Preclinical Validation of a Monochrome Real-Time Multiplex Assay for Translocations in Childhood Acute Lymphoblastic Leukemia

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ABSTRACT

Purpose: The purpose is to develop a real-time multiplex reverse transcription-PCR assay for detection and quantification of leukemia-specific chimeric transcripts that identify the genetic subgroups of acute lymphoblastic leukemias (ALLs) proposed by the WHO classification.

Experimental Design: Real-time multiplex assay for t(12;21), t(4;11), and t(1;19) with hypoxanthine phosphoribosyltransferase as internal standard used in tandem with a new real time quantitative-RT-PCR assay for the t(9;22). This new strategy was designed to yield an amplicon from each translocation with a distinct melting peak allowing dependable identification using only Sybr green I, without any need for expensive hybridization probes.

Results: We validated this method with 92 primary ALLs and identified 4 E2A-PBX1, 4 mBCL-ABL, and 10 TEL-AML1. When compared with conventional RT-PCRs and Southern blot analyses, 100% concordance was obtained. During the course of these studies, we found marked variations in the levels of the TEL-AML1 transcripts in individual patients. We, therefore, extended the study to accurately and reproducibly determine TEL-AML1 mRNA levels in 47 additional patients with t(12;21). The results indicated that the level of expression of TEL-AML1 varied among individual patients, and it was independent of the WBC count.

Conclusions: Our new real-time multiplex assay can be used for rapid, simple, and reliable classification of pediatric ALL. Its reproducible quantification results should also facilitate studies on minimal residual disease. The observed variation in TEL-AML1 transcript levels is of interest because it could reflect biological and/or clinical heterogeneity in the behavior of these leukemias.

INTRODUCTION

Childhood precursor B cell ALLs frequently carry specific chromosomal translocations (1–3). Although >50 distinct rearrangements have been described, four, i.e., t(12;21), t(1;19), t(9;22), and t(4;11), constitute the most frequently observed abnormalities.

These recurrent chromosomal translocations, which typically lead to the formation of fusion genes and their expression as chimeric transcripts and proteins (4), are of relevance in leukemogenesis because they influence the expression of key regulatory genes in the hematopoietic system. It is now generally accepted that these four chromosomal translocations have therapeutic importance for risk assessment in current multiagent chemotherapy protocols because they are predictors of prognosis (5–8).

The identification of these four translocations is thus important not only for understanding the biology of ALL but also as a guide for patient management. The recognition of this has allowed precursor B ALL to be subdivided into molecular entities in the recently proposed WHO classification (9). The presence of t(12;21) is associated with good response and detected in 20–25% of pediatric ALL (10). The t(1;19) is observed in 5–6% of all ALLs but constitutes 25% of the pre-B subgroup and predicts intermediate response (11). The other two translocations, t(9;22) and t(4;11), are each observed in 5–6%, and both associate with poor response but the latter occurs mostly in infant patients (12, 13).

Fluorescence in situ hybridization, Southern blot, or RT-PCR strategies can successfully detect these translocations. However, all these methods are sample/time consuming and are not accurate for quantification purposes. Except for RT-PCR, they are directed toward one specific rearrangement at a time. More recently, real-time amplification assays have been developed and they have several advantages (14). Reactions are characterized by fluorescence appearance during the exponential phase of amplification when none of the reagents is limiting and, therefore, allowing a more precise quantification. Because this is based on fluorescence detection allows analysis of minimal amounts of template with high sensitivity. The real-time system also obviates post-PCR manipulation preventing car-

2 The abbreviations used are: ALL, acute lymphoblastic leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBL, peripheral blood lymphocyte; HPRT, hypoxanthine phosphoribosyltransferase; RT-PCR, reverse transcription-PCR.
ryover contaminations, which is critical in a clinical setting. Although recent reports have dealt with real-time PCR for BCR-ABL (15), E2A-PBX1 (16), and TEL-AML1 (17, 18), they are single PCRs based on the Taqman technology that requires the use of dual-labeled fluorogenic-specific oligonucleotides. None of the reported assays is designed to interrogate multiple translocations in a single reaction.

Consequently, our aim was to develop a real-time multiplex strategy to determine the presence of the chimera transcripts, which will also allow quantification.

We report here a simple, robust, and reproducible new multiplex system based on real-time RT-PCR to detect all of the four translocations necessary for the identification of the major genetic subgroups of ALL. The additional advantage is that it is a monochrome assay that does not require hybridization probes and is hence less expensive. We also show that the system is highly precise for quantification purposes using the TEL-AML1 transcript as an example. This analysis further demonstrated a notable interpatient variation in the expression level of TEL-AML1, which could not be accounted for by the leukemia burden. Because this variation appears to be an intrinsic factor, it may influence the biology of this ALL and, consequently, have clinical significance.

MATERIALS AND METHODS

Biological Samples. We have used the following leukemia cell lines that carry chromosomal translocations to develop the multiplex assay: REH with the t(12;21)(p13;q22); 697 with the t(1;19)(q23;p13); SupB15 with the t(9;22)(q34;q11); and RS4;11 with the t(4;11)(q21;q23). Cells were grown at 37°C in a 5% CO2 atmosphere in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal bovine serum, containing also 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Samples from 92 children with primary precursor B cell ALL were also analyzed. The median age of the patients was 5 years (range, 0 to 14 years). Bone marrow aspirates or peripheral blood samples were collected at the time of routine diagnostic procedures. Written informed consent was obtained from the guardians under approved Institutional Review Board projects. Fifty-two samples were collected from the All India Institute of Medical Sciences (New Delhi, India), and 40 were collected from the King Fahad National Centre for Children’s Cancer Research, King Faisal Specialist Hospital and Research Centre (Riyadh, Saudi Arabia). Forty-seven additional ALLs carrying a 12;21 translocation were available from Istanbul University (Istanbul, Turkey) and were specifically analyzed to evaluate the level of TEL-AML1 expression.

RNA Extraction and cDNA Synthesis. Patients’ mononuclear cells were obtained from ficoll-hypaque density gradient centrifugation and lysed with Trizol (Invitrogen Life Technologies, Inc., Carlsbad, CA) for RNA extraction. The quality of RNA was checked by absorbance at 260 and 280 nm and gel electrophoresis. Total RNA (2–3 μg) from patient samples or cultured cell lines was reverse transcribed using the Superscript first strand system for RT-PCR (Invitrogen, Carlsbad, CA) and random hexamers.

End Point RT-PCR for the Four most Frequent Translocations. The quality and integrity of cDNAs were tested by initially amplifying the housekeeping gene GAPDH. All samples were then analyzed by standard RT-PCR reactions for each of the four rearrangements. The primers used are shown in Table 1.

Once the RT-PCR reactions were standardized, a multiplex RT-PCR reaction was developed. In a 50-μl reaction, all four sets of primers were included at 30–100 pmols and 1.5 mM MgCl2 in a program of 4 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 56°C, 1 min at 72°C, and finally 10 min at 72°C. Each amplicon was designed to contain the specific junction giving a different size band after 4% agarose gel electrophoresis (Table 1). This easy identification of the translocation present was used to test all patient samples.

Multiplex Real-Time RT-PCR. The real-time assay was based on the Lightcycler instrument (Roche, Mannheim, Germany) using fluorogenic Sybr Green I. The PCR reactions were carried out in 20 μl, including 10–15 pmols of each primer, 2.5 mM MgCl2 and reagents from the FastStart DNA master Sybr green I kit (Roche). The program consisted of 10 min at 95°C followed by a touchdown setting of 32 cycles of 10 s at 95°C, 5 s at 62–58°C and 10 s at 72°C and a final cycle of 20 s at 97°C, 15 s at 65°C, 0 s at 98°C, and cooling to 40°C.

All experiments were conducted multiple times (at least three) using the appropriate positive, negative, and no template controls. The quality and integrity of cDNAs were tested by standard RT-PCR reactions for each of the four rearrangements. The primers used are shown in Table 1.

Table 1 Primers and major amplicons’ identities

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primer F</th>
<th>Primer R</th>
<th>Size (bp)</th>
<th>Melting peak (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEL-AML1</td>
<td>aagcccatcacaacctctctca (TEL, exon 5)</td>
<td>tggaggccgctggctgaagc (AML1, exon 3)</td>
<td>181</td>
<td>86 ± 0.5</td>
</tr>
<tr>
<td>E2A-PBX1</td>
<td>gcccaggggcgttgccctcaggttt (E2A, exon 12)</td>
<td>cagccctttcgcctaacag (PBX1, exon 2)</td>
<td>275</td>
<td>93.9 ± 0.4</td>
</tr>
<tr>
<td>MLL-AF4</td>
<td>aagatccagtccagagcagagc (MLL, exons 8 to 9)</td>
<td>actgtgagagtccttttgaggg (AF4, exons 5 to 6)</td>
<td>358</td>
<td>83.6 ± 0.3</td>
</tr>
<tr>
<td>mBCR-ABL</td>
<td>accttacactccagcgaggagaacctct (BCR, exon 1)</td>
<td>tcacctgqccacaaatcatacagty (ABL, exon 2)</td>
<td>418</td>
<td>92.2 ± 0.3</td>
</tr>
<tr>
<td>HPRT</td>
<td>gttggatataagccagactttgttg</td>
<td>acctcaacttggaactctcatcttaggc</td>
<td>164</td>
<td>80.8 ± 0.3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ccggagatctgcatcaatgg</td>
<td>catggttcacacccactgagc</td>
<td>221</td>
<td>89.1 ± 0.3</td>
</tr>
</tbody>
</table>

* For the major transcript.
controls. Fluorescence emission spectra were monitored in real time for amplification kinetics, and melting curve analyses were performed to assess the specificity of the amplified products.

**Quantitative Real-Time RT-PCR.** PCR products were measured by the threshold cycle (or crossing point, Cp) at which fluorescence became detectable above the baseline. The Cp was used for kinetic analysis and was proportional to the initial number of target copies in the sample.

We calculated the relative expression of TEL-AML1 transcripts as the ratio between the level of TEL-AML1 and the level of GAPDH. For this purpose, we generated standard curves by measuring the Cp values for GAPDH transcripts from serial dilutions of REH cDNA. The TEL-AML1 amplicon was cloned in a TA vector (Invitrogen), and serial 10-fold dilutions of this plasmid, corresponding to known copy numbers, were run in triplicates in the Lightcycler. For statistical correlations with clinical features, the Pearson χ² test and the Kaplan-Meier analysis were used.

**Southern Blot Analyses.** Ten μg of genomic DNA from patient samples, PBLs from normal individuals and control cell lines, REH and RS4;11, were independently digested with BamHI and HindIII. Restricted fragments were separated in a 0.8% agarose gel and transferred to nylon membranes. Blots were hybridized with ³²P-radiolabelled TEL and MLL probes (19).

**RESULTS**

**Identification of Translocations by Real-Time RT-PCR.** We initially established conventional end point RT-PCR reactions individually for the four most common translocations in childhood ALL that define clinical subgroups (3, 9). Primers were chosen to amplify all of the breakpoints and splice variants...
known (20), except for infrequent MLL-AF4 rearrangements (<15%) and an extremely rare E2A-PBX1 chimera (21). These specific primers were designed to give amplicons of different sizes (Table 1), allowing us to subsequently combine them in a single multiplex reaction and identify them by the size of the band in 4% agarose gels. The identity of each amplicon was additionally confirmed by hybridization to internal oligonucleotide probes (data not shown).

We then translated the individual amplifications to the Lightcyler system (Roche) using Sybr Green I. We had determined a MgCl₂ concentration of 2.5 mM as optimum for all of the four reactions. To successfully multiplex the system, we screened for conditions that would diminish primer-dimer formation and the signals resulting from them. To achieve this, we standardized the assays using a temperature release TaqDNA polymerase and a temperature touchdown amplification program consisting of 32 cycles. As shown in Fig. 1, A–C and Table 1, each amplicon yielded a unique, characteristic, and reproduc-

Fig. 2 Validation of the assay using ALL patient samples. Positive control cell lines were included in each run, and the signals obtained are indicated. A, real-time multiplex RT-PCR, including the internal standard. Samples with none of these three translocations show amplification of HPRT and samples with t(12;21) or t(1;19) show the respective peak. B, tandem reaction for mBCR-ABL. Positive samples clearly show the characteristic peak. We have specifically included a small fraction of negative patients that demonstrate nonspecific, low signals.
We have tested and validated the assay for the MBCR-PCR product was not altered in the multiplex reaction. However, reaction maintaining the specificity and reproducibility of each operated separately as a tandem assay (Fig. 2). mised in multiple combinations and thus, this reaction was developed demonstrating that the Sybr green I assay was specific enough to identify these alternative transcripts.

splicing has been reported. As an example, the variant isoforms of splice variants could be distinguished (Fig. 1). Analysis using a MLL probe. One patient demonstrated an 11q rearrangement by Southern blot. The next step was to approach this by testing the translocations in different pairs and then combining them by gradually increasing the number of primer sets included in the reaction. Fig. 1D shows that three fusion transcripts could be readily detected and identified in a multiplex reaction maintaining the specificity and reproducibility of each melting peak. It is noteworthy that the temperature profile of each PCR product was not altered in the multiplex reaction. However, the amplicon for the mBCR-ABL chimera was always compromised in multiple combinations and thus, this reaction was developed separately as a tandem assay (Fig. 2B).

Application of the Tandem Real-Time RT-PCR to Primary Leukemias. We have tested and validated the assay with bone marrow aspirates or peripheral blood samples from 92 pediatric patients with precursor B cell ALL (Fig. 2). This study was conducted blindly, without previous knowledge of the presence of translocations in the samples.

Although cDNAs have been initially screened for GAPDH expression as a quality control, it was important to include an internal control (housekeeping gene) in the multiplex reaction to ensure PCR ability for all cDNA templates. We have hence included GAPDH primers in the multiplex reaction, but this addition repeatedly compromised amplification of two chimeric transcripts, TEL-AML1 and MLL-AF4. Two other housekeeping genes, phospholipase A2 and β-actin, were similarly compromised. Three other genes, TATA box binding protein, α-tubulin, and β2-microglobulin, were properly amplified in the combined reaction, but the melting peak of the amplicon was very similar to the peak for the MLL-AF4 PCR product interfering with interpretation of results. Finally, one gene, HPRT1, could be established in the multiplex reaction and yielded an amplicon with a different melting peak (Table 1, Fig. 2A).

The PCR conditions were optimized to minimize primer-dimers when using clinical samples. We determined that 32 cycles of amplification produced a signal from each sample, coming either from a fusion transcript (if present) or from the housekeeping gene HPRT, without any background. This observation was additionally confirmed by agarose gel electrophoresis.

The use of the multiplex tandem real-time RT-PCR allowed us to also report differences in the distribution of translocations in two different non-Western ethnic groups (Saudi Arabia, n = 40 and India, n = 52). The clinical characteristics of the patients that carried translocations are depicted in Table 2. It appears that although E2A-PBX1 and BCR-ABL ALLs are underrepresented in Saudi Arabia (0 of 40), TEL-AML1 ALL is rarely detected in India (Table 3).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>WBC (×10⁹/liter)</th>
<th>Fusion gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>3</td>
<td>13.5</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>5</td>
<td>4.26</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>3</td>
<td>3.11</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>2</td>
<td>31</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>6</td>
<td>4.93</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>5</td>
<td>5.92</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>2</td>
<td>11.7</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>4</td>
<td>5.67</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>6</td>
<td>27.3</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>6</td>
<td>6.7</td>
<td>TEL-AML1</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>3</td>
<td>400</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>8</td>
<td>21.3</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>9</td>
<td>127</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>11</td>
<td>120</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>7</td>
<td>132</td>
<td>E2A-PBX1</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>4</td>
<td>181</td>
<td>E2A-PBX1</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>9</td>
<td>66.7</td>
<td>E2A-PBX1</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>11</td>
<td>254</td>
<td>E2A-PBX1</td>
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<table>
<thead>
<tr>
<th>ARABIA (n = 40)</th>
<th>TEL-AML1</th>
<th>E2A-PBX1</th>
<th>mBCR-ABL</th>
<th>MLL-AF4</th>
<th>None/other</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>32²</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 2 Clinical characteristics of the patients carrying translocations

<table>
<thead>
<tr>
<th>Country</th>
<th>TEL-AML1</th>
<th>E2A-PBX1</th>
<th>mBCR-ABL</th>
<th>MLL-AF4</th>
<th>None/other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saudi Arabia</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>32²</td>
</tr>
<tr>
<td>India</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 3 Distribution of the four translocations in precursor B cell ALL from non-Western countries

Fig. 3 Southern blot analysis of BamHI digested DNAs. A, hybridization of two blots with an MLL probe. PBLs from a healthy control were used as a normal, germ-line (GL) control. RS4;11 demonstrates a rearranged band (+) because of a 4;11 translocation. Lane 2 shows rearrangement of the MLL gene (+). B, another blot was hybridized with a TEL probe. PBL shows the GL band and REH shows a rearranged band (+). Lanes 2, 3, 4, and 8 show rearranged bands indicated with *, demonstrating t(12;21).
and real-time multiplex data. In some cases there was an exact concordance between Southern blot analysis and the RT-PCR data. Therefore, in these studied cases we used the RT-PCR data. Although this would have detected any of the known 4;11 breakpoints, no product was obtained from the sample. Four of the 16 patients carrying the 12;21 translocation in two independent experiments. Four of these patients expressed the smaller 4;11 breakpoint translocation.

**Quantitative Analysis of TEL-AML1 Transcripts.** During the qualitative analysis of samples for the presence of translocations, it appeared from the kinetics of the reactions that the levels of expression of the TEL-AML1 fusion transcript varied between patients. To appropriately expand this analysis, we developed assays to specifically quantify the leukemia-specific transcript levels in relation to GAPDH expression as the endogenous RNA control. Each TEL-AML1-positive sample was independently analyzed for the levels of these two transcripts.

A real-time RT-PCR for GAPDH was established with a characteristic melting profile (89.1 ± 0.3°C). No genomic DNA amplification was observed as confirmed by the single melting peak detected and the absence of the DNA band (440 bp) after running the products in an agarose gel (data not shown).

The standard curve displays the linear relation between the Ct and the logarithm of the template concentration. To generate a calibration curve for GAPDH transcripts, five independent preparations of cDNA from the REH cell line were pooled, purified, and serially diluted in water (10-fold). Each RNA concentration was extrapolated to indicate number of cells considering 5 pg of total RNA/cell. Each data point was collected in triplicate (Fig. 4).

We cloned the TEL-AML1 amplicon in a TA vector (pCR2.1-TEL-AML1) and used known amounts of this plasmid to generate a standard curve for the fusion template, measured as copy number. Ten-fold dilutions of plasmid DNA in water were made to represent values in the range of those previously observed from patients, which for this calibrator represented 4.6 × 10^2 to 4.6 × 10^7 copies. Data were obtained from triplicates with r = 0.98 and error = 0.226. This standard curve indicates a linear detection of the TEL-AML1 transcript over at least five logs.

We calculated the interexperiment and intraexperiment variability using five independent preparations of REH cDNA and their corresponding dilutions run in two experiments in the Lightcycler for TEL-AML1 and GAPDH. TEL-AML1 transcript values ranged from 50 to 101 when normalized to GAPDH. Intraexperiment variability was higher than interexperiment variability, however, these variations were at the most 2-fold.

Having established the parameters of TEL-AML1 quantification, we analyzed 47 additional cDNAs from Turkish patients carrying the 12;21 translocation in two independent experiments. Four of these patients expressed the smaller fusion (at exon 3 of AML1). We calculated the number of TEL-AML1 transcripts/cell from this cohort and observed that the level of expression of TEL-AML1 varied widely from 0.4 to 1429 with a median of 198 (Fig. 6A). Obviously, these differences could not be accounted for only by the experimental deviation of 2-fold. We, therefore, correlated the level of TEL-AML1 with the WBC counts of the patients at presentation as a measure of leukemia burden. As shown in Fig. 6B, no correlation was apparent even when only the major group, i.e., patients with <30 × 10^9/liter, was analyzed. However, three groups of patients could be discerned: (a) majority clustered within a level of expression between 100 and 600 (34 of 47, 72%); (b) small proportion expressed lower values, <100 (6 of 47, 13%); and (c) small group expressed higher levels, >600 (7 of 47, 15%). Although the number of patients in each group is small, we correlated these three categories with clinical data. We did not find statistically significant association with gender, age, hemoglobin, platelet count, or presence of hepatomegaly or splenomegaly (Pearson χ^2 test). Similarly, overall survival was not significantly different between the groups (83.33, 91.83, and 100% for the low, moderate, and high expressing categories, respectively).
DISCUSSION

The presence of translocations in ALL not only influences the biology of the leukemic clone but also predicts clinical outcome. Therefore, we developed a simple real-time RT-PCR multiplex assay to detect the four most common rearrangements in childhood precursor B ALL. There are recent reports based on real-time RT-PCR to detect some of these translocations (15–18), but all these assays detect a single translocation and use dual color fluorochromes in the Taqman system. Therefore, the major advantages of this newly developed test are that it is a multiplex reaction and uses only Sybr green I. This monochrome assay allows reliable identification of the amplicons based on their characteristic melting peak.

We tested several primer pairs to arrive at the sets that reproducibly amplified a segment with a characteristic peak and very little variability (0.3–0.5°C), allowing proper identification (Table 1) both singly and in a multiplex reaction. Three of the translocations were easily combined (Figs. 1 and 2A), but all these assays detect a single translocation and use dual color fluorochromes in the Taqman system. Therefore, the major advantages of this newly developed test are that it is a multiplex reaction and uses only Sybr green I. This monochrome assay allows reliable identification of the amplicons based on their characteristic melting peak.

The primers were designed to amplify the two known variants of TEL-AML1, mBCR-ABL, and E2A-PBX1. MLL-AF4 has been reported with at least 10 different rearrangements, and the primers used here amplify 6 of them representing >85% of all t(4;11) (20). We have also tested another AF4 primer (in exon 8), which will expand the amplifiable rearrangements to >95%. Interestingly, this new amplicon has a very similar melting peak (83.9 ± 0.5) to the previous MLL-AF4 PCR product and can be easily interchanged. We attempted to use another set of primers that will amplify all known 4;11 variants (exon 8 of each gene), but this amplification was compromised in the multiplex reaction.

To have an internal control in the multiplex reaction, seven housekeeping genes were tested and only one, HPRT, yielded an appropriate melting peak not overlapping with the ones from fusion transcripts in the multiplex reaction (Fig. 2A). This was important when testing samples from patients for the potential translation to the clinical setting. Every sample yields a specific product from the housekeeping gene or from the fusion transcript if a translocation is present ensuring PCR ability of every cDNA and reconfirming the high fidelity of the system.

In fact, this assay was validated with 89 ALL pediatric cases from two non-Western countries. Although the numbers of patients were small and the study was not meant to be a comprehensive epidemiological study, it appears that in comparison to the West, E2A-PBX1 and BCR-ABL ALLs were underrepresented in Saudi Arabia (0 of 40) but not in India (4 of 52) (Table 3). One possible explanation is that although our cohort of Indian patients had a median age of 7 years, the median age for the Arab patients studied was 3 years. The t(1;19) as well as the t(9;22) most frequently occur in older patients (11, 12). On the other hand, TEL-AML1 occurred at a significantly low frequency in ALL from India (4%) but not in Saudi Arabia (20%), reconfirming previous data (19). Additional studies of larger number of patients are warranted to confirm these results.
observed variable levels of the fusion transcript beyond the experimental deviation (Fig. 6A). This was somewhat surprising because this translocation retains the TEL promoter, and therefore, it would be expected to be consistently regulated in all TEL-AML1 leukemias. Our observation then could indicate heterogeneity of other factors influencing the transcript levels. Three groups of patients appear to exist, a cluster (72%) with arbitrary expression of 100–600, low expressors (13%) and high expressors (15%). No statistical association with WBC (Fig. 6B), gender, age, hemoglobin, platelets, organomegaly, or overall survival was detected. Because this variation appears to be an intrinsic factor, it may influence the biology of this ALL. Additional correlations may elucidate the clinical significance of this observation.

Previous reports have quantified TEL-AML1 levels in a few cases to study minimal residual disease and, consequently, the kinetics of disappearance of leukemic transcripts during therapy (28–30). Although different TEL-AML1 levels at presentation were not specifically highlighted, Pallisgaard et al. (17) reported up to 14-fold variation in 12 patients. From the data of Drunat et al. (29), 6.5-fold differences can be deduced and this variation appeared to be independent of the percentage of cycling cells.

In conclusion, our real time quantitative-RT-PCR using Sybr green I represents a robust, reliable, sensitive, and cost effective technique for quantitative PCR analyses. The turnover time is short, and the capacity for sample throughput is high. Furthermore, this strategy allows correlation of outcome not only with the type of gene rearrangements but also has the simultaneous potential of quantifying the expression of chimeric genes in ALL at presentation, making the follow up of minimal residual disease feasible.

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