis of B-ALL was performed based on standard FAB classification (Miller *et al*. 1981) and immunophenotyping by Flowcytometer (BD Biosciences, San Jose, USA) using a panel of monoclonal antibodies.

Isolation of RNA and cDNA synthesis

Lymphocytes were isolated from PB/BM by the Ficoll–Paque PLUS (Amersham Biosciences, Uppsala, Sweden), washed twice with diethyl pyrocarbonate treated PBS and 10^7 cells suspended in 1 mL of Trizol reagent (Gibco-BRL, Life Technologies) and stored at −70°C until use. Total RNA was isolated from the thawed cells by Trizol method according to manufacturer's instructions and the pellet was resuspended in RNA secure solution (Ambion,

*For correspondence. E-mail: Nirmala Nancy, nirc18@gmail.com; Natarajan Sudhakar, nsudha79@gmail.com.

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St Austin, USA). The total RNA was run on a 1% agarose gel containing ethidium bromide to visualize integrity of 28S and 18S bands and quantitated in Genequant (Amersham Pharmacia, Uppsala, Sweden). Two μg of total RNA was reverse transcribed to cDNA with 5× MMLV reaction buffer, 2.5 μg random hexamers (Amersham Pharmacia), 125 μM dNTP and 100 U MMLV reverse transcriptase for 90 min at 37°C and the cDNA then used for PCR studies (Van Dongen *et al*. 1999).

RT-PCR analysis of E2A-PBX1, TEL-AML1, mBCR-ABL and MLL-AF4 fusion gene transcripts

Two μL of cDNA was PCR amplified for *ABL* (296 bp) gene to check the integrity of cDNA. Then 2 μL of cDNA was amplified in a 25 μL reaction volume for the detection of fusion gene transcripts. PCR reaction was performed using 10× PCR buffer, 200 μM dNTPs, 10 pmol of forward and reverse primers (*E2A-PBX1, TEL-AML1, mBCR-ABL and MLL-AF4*) and 1 U *Taq* polymerase (AB gene, Epsom, UK). The primer sequences are given as follows: *ABL* forward: 5′-GGCCAGTAGCATCTGACTTTG-3′; reverse; 5′-ATGGTACCAGGAGTGTTTCTCC-3′; *mBCR*: 5′-ACCTCACCTCCAGCGAGGAGGACTT-3′, *ABL*-5′-TCCACTGGCCACAAAATCATACAGT-3′ (Siraj *et al.* 2002); *E2A*: 5′-GCCACGGGGCGCTGGCCTCAGGTTT-3′; *PBX1*: 5′-CCGCATCAGCTGGGGGTCTGTGGGT-3′ Siraj *et al.* 2002); *TEL*: 5′-GAACCACATCATGGTCTCTG-3′; *AML1*: 5′-AGCGGCAACGCCTCGCTCAT-3′; *MLL*: 5′-CCTGAATCCAAACAGGCCACCACT; AF4: 5′-GTCACTGAGCTGAAGGTCGTCTTCG-3′ (Repp *et al.* 1995). After an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min was performed in a Perkin Elmer 480 Thermal cycler. The PCR products were run on a 3% agarose gel to visualize the amplified products of fusion gene transcripts. Plasmids containing the fusion gene inserts were used as positive controls.

Real-time PCR detection of E2A-PBX1, TEL-AML1 and mBCR-ABL fusion gene transcripts

In a singleplex reaction, 1 μL of cDNA was PCR amplified using TaqMan Universal Master mix (Applied Biosystems, Foster City, USA) with TaqMan probe and primers for GAPDH (Applied Biosystems, Foster City, USA) in a ABI 7000 Sequence Detection System. *E2A-PBX1, mBCR-ABL* and *TEL-AML1* were amplified using SYBR Green Master mix (Applied Biosystems, Foster City, USA) by following the same PCR thermal conditions as used for RT-PCR. The primer sequences are given as follows; mBCR : 5′-ATCGTGGGCGTCCGCAAGAC-3′; ABL:5′-GCTCAAAGTCAGATGCTACTG-3′; E2A :5′-GCCACGGGGCGCTGGCCTCAGGTTT-3′; PBX1 :5′-CCGCATCAGCTGGGGGTCTGTGGGT-3′ (Siraj *et al.* 2002); TEL: 5′-TGGCTTACATGAACCACATCATGG-3′; AML1 : 5′-GGAAGGCGGCGTGAAGC-3′ (Seeger *et al.* 2001). The dissociation curves for *E2A-PBX1, mBCR-ABL* and *TEL-AML1* were analysed at the end of the PCR to confirm the identity of the PCR products. Positive control (cell lines harbouring the translocations) and negative control (no cDNA) were included.

PCR product was sequenced to confirm the presence of breakpoints in fusion gene and check for the presence of any variants in the fusion region.

Results

Common ALL (cALL) constituted 75% (48/64) of the patients comprising 38/48 (80%) pediatric (0–15 years) and 10 (20%) adolescents and young adults (16–25 years). Pre-B constituted 12 (19%) patients including nine (75%) pediatric and three (25%) adolescents and young adults (AYA). Of the three pro-B ALL patients, two were pediatric and one was a young adult. One pediatric B-ALL patient was also included in the study. The male-to-female ratio of the patients was 1.6 : 1 (39 males and 25 females). In the pediatric age group, 30 were males and 20 females. In the AYA group nine were males and five females.

Complementary DNA (cDNA) synthesized from 64 B-ALL samples amplified for *ABL* housekeeping gene (amplicon size 296 bp). Eleven of the 64 (17.18%) samples revealed one of the fusion gene transcript by RT-PCR. Fusion genes were detected in nine pediatric and two AYA patients. Four patients (6.25%) showed *E2A-PBX1* fusion gene and *TEL-AML1* fusion gene respectively, two (3.1%) *mBCR-ABL* fusion gene and *MLL-AF4* fusion gene were detected in a one-year-old female infant (figure 1a). The details of the fusion gene transcripts with clinical parameters and immunophenotype are depicted in table 1. Among the four patients showing *E2A-PBX1* fusion gene, three were cALL and one patient was a Pre-B. A similar immunophenotype pattern was observed in four patients of *TEL-AML1* fusion gene also. Both the patients showing *mBCR-ABL* fusion gene were cALL and an infant with MLL-AF4 fusion gene revealed pro-B immunophenotype.

The melting curves produced for *mBCR-ABL, E2A-PBX1* and *TEL-AML1* are shown in figure 1b. The T_m of *mBCR-ABL* was 86.5°C, *E2A-PBX1* was 90.60°C and *TEL-AML1* was 84.2°C. At these T_m, the PCR products produced a melting curve with a single peak. The fusion gene transcripts *E2A-PBX1, TEL-AML1* and *mBCR-ABL* detected by RT-PCR analysis were confirmed by real-time PCR analysis and sequencing.

Sequencing of the *E2A-PBX1* positive samples revealed a constant junction of exon 13 of *E2A* gene to the exon 2 of *PBX1*. Similarly, the *TEL-AML1* fusion transcripts showed, joining of exon 5 of *TEL* to the exon 2 of *AML1* gene. In the two *mBCR-ABL* fusion gene transcripts, the breakpoint in exon a2 of *ABL* gene joined to the first exon of *BCR* gene

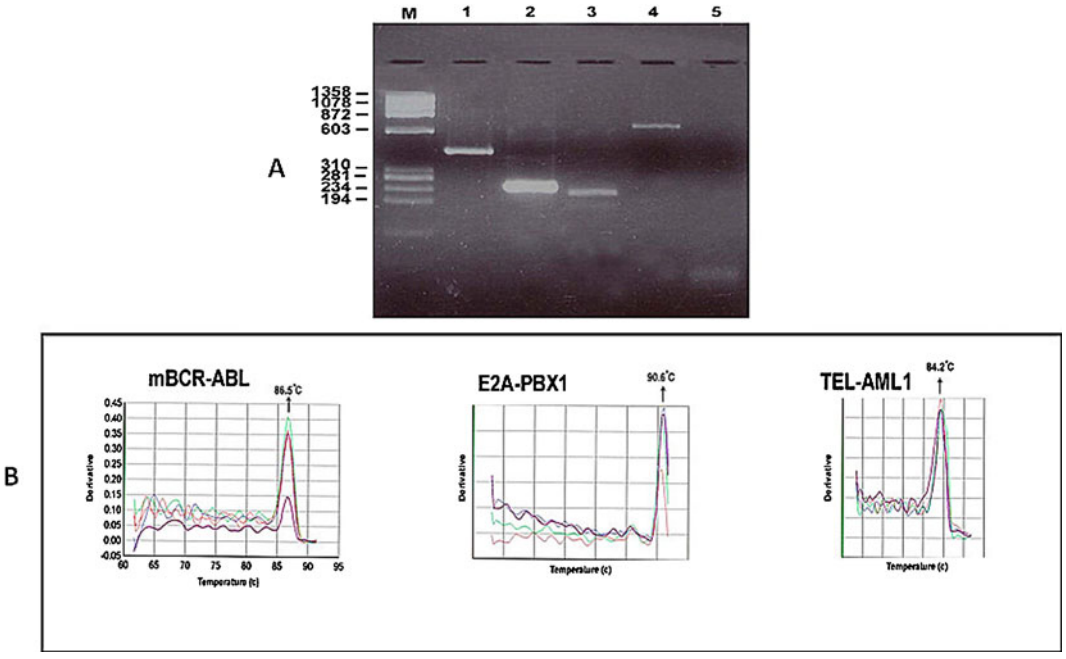

Figure 1. (a) RT-PCR analysis of fusion gene transcripts: lanes 1 to 4 illustrates amplified PCR products of *mBCR-ABL* (425 bp), *E2A-PBX1*(245 bp), *TEL-AML1* (180 bp), *MLL-AF4* (633 bp) fusion gene transcripts; lane M shows ΦX *Hae*III digest run on a 3% agarose gel and lane 5 is a negative control. (b) Melting curve analysis of *mBCR-ABL*, *E2A-PBX1* and *TEL-AML1* fusion gene transcripts detected by real-time PCR: cDNA samples were amplified in real-time PCR with specific set of primers and melting curve analysis was performed to confirm the identity of the PCR products. PCR primers were chosen to give a distinct melting curve in real-time PCR. The T_m of *mBCR-ABL* was 86.5°C, *E2A-PBX1* was 90.60°C and *TEL-AML1* was 84.2°C.

Table 1. Relative frequencies and clinical characteristics of fusion gene transcripts in B-ALL patients.

Clinical characteristics	*E2A-PBX1* ($n = 4$)	*TEL-AML1* ($n = 4$)	*mBCR-ABL* ($n = 2$)	*MLL-AF4* ($n = 1$)	None ($n = 53$)
Frequency (%)	6.5	6.5	3.2	1.6	81.9
Age					
Range (yrs)	4–21	3–9	8–11	–	2–25
Median age	15.5 yrs	5 yrs	9.5 yrs	–	8 yrs
WBC count (10°/L)					
Range	2.7–40	3.4–100	199–225	–	0.4–199
Mean	22	39	212	–	35.5
Gender					
Male	4	1	1	0	30
Female	0	3	1	1	20
Male-to-female ratio	4 : 0	1 : 3	1 : 1	–	1.5 : 1

(e1–a2). The *MLL-AF4* showed joining of exon 5 of *MLL* gene to exon 4 of *AF4* gene. No variants in the breakpoint region were detected (figure 2).

Discussion

In our RT-PCR and real-time PCR analysis of fusion genes, 17.18% of the patients revealed chromosomal translocation and fusion gene transcripts. *E2A-PBX1* detected in four patients constituted 6.25% of the B lineage ALL. The *E2A-PBX1* translocation seen more frequently in nonCaucasians constitute 5–6% of translocations seen in childhood ALL and 3% of adult ALL (Crist *et al*. 1990; Faderl *et al*. 1998). In a study of Mexican patients, 6 of 52 (11.5%) childhood ALL patients expressed the *E2A-PBX1* fusion gene transcript that shows a high prevalence of that fusion gene compared to other populations (Jiménez-Morales *et al*. 2008). All the four patients showing *E2A-PBX1* fusion gene in the present study were males. The association of males with this fusion gene

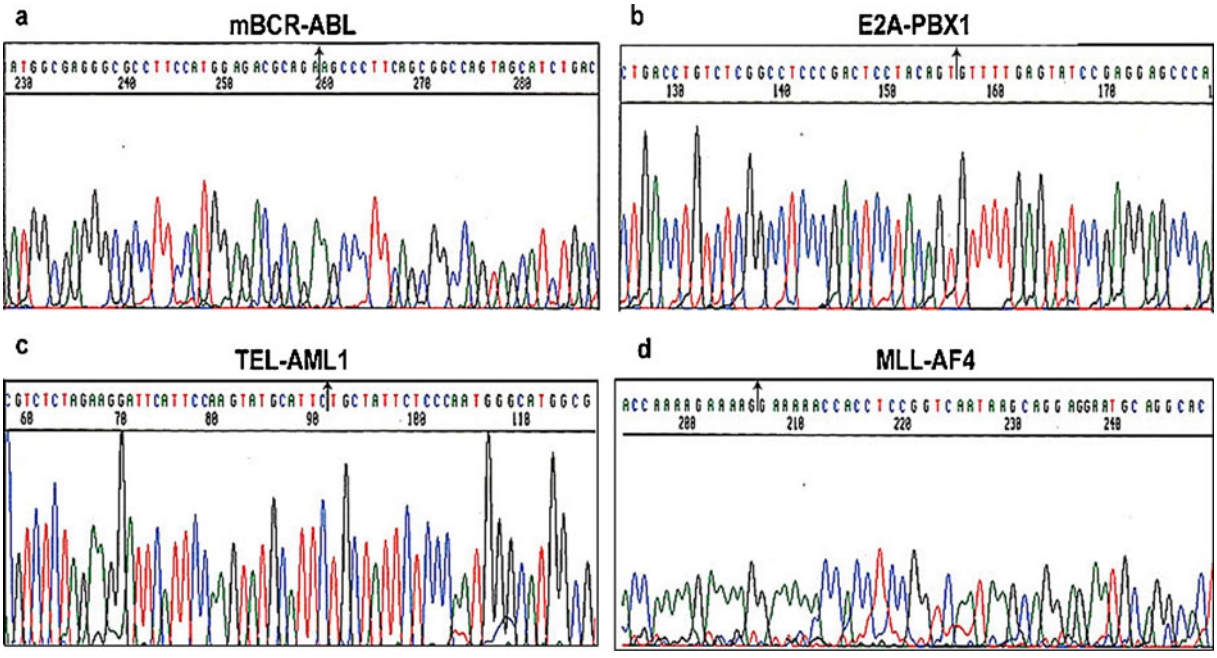

Figure 2. Sequencing result of the amplified PCR products of *mBCR-ABL*, *E2A-PBX1*, *TEL-AML1* and *MLL-AF4* fusion gene transcripts are depicted in figure. Break point and the fusion region in the sequence are marked by an arrow.

has to be confirmed in larger number of patients. Usually patients with this fusion gene show high WBC count, but in our study the mean WBC count in patients with *E2A-PBX1* fusion gene transcripts was less compared to other translocations (table 1). The median age of the *E2A-PBX1* positive patients (15.5 years) was considerably high when compared to the patients not showing any of the other analysed fusion genes (8 years). Thus, the occurrence of *E2A-PBX1* fusion gene is more common in AYA of B-ALL.

TEL-AML1 is the most common translocation seen in pediatric malignancy, reported in 25% of the childhood ALL in the west and predictive of a favourable outcome (Romana *et al*. 1995; Loh and Rubnitz 2002). In our study, we have detected this translocation in only four (6.25%) pediatric patients. A meta-analysis depicted the frequency of this translocation in 13.4% (177/1321, range 9–23%) of childhood ALL in Far East countries (Liang *et al*. 2010). In Taiwanese children this translocation was detected in 17.6% of B-ALL patients (Yang *et al*. 2010). *TEL-AML1* fusion gene is possibly an initiating event for ALL in children, originating in CD19+ progenitor cells and *in utero* (Wiemels *et al*. 1999). In contrast to the *E2A-PBX1* fusion gene, the prevalence of *TEL-AML1* fusion gene was more in females.

In this study, only two (3.1%) children revealed *mBCR-ABL* fusion gene transcript. The chimeric mBCR-ABL protein with increased tyrosine kinase activity induces resistance to apoptosis, growth factor interactions and alters cell–cell and cell–matrix interaction (Bedi *et al*. 1994). Hence patients with mBCR-*ABL* fusion gene have a high WBC count (mean WBC count = 212 × 10^9/L) compared with the *mBCR-ABL* negative patients. Sazawal *et al*. (2004) reported 2.8% of pediatric and 14.7% of adult ALL patients with this translocation. In the present study *mBCR-ABL* was not detected in AYA patients.

MLL-AF4 gene transcript was detected in a CD19+ one-year-old infant and this fusion gene has been reported for the first time in a B-ALL patient from India. Since the amplicon size of *MLL-AF4* is >600 bp, only RT-PCR analysis was performed and the samples were not amplified in real-time PCR. Microarray studies have revealed that *MLL* rearranged leukaemias display a characteristic gene expression profile which distinguishes them from other childhood ALL subtypes and make them a unique biological entity (Armstrong *et al*. 2002).

A study of 259 pre-B ALL samples from two major cancer centres in India revealed 5% of *mBCR-ABL*, 7% of *E2A-PBX1* and *TEL-AML1* fusion gene respectively with the absence of *MLL-AF4* (Siraj *et al*. 2002). Sazawal *et al*. (2004) in their study of 69 B-lineage ALL patients detected *E2A-PBX1* in 5.7% of patients with the absence of *TEL-AML1* and *MLL-AF4* fusion gene transcripts (Sazawal *et al*. 2004). In a similar study by Hill *et al*. (2005) in 42 B-lineage ALL, only two patients were positive for *TEL-AML1* and none for *E2A-PBX1* and *MLL-AF4* (Hill *et al*. 2005). In our study of 64 B-lineage ALL patients, we found that the pattern and frequency of the fusion gene transcripts differs from the findings in Western literature. Thus our study reveals a lower frequency of *TEL-AML1* (6.25%) fusion gene in childhood ALL

and absence of *mBCR-ABL* fusion gene in young adult ALL patients. The results were confirmed by RT-PCR, real-time PCR and sequencing.

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