Background and Objectives. We report a new real-time reverse transcription polymerase chain reaction (RT-PCR) method for quantification of TEL-AML1 transcripts. The method is based on hybridization probe (HybProbe) chemistry applied in LightCycler equipment. The study group comprised 44 successive cases of pediatric acute lymphoblastic leukemia (P-ALL).

Design and Methods. The quantitative estimation of TEL-AML1 transcripts was performed in 10 P-ALL TEL-AML1-positive patients. The PCR was performed in capillary tubes in 10 µL final volumes using two sets of primers: M1, which detects the long (L-form) and short (S-form) transcripts, and M2, specific for the L-form. The fluorescently labeled HybProbes (TEL3FL and TEL5LC) hybridize to the TEL region. TEL-AML1 expression was normalized relative to the levels of AML1 transcripts. Standard curves were prepared using serial dilutions of plasmids with TEL-AML1 or AML1 inserts.

Results. The sensitivity attained allowed the detection of TEL-AML1 transcripts at a 10⁻⁴ dilution of a cDNA sample from a patient at diagnosis. The within-assay coefficient of variation (CV) for TEL-AML1 was 7.0% and the between-assay CV was 13%. Levels of TEL-AML1 transcript and the TEL-AML1/AML1 ratio decreased by more than four log units (p < 0.001) during or after the course of initial treatment. Most of the patients who achieved complete remission after 5-6 months of initial treatment were TEL-AML1 negative, although some positive samples with negligible amounts of TEL-AML1 transcripts were still detected.

Interpretation and Conclusions. This method has the sensitivity and reliability required to monitor the presence of minimal residual disease, and could be a powerful tool in monitoring the efficacy of the response to chemotherapy.

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Key words: real-time PCR, TEL-AML1, t(12;21), pediatric acute lymphoblastic leukemia, minimal residual disease
transcripts were detected at the time of diagnosis, but patients usually became TEL-AML-negative soon after chemotherapy or allogeneic bone marrow transplantation.5

To date, the molecular monitoring of minimal residual disease (MRD) in acute leukemia has mostly been done by qualitative methods based on reverse transcription (RT) followed by nested PCR (RT-PCR)5,7 or quantitative RT-PCR procedures based on competitive assays.3 With the advent of real-time PCR technology, two quantitative real-time RT-PCR methods for TEL-AML1 fusion transcripts based on the use TaqMan™ probes with ABI PRISM 7700 equipment have so far been reported.12,13 However, to our knowledge, no real-time quantitative method based on hybridization probe (HybProbe) chemistry using the LightCycler apparatus (Roche) has yet been reported. HybProbe chemistry consists in two adjacent probes in a head-to-tail orientation labeled with fluorescent dyes at their opposite ends.

We report a new method for TEL-AML1 quantification adapted to the LightCycler equipment using HybProbe chemistry. The results obtained in the clinical assessment of 10 TEL-AML1-positive children with P-ALL confirm that the method has the reliability and sensitivity required for clinical practice. Furthermore, the high-speed thermal cycling of the LightCycler makes it possible to complete the analysis in only 50 min.

### Design and Methods

#### Patients

The quantitative study was performed in 10 TEL-AML1 positive patients identified by our conventional qualitative RT-PCR method from 44 consecutive cases of P-ALL (31 B-cell type, seven pre-B-cell, four T-cell type and two biphenotypic ALL; 37 at first diagnosis and seven at relapse) examined in our laboratory from 1998 to 2000. The patients, 17 boys and 27 girls, had a median age (range) of 4.5 (0.7–13.5) years and came from the hospitals Universitario La Fe (Valencia), Virgen de la Arrixaca (Murcia), and General de Alicante (Alicante).

### Table 1. Biological characteristics of the pediatric B-cell precursor acute lymphoblastic leukemias which were TEL-AML1 PCR positive.

<table>
<thead>
<tr>
<th>Pts</th>
<th>Gender/ Age (yrs)</th>
<th>Immuno- phenotype</th>
<th>Moment</th>
<th>Treatment</th>
<th>Sample</th>
<th>TEL-AML1 form</th>
<th>TEL-AML1 copies/µL cDNA</th>
<th>AML1 copies/µL cDNA</th>
<th>TEL-AML1/ AML1</th>
<th>FISH</th>
<th>WBC (×10^9/L)</th>
<th>DNA index</th>
<th>Clinical outcome (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/10.9</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-94</td>
<td>PB</td>
<td>L</td>
<td>472,708</td>
<td>3,858,600</td>
<td>0.122</td>
<td>ND</td>
<td>26.9</td>
<td>1.12</td>
<td>CR off therapy, (+27.43)</td>
</tr>
<tr>
<td>2</td>
<td>M/8.0</td>
<td>B-ALL</td>
<td>R</td>
<td>SHOP-REC-94</td>
<td>BM</td>
<td>L</td>
<td>267,034</td>
<td>2,108,188</td>
<td>0.127</td>
<td>ND</td>
<td>5.1</td>
<td>1.0</td>
<td>Testicular relapse, (+24.13)</td>
</tr>
<tr>
<td>3</td>
<td>F/6.0</td>
<td>B-ALL</td>
<td>D</td>
<td>PETHEMA-96</td>
<td>BM</td>
<td>L</td>
<td>109,976</td>
<td>281,076</td>
<td>0.391</td>
<td>ND</td>
<td>26.6</td>
<td>ND</td>
<td>CR on maintenance, (+18.37)</td>
</tr>
<tr>
<td>4</td>
<td>F/1.2</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-94</td>
<td>PB</td>
<td>L</td>
<td>531,236</td>
<td>695,964</td>
<td>0.763</td>
<td>ND</td>
<td>32</td>
<td>1.67</td>
<td>CR on maintenance, (+6.83)</td>
</tr>
<tr>
<td>5</td>
<td>F/3.3</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-99</td>
<td>PB</td>
<td>L</td>
<td>2,154,444</td>
<td>7,427,392</td>
<td>0.290</td>
<td>Positive (93/100)</td>
<td>58</td>
<td>1.0</td>
<td>CR on maintenance, (+6.83)</td>
</tr>
<tr>
<td>6</td>
<td>M/6.0</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-99</td>
<td>BM</td>
<td>L</td>
<td>445,922</td>
<td>3,845,500</td>
<td>0.115</td>
<td>Positive (190/200)+21.del(11)</td>
<td>52.5</td>
<td>1.0</td>
<td>CR on maintenance, (+5.87)</td>
</tr>
<tr>
<td>7</td>
<td>M/6.5</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-99</td>
<td>BM</td>
<td>L</td>
<td>38,468</td>
<td>241,664</td>
<td>0.159</td>
<td>Positive (99/100)</td>
<td>11.2</td>
<td>1.0</td>
<td>CR on treatment, (+1.67)</td>
</tr>
<tr>
<td>8</td>
<td>M/2.7</td>
<td>B-ALL</td>
<td>R</td>
<td>ALL-REZ-BMF-96</td>
<td>BM</td>
<td>L</td>
<td>480,968</td>
<td>1,319,712</td>
<td>0.364</td>
<td>Positive (90/100)</td>
<td>80</td>
<td>1.0</td>
<td>CR on treatment, (+0.67)</td>
</tr>
<tr>
<td>9</td>
<td>F/1.4</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-99</td>
<td>PB</td>
<td>L</td>
<td>48,498</td>
<td>301,068</td>
<td>0.161</td>
<td>Positive (80/100)</td>
<td>135</td>
<td>ND</td>
<td>On treatment, (0)</td>
</tr>
<tr>
<td>10</td>
<td>F/4.0</td>
<td>B-ALL</td>
<td>D</td>
<td>SHOP-99</td>
<td>BM</td>
<td>L</td>
<td>216,294</td>
<td>1,248,440</td>
<td>0.173</td>
<td>ND</td>
<td>11</td>
<td>ND</td>
<td>On treatment, (0)</td>
</tr>
</tbody>
</table>

Abbreviations.- CR= Complete remission, D= Diagnosis; F= Female, L=L-form, M= Male, ND= Not done; Pts= Patients, R= Relapse.
The TEL-AML1 positive group consisted of five boys and five girls (Table 1), with a median age (range) of five (1.2–10.9) years. Eight of these patients were studied at diagnosis, and two at first relapse. In two patients, only the samples taken at diagnosis were available. The remaining eight patients were monitored quantitatively for TEL-AML1 transcripts during the clinical course of their disease (median time: 6.8 months; range: 0.57–27.43 months).

Treatment

Newly diagnosed patients included in this study were treated with three different therapy protocols: SHOP-94, SHOP-99 (Spanish Leukemia Group), and PETHEMA-96 (Table 1).

Briefly, induction to remission consisted of a five-drug combination treatment (vincristine, daunorubicin, prednisone, L-asparaginase, and triple intrathecal therapy) plus cyclophosphamide (SHOP-94, PETHEMA-96) or cyclophosphamide and intermediate-dose methotrexate (SHOP-99) in high-risk patients. Consolidation treatment included 6-mercaptopurine, IV methotrexate (3 g/m²), high-dose ARAC, and triple intrathecal therapy (TIT) in the three protocols, plus teniposide (only in PETHEMA-96). One patient in relapse was treated with SHOP-REC-94 (Spanish relapsed-ALL protocol) described (briefly) as follows. Induction to remission: dexamethasone, vincristine, mitoxantrone, ifosfamide, etoposide, ARAC, and TIT; consolidation-1: methotrexate (5 g/m²), HD-ARAC, TIT, and thioguanine; consolidation-2: dexamethasone, vin desine, ifosfamide, mitoxantrene, etoposide, low-dose ARAC, and TIT. After this phase, patients usually undergo stem-cell transplantation but multi-chemotherapy rotation maintenance may also be administered. The other relapsed patient was treated with the ALL-REZ BFM-96 protocol.14,15

Samples

The samples from other hospitals were sent to our laboratory in a cool transport container at 4°C and they were delivered on the same day as the sample has been collected.

Either a bone marrow (BM) aspirate or a peripheral blood sample (PB) was collected in a tube containing ethylenediaminetetra-acetic acid (EDTA) as anticoagulant. A total of 61 samples (40 BM and 21 PB) were analyzed, 44 at the time of active disease (25 BM and 19 PB). Quantitative assessment of TEL-AML1 transcripts was performed on 27 samples (21 BM and 6 PB) from the 10 TEL-AML1 positive patients, with a median of three samples per patient (range: 1–5).

Reagents

- Taq Start™ antibody (PT 1576-1)(Clontech, Palo Alto, CA, USA, Cat. No.5400-1);
- Light Cycler™-DNA Master Hybridization Probes (Roche Molecular Biochemicals, Indianapolis, IN, USA, Cat. No. 2015102);
- FastStart Light Cycler™-DN Master Hybridization Probes (Roche Molecular Biochemicals, Mannheim, Germany, Cat. No. 3003248);
- Uracil-DNA-glycosylase, heat-labile 1U/mL (Roche Molecular Biochemicals, Mannheim, Germany, Cat. No. 1775367);
- Light Cycler™-Capillaries 8¥96 (Roche Molecular Biochemicals, Cat. No. 190939).

RNA isolation

Mononuclear cells were isolated from EDTA-anticoagulated BM or PB by Lymphoprep (Nycomet, Pharma AS) density gradient centrifugation. The cells collected (mean number 5×10⁶) were resuspended in guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, [pH 7], containing 5 g/L sarcosyl and 0.1 M 2-mercaptoethanol) and stored at –80°C until RNA extraction. RNA was extracted following the guanidinium thiocyanate, phenol–chloroform procedure described by Chomczynski and Sacchi.16

Cloning of PCR products

Quantification involved the use of standard curves that were prepared with plasmid constructs containing the L-form of the TEL-AML1 fusion transcript. These constructs [pcr II-TOPO (TEL-AML1)] were prepared cloning the PCR products obtained amplifying a patient’s sample with the TEL2 and AML1-R3 primers of Satake et al.5 into the pCR®II-TOPO vector (TOPO™ Cloning®, Invitrogen BV). We also cloned the PCR product of AML1 gene obtained using the primers described by Kozu et al.17 to construct the plasmid pCR II-TOPO (AML1).

cDNA synthesis

One microgram of RNA was reverse transcribed into cDNA in a 25 μL reaction volume, using MoM uLV-reverse transcriptase (Promega, Madison,
and random hexamer primers. The RNA was first incubated at 70°C for 5 min, and then the reagent mixture (MoMuLV transcription buffer containing 0.5 mM dNTP, 25 U RNasin and 200 U MoMuLV) was added. The MoMuLV was left to act for 60 min at 42°C and finally denatured by heating at 95°C for 5 min.

Qualitative detection of TEL-AML1 transcripts
The qualitative RT-PCR method followed here was that reported by Satake et al.,5 which consists of a nested PCR using one of the forward primers (TEL1 or TEL2) that bind to exon 5 of TEL, in combination with one of the corresponding reverse primers (AML1-R41 or AML1-R42) that bind exon 4 of AML1. Splicing of exon 3 was assessed by a hemi-nested PCR using the forward primers TEL1 and TEL2 in combination with the reverse primer AML1-R3, which binds exon 3. The PCR products were analyzed by agarose gel electrophoresis.

Quantitation of TEL-AML1 transcripts
Standard curve. The TEL-AM L1 and AM L1 standard curves were prepared using 10-fold serial dilutions of the respective PCR II-TOPO (TEL-AM L1) and PCR II-TOPO (AM L1) plasmids, in aqueous heterologous DNA. For TEL-AM L1, we used seven 10-fold serial dilutions in a range from 45,000,000 to 5 copies, and for AM L1 six 10-fold serial dilutions from 16,750,000 to 168 copies.

Real-time PCR. For TEL-AM L1 transcripts we used the TEL2 forward primer of Satake et al.,3 which binds the TEL gene, in combination with either the AM L1-R3 reverse primer, which binds exon three of AM L1 (M1 primer-set), or the X2AM L1 primer, which binds AM L exon two (M2 primer-set) (Table 2 and Figure 1). The M2 primer-set (TEL2+X2AM L) is specific to the long transcript (L-form), since amplification of this PCR product requires the presence of exon 2.2 and the short transcript (S-form) could not be amplified due to its lack from exon 2.6 The M1 primer-set detects both the L- and S-forms, although two alternative splicing products are detected for the L-form: a larger one that includes exon 2 and a shorter one from which 39 nucleotides of exon 2 are removed.6 The existence of two splicing fragments in the L-form explains how, in the present work, due to the lack of S-forms in our series, the use of the M1 primer-set had a secondary role limited to the characterization of breakpoint isoforms (L or S forms). The fluorogenic 3' hybridization probe (TEL 3FL), labeled with fluorescein, and the 5' probe (TEL 5LC), labeled with LC Red640, compatible with both combinations of primer-sets (M1 and M2), were designed and synthesized by TIB MolBiol (Berlin, Germany) (Table 3). Both probes hybridize to regions of the amplified TEL fragment (Figure 1).

TEL-AM L1 was normalized with AM L1 which was taken as the internal reference gene according to the method of Kozu et al.17 The procedure for AM L quantification has already been reported.18 Briefly, it consists of the amplification of the AM L gene using the AM L1C(+)- and AM L1E(−) primers and the probes AM L 3FL, fluorescein-labeled at the 3’ end, and AM L 5LC, labeled with LC Red640 at the 5’ end and phosphate-blocked at its 3’ end, designed and synthesized by TIB MolBiol (Table 2).

PCR was performed in 10 µL final volumes, using 1 µL FastStart Light Cycler™-DNA M aster Hybridiza-
Real time PCR quantification of fusion transcripts using fluorescently labeled probes

...tion Probes mastermix (buffer, dNTPs in which dTTP is replaced by dUTP, MgCl₂, and the modified inactive Taq DNA polymerase)×10.

The TEL-AML1 primer-sets, M1 and M2, were used at final concentrations of 0.40 µM, and those for AML1 (AML1C(+) and AML1E(−)) at a final concentration of 0.5 µM. The fluorescent probes for TEL-AML1 and AML1 were used at final concentrations of 0.2 µM.

MgCl₂ was used at final concentrations of 5 mM and 3 mM for the quantification of TEL-AML1 and AML1 transcripts, respectively. We also added 0.5 U of heat labile uracil-DNA glycosylase (UDG) to preclude possible contamination of the PCR by previous reactions.

Two microliters of each point of the standard curve, or of the cDNA samples, were used in both the TEL-AML1 and AML1 assays.

The PCR program followed for TEL-AML1 amplification consisted of three steps. The first step involved an incubation at 32°C for 5 min, to allow

Figure 1. Schematic representation of the placement of primers and hybridizations used in the quantitative detection of (A) TEL-AML1 fusion transcripts and (B) AML1 quantification. Circles indicate the localization of the fluorescent labels (FL, fluorescein; and LC Red640). Numbers in boxes indicate exons.

Figure 2. (A) TEL-AML1 standard curve calculated from 10-fold dilutions of the plasmid pCR II-TOPO (TEL-AML1); where F2/F1 are the fluorescence intensity readings measured at channel ratio F2 (640 nm)/F1 (530 nm). (B) Straight line obtained from the cycle threshold (Cₜ) and concentration at each point on the standard curve.
the UDG to degrade any possible contamination from previous PCRs. This was followed by heating at 94°C for 10 min to activate the Taq DNA polymerase. Amplification was performed for 45 cycles, each one comprising annealing at 61°C for 10 s, elongation at 72°C for 9 s, and denaturation at 96°C for 2 sec. Fluorescence was measured at the end of the annealing step at F2 (640 nm)/F1 (530 nm) channel ratio. Calculations were performed with the software (LightCycler 3) provided with the LightCycler apparatus.

The PCR program for the AML1 control gene has already been reported, although AML1 can be satisfactorily amplified using the PCR program described for TEL-AML1.

The software included with the equipment, based on the established relationship between the cycle threshold (CT) and the logarithm of the initial number of target copies (N) present in the sample, fits an empirical straight-line with the points of the standard curve. This allows estimation of N for each sample on the basis of its CT, for both TEL-AML1 and the control gene AML1. N was expressed in terms of copies of TEL-AML1 plasmid per microliter of cDNA. Normalized levels were calculated as the ratio TEL-AML1/AML1.

Statistical analysis

For quantitative data, non-parametric tests were applied using a Mann-Whitney U test when two groups were compared and a Kruskal-Wallis H test for more than two groups. Values of p < 0.05 were considered statistically significant. The statistical calculations were performed with the statistical package SPSS 8.0.

Results

Study of the method

Standard curve. All the standard curves were generated using the M2 primer-set. The regression coefficients calculated for five consecutive TEL-AML1 standard curves were all -1.0. We estimated a mean ± SD slope of -3.26±0.15, with mean ± SD intercept of 39.16±1.09 (Figure 2). The mean CT for each point in the five consecutive TEL-AML1 standard curves were almost constant with SD < 0.7 cycles for all the points, except for the lowest point (5 plasmid copies), which showed a SD of 1.28 cycles. For the five AML standard curves, we calculated a slope of -4.61±0.1, and an intercept of 42.48±3.61.

Quality of amplified products. Some of the PCR products were checked by electrophoresis on a 2% agarose minigel, verifying the absence of artifacts and that the amplified products corresponded to their expected sizes (with M1 two splicing products of 214-bp and 175-bp were obtained, whereas with the M2 primer-set a single fragment of 134-bp was produced) (Figure 3).

Sensitivity. The PCR sensitivity was assessed by analyzing three times a series of 10-fold aqueous dilutions (range: 10⁻² to 10⁻⁵) of a TEL-AML1-positive cDNA sample taken from a patient at diagnosis. The M2 primer-set allowed the amplification of TEL-AML1 transcripts at a dilution of 10⁻⁴ and, occasionally, at 10⁻⁵ dilution. On the other hand, studies of absolute sensitivity using dilutions of the pCR II-TOPO (TEL-AML1) plasmid for points on the standard curve confirmed that the method can detect up to 5 TEL-AML1 insert copies.

Reliability of the assay

Within-assay reproducibility was studied by repeating the analysis of the same sample 10 times in the same assay. For the TEL-AML1 Ct, a mean±SD of 23.10±0.20 cycles was estimated, which reflects a CV of 0.6%. These results correspond to 536,428±38,775 copies/μL cDNA (CV = 7.0%). For the AML1 Ct, we obtained a mean±SD of 21.38±0.1 cycles (CV= 0.46%), or 249,216±17,464 AML1 copies/μL cDNA (CV = 7.0%). Between-assay reproducibility was studied by repeating, in seven successive assays, the analysis of three cDNA samples with high, intermediate, and low levels of TEL-AML1 transcripts. In the sample with the highest levels of TEL-AML1...
transcript, we estimated for CT a mean ± SD of 23.1±0.2 cycles (CV = 0.6%), which converts to 2,404,520±315,122 copies TEL-AML1/µL cDNA (CV = 13%). In the sample with intermediate levels of TEL-AML1 transcript, we calculated a mean CT ±SD of 29.1±0.5 cycles (CV = 1.6%) and 17,743±4,700 copies TEL-AML1/µL cDNA (CV = 26%). In the samples with the lowest levels of TEL-AML1, the mean CT ±SD was 34.8±1.3 cycles (CV = 3.8%) and 823±470 copies TEL-AML1/µL cDNA (CV = 49%). The between-assay reproducibility for AML1 was assessed by analyzing, in consecutive assays, a cDNA sample of Kasumi-1 cell line at a 10−2 aqueous dilution. For AML1, we obtained a mean CT of 20.17 ± 0.26 cycles, with a CV of 1.29%, or 320,724 ± 39,176 copies/µL cDNA, which reflects a CV of 12%.

TEL-AML1 rearrangement in ALL

We detected L-form TEL-AML1 transcripts in 10 of the 44 children with ALL (22.7%) (Table 1). FISH studies confirmed the presence of the TEL-AML1 rearrangement in more than 90% of the cells in the five patients on whom the study was performed (Table 1).

The group of 10 patients with TEL-AML1-positive P-ALL (Table 1) had a median (range) of 5 (1.2–10.9) years, 29×10⁹ (5–135) WBC/L and 1 (1–1.67) for DNA index. These data are not statistically different from the corresponding parameters of the 34 patients TEL-AML1-negative P-ALL patients, who had a median (range) of 4.5 (0.7–13.5) years, 10×10⁹ (1–280) WBC/L and 1.0 (0.82–1.6) for DNA index. Although no statistical significance was found, the median WBC count of TEL-AML1-positive patients was clearly higher than that of the TEL-AML1-negative group.

Quantitative monitoring of TEL-AML1 transcripts

The TEL-AML1 transcripts at the time of active disease (eight patients at diagnosis and two in relapse) showed a median (range) of 356,478 (38,468–2,154,444) copies/µL cDNA, and median (range) of 0.167 (0.115–0.763) when normalized by the reference gene AML1. Although we did not compare the TEL-AML1 levels in PB and BM in the same patients, we did not find statistical differences between the results obtained in PB and BM among the different samples (4 PB vs 6 BM) patients at the moment of diagnosis for either copies of TEL-AML1/µL cDNA or its ratio with AML1.

The TEL-ALM1 transcripts and the TEL-AML1/AML1 ratio decreased significantly (p=0.00), by approximately four logs in the 8 samples collected during or early after induction, to reach a median (range) of 94 (0–1,888) copies/µL cDNA and median (range) of 0.00015 (0.0–0.025) for the TEL-AML1/AML1 ratio. The levels of TEL-AML1 in the 9 samples from patients during maintenance were almost negligible, with a median (range) of 1.5 (0.0–118) copies/µL cDNA and a median (range) of 0.0 (0.0–0.0018) for the TEL-AM1/AML1 ratio.

The AML1 reference gene decreased (p = 0.052) from a median (range) of 1,284,076 (241,664–7,427,392) copies/µL in the samples obtained at the moment of active disease to 285,525 (75,905–728,970) copies/µL in the samples taken during induction or early thereafter and a median (range) of 340,280 (17,625–2,966,850) copies/µL in the samples collected during maintenance.

TEL-AML1 transcript levels and the course of the disease

TEL-AML1 transcripts were detected in four of eight samples (50%) and in three of five patients...
(60%) in the course of initial treatment or shortly thereafter (Figure 4), although the levels of TEL-AML1 transcripts were very low, since all but one of the transcripts of these samples were detected after 35 cycles (median = 36.3 cycles).

TEL-AML1 transcripts were detected in three of 9 samples (33%) and in two of five patients (40%) on maintenance treatment (Figure 4). The positive samples were two consecutive samples from the same patient (Pt #3, Figure 4), collected after 13 and 18 months of follow-up. The other positive sample was collected from another patient (Pt #1, Figure 4) at the end of the maintenance regime. Nevertheless, all these transcripts appeared near the detection limit of 41 cycles (median = 37.7 cycles).

The individual follow-ups of the patients included in the study showed rapid reduction of TEL-AML1 transcripts during or after the induction treatment (Figure 4). So that the majority of samples collected after 5 months were either negative (7 of 10 samples) or had negligible levels of transcript (3 of 10 samples).

Discussion

The first real-time PCR studies of TEL-AML1 quantification were developed on the ABI/Prism™ 770012,13 using TaqMan™ technology. The method developed here is based on HyProbe technology used in LightCycler equipment. Although TaqMan™ probes can be satisfactorily used in the LightCycler,21 the use of HyProbes is clearly superior to the use of TaqMan™ in this equipment, since HyProbes do not require the long annealing-elongation incubation times (≥ 1 min) needed to achieve complete 5' nuclease degradation of the TaqMan™ probe, this allowing the entire PCR program to be accomplished in less than 50 min.

The data recorded for the TEL-AML1 standard curves support the high reproducibility in successive assays, yielding very consistent Ct values for each one of the points.

The sensitivity achieved with the method, which can amplify the cDNA from a sample at diagnosis up to a 10⁻⁴ dilution and occasionally reaching 10⁻⁵ dilution, is similar to that achieved by Ballerini et al.,12 using the TaqMan™ probe in the ABI/Prism 7700, but one log lower than that reported by Pallisgaard et al.13

The within-assay CV of 0.6% for Ct and 7.0% for TEL-AML1 transcripts are also equivalent to those reported by Ballerini et al.12 This precision seems to be sufficient to monitor samples reliably from each patient. However, the between-assay CV of TEL-AML1 concentration increases considerably in parallel with Ct, from a CV of 13% at Ct of 23 cycles to 50% at Ct of 35. Even though the CV were very high, they are similar to those reported in other studies.22,23 The increase in CV with Ct limits the quantitative reliability of the method to samples with a Ct value lower than 30 cycles, being semiquantitative for a Ct between 30 and 35 cycles, qualitative from 35 to 40 cycles and negative for Ct > 40 cycles.

We detected TEL-AML1 transcripts in 10 of the 44 P-ALL patients studied, which represents an incidence of 22.7%, in accordance with the range reported in previous studies,24 but clearly different from the data reported by García Sanz et al.25 in Spain who, in a study of a series of 101 cases of ALL (63 adults and 38 children) from the central part of Spain, were unable to detect TEL-AML1 by FISH and RT-PCR. Our results clearly indicate that the data reported by those authors are by no means representative of Spain and reinforce the evidence of the existence of geographic variations in the genotype of acute lymphoblastic leukemia.

The concentration of TEL-AML1 transcripts, or its normalized value with AM1L, showed an approximately four log decrease during or after the course of the initial treatment. Nevertheless, we still found TEL-AML1 transcripts in four of eight samples (four of five patients) collected during or early after the treatment. However, the levels of transcripts detected at this time were all very low and the results obtained could be considered only as qualitative (Ct > 35 cycles). The four log reduction in TEL-AML1 transcripts obtained in response to treatment is even higher than that reported in the study by Ballerini et al.,12 in a series of four patients. Besides, the present study corroborates the idea suggested by Ballerini et al. that the quantitative assessment of TEL-AML1 transcripts might be useful in monitoring the efficacy of the response to the therapy regimen applied.

The reduction of the expression levels of the reference gene AM1L from the moment of active disease to soon after the treatment questions the suitability of this gene for normalizing TEL-AML1 transcripts. In this respect it could be better to assay alternative reference genes such as that for β2-like globulin13 since they have similar expression levels in leukemic and normal cells.

The results reported by Satake et al.15 using qualitative nested PCR in a series of seven patients, with follow-up at ≤ 48 months, showed that the patients who responded to treatment became PCR-negative within the first month, and that samples...
collected after eight months of follow-up were PCR-negative. Our study revealed that the patients usually reverted to being TEL-AML1-negative within the six-month treatment, and that, after this period, the few positive samples observed were all beyond the limits of the sensitivity of the assay (>35 cycles). All the patients included in the study had a six-month follow-up, but one who suffered testicular relapse (Pt #2), remained in CR.

The evaluation of the method and the initial results obtained for the 10 TEL-AML1-positive patients with P-ALL indicate that the quantitative method established here has the sensitivity and reliability required to monitor the presence of MRD in P-ALL using TEL-AML1 transcripts. Moreover, the reduction in the transcripts during or after treatment facilitates the use of this quantification in monitoring the efficacy of the therapeutic regimes applied. However while the potential of the quantification of TEL-AML1 transcripts by real time PCR might be important, both the small number of patients of our series and the short follow-up hamper the analysis of the clinical impact of TEL-AML1 quantification.

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PB and EB were responsible for the design of the method and the study and for drafting the manuscript. JC was in charge of the FISH studies and also contributed to the production of the manuscript. EL and IM were in charge of performing the PCR analyses of the patients. MAS was responsible for the critical evaluation of the present paper and the funding. AV, JMF, CE, MT, VF and MB participated in the final processing of the manuscript and collected the follow-up data from the patients. The order of authorship reflects the authors’ contribution to the study and/or their participation in the processing of the manuscript.

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