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Rapid quantitative detection of *TEL-AML1* fusion transcripts in pediatric acute lymphoblastic leukemia by real-time reverse transcription polymerase chain reaction using fluorescently labeled probes

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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 (*TEL* 3FL and *TEL* 5LC) 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.

Key words: real-time PCR, TEL-AML1, t(12;21), pediatric acute lymphoblastic leukemia, minimal residual disease

The *TEL/ETV6* (1p12) *AML1/CBFα2* (21q22) rearrangement, caused as a consequence of the t(12;21)(p13;q22) cryptic translocation, is by far the most frequent genetic lesion in pediatric acute lymphoblastic leukemia (P-ALL)[1] occurring in 20–30% of P-ALL[2] and in 3% of adult ALL.[3] Furthermore, t(12;21) usually escapes cytogenetic detection, and can only be detected by molecular procedures or fluorescence *in situ* hybridization (FISH).[1]

The breakpoint at *TEL* occurs invariably in intron 5 and three translocation breakpoints in *AML1* have been described,[4,5] although two are more common. In the predominant form, the long transcript (L-form), the *TEL* fragment is fused with the first intron of the *AML*.[4,6,7] In the minor or short form (S-form), the *TEL* gene is fused to the second intron of *AML1*. The presence of the breakpoint in *AML1* intron 4 is very uncommon.[8,9]

The prognosis of P-ALL patients with *TEL-AML1* varies among different series. Whereas some investigators have reported a better clinical outcome for this subtype of leukemia,[2,9,10] others could not confirm such observations.[11] *TEL-AML1* fusion

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.[5] 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[5] 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.

Pts	*Gender/ Age (yrs)*	*Immuno-phenotype*	*Moment*	*Treatment*	*Sample*	*TEL-AML1 form*	*TEL-AML1 copies/μL cDNA*	*AML1 copies/μL cDNA*	*TEL-AML1/ AML1*	*FISH*	*WBC (×10⁹/L)*	*DNA Index*	*Clinical outcome (months)*
1	M/10.9	B-ALL	D	SHOP-94	PB	L	472,708	3,858,600	0.122	ND	26.9	1.12	CR off therapy, (+27.43)
2	M/8.0	B-ALL	R	SHOP-REC-94	BM	L	267,034	2,108,188	0.127	ND	5.1	1.0	Testicular relapse, (+24.13)
3	F/6.0	B-ALL	D	PETHEMA-96	BM	L	109,976	281,076	0.391	ND	26.6	ND	CR on maintenance, (+18.37)
4	F/1.2	B-ALL	D	SHOP-94	PB	L	531,236	695,964	0.763	ND	32	1.67	CR on maintenance, (+8.33)
5	F/3.3	B-ALL	D	SHOP-99	PB	L	2,154,444	7,427,392	0.290	Positive (93/100)	58	1.0	CR on maintenance, (+6.83)
6	M/6.0	B-ALL	D	SHOP-99	BM	L	445,922	3,847,500	0.115	Positive (190/200) +21,del(tel)	52.5	1.0	CR on maintenance, (+5.87)
7	M/6.5	B-ALL	D	SHOP-99	BM	L	38,468	241,664	0.159	Positive (99/100)	11.2	1.0	CR on on treatment, (+1.67)
8	M/2.7	B-ALL	R	ALL-REZ BMF-96	BM	L	480,968	1,319,712	0.364	Positive (90/100)	80	1.0	CR on treatment, (+0.67)
9	F/1.4	B-ALL	D	SHOP-99	PB	L	48,498	301,608	0.161	Positive (80/100)	135	ND	On treatment, (0)
10	F/4.0	B-ALL	D	SHOP-99	BM	L	216,294	1,248,440	0.173	ND	11	ND	On treatment, (0)

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/m^2), high-dose ARAC, and triple intrathecal therapy (TIT) in the three protocols, plus teniposide (only in PETHEMA-96). The maintenance phase was based on daily oral 6-mercaptopurine and weekly oral/intramuscular methotrexate with pulses of vincristine/prednisone (SHOP-94), vincristine/prednisone plus cyclophosphamide or L-asparaginase (SHOP-99), or vincristine/prednisolone/L-asparaginase (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/m^2), HD-ARAC, TIT, and thioguanine; consolidation-2: dexamethasone, vindesine, ifosfamide, mitoxantrone, etoposide, low-dose ARAC, and TIT. After this phase, patients usually undergo stem-cell transplantation but multichemotherapy 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).
- TOPO™ TA Cloning® kit (Invitrogen BV, 970 VT Groningen, The Netherlands, Cat. No. K4600-01).

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 5×10^6) were resuspended in guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, [pH 7], containing 5 g/L sarcosyl and 0.1 M2-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 MoMuLV-reverse transcriptase (Promega, Madison,

Table 2. Primers and probes used.

Primers/probes	*Sequence*	*Gene (Accession number) nt*
Primers		
TEL-AML1		
TEL2 (+)	AACCTCTCTCCATCggGAAGA	TEL (U11732) 937-956
AML1-R3 (-)	AgCggCAACgCCTCgCTCAT	AML1 (D43969) 615-596
X2AML(-)	ggACgTCTCTAgAAggATTCAT	AML1 (D43969) 538-517
AML1		
AML1C (+)	gAgggAAAAgCTTCACTCTg	AML1(D43969) 950-969
AML1E (-)	gCCgCAgCTgCTCCAgTTCA	AML1(D43969) 1141-1122
Probes		
TEL-AML1		
TEL 3FL	TggTCTCTgTCTCCCCgCCTgAAgA X	TEL (U11732) 580-604
TEL 5LC	LC Red640-ACgCCATgCCCATTgggAGAATAgC ph	TEL (U11732) 607-630
AML1		
AML1 3FL	AACCCACCgCAAgTCgCCACCT X	AML1(D43969) 988-1109
AML1 5LC	LC Red640-CCACAgAgCCATCAAAATCACAgTggATgg ph	AML1(D43969) 1011-1040

Abbreviations.- nt= nucelotide number.

WI, USA), 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-AML1* and *AML1* standard curves were prepared using 10-fold serial dilutions of the respective pCR II-TOPO (*TEL-AML1*) and pCR II-TOPO (*AML1*) plasmids, in aqueous heterologous DNA. For *TEL-AML1*, we used seven 10-fold serial dilutions in a range from 45,000,000 to 5 copies, and for *AML1* six 10-fold serial dilutions from 16,756,000 to 168 copies.

Real-time PCR. For *TEL-AML1* transcripts we used the *TEL2* forward primer of Satake *et al.*,[5] which binds the *TEL* gene, in combination with either the *AML1*-R3 reverse primer, which binds exon three of *AML1* (M1 primer-set), or the *X2AML1* primer, which binds AML exon two (M2 primer-set) (Table 2 and Figure 1). The M2 primer-set (*TEL2+X2AML*) is specific to the long transcript (L-form), since amplification of this PCR product requires the presence of exon 2[4,7] 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-AML1 was normalized with *AML1* which was taken as the internal reference gene according to the method of Kozu *et al.*[17] The procedure for *AML1* quantification has already been reported.[18] Briefly, it consists of the amplification of the *AML1* gene using the AML1C(+) and AML1E(–) primers and the probes AML 3FL, fluorescein-labeled at the 3' end, and *AML* 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 Master Hybridiza-

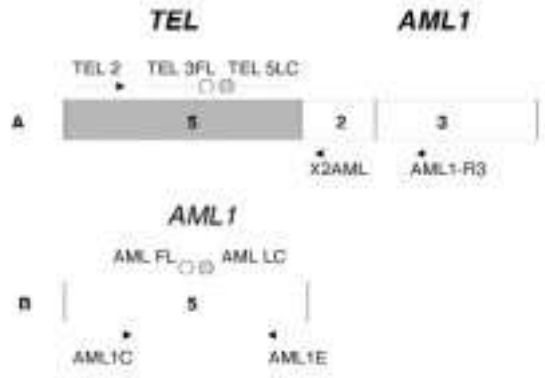

Figure 1. Schematic representation of the placement of primers and hybridizations used in the quantitative detection of (A) *TEL-AML1* fusion transcripts and (B) *AML1* quantification. Circles indicate the localization of the fluorescent labels (FL, fluorescein; and LC Red640). Numbers in boxes indicate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

tion Probes mastermix (buffer, dNTPs in which dTTP is replaced by dUTP, $MgCl_2$, 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 *AML1*E(–)] at a final concentration of 0.5 μM. The fluorescent probes for *TEL-AML1* and *AML* were used at final concentrations of 0.2 μM.

$MgCl_2$ 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

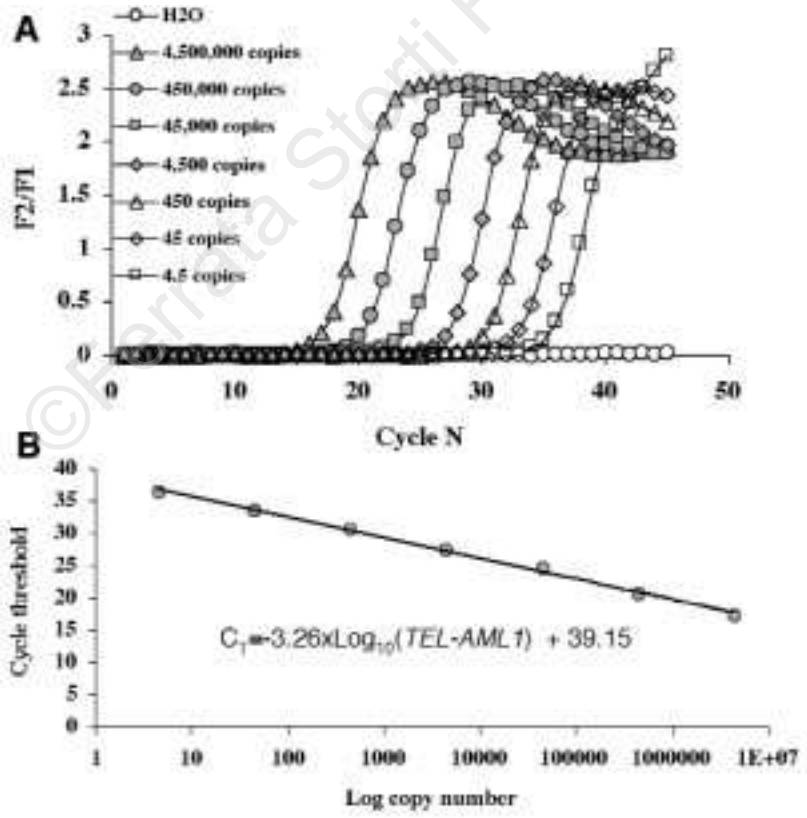

Figure 2. (A) *TEL-AML1* standard curve calculated from 10-fold dilutions of the plasmid pCR II-TOPO (*TEL-AML1*); where F2/F1 are the fluorescence intensity readings measured at channel ratio F2 (640 nm)/F1 (530 nm). (B) Straight line obtained from the cycle threshold (C_T) and concentration at each 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,[18] 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 (C_T) and the logarithm of the initial number of target copies (N) present in the sample,[19,20] 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 C_T, 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 C_T for each point in the five consecutive *TEL-AML1* standards 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).

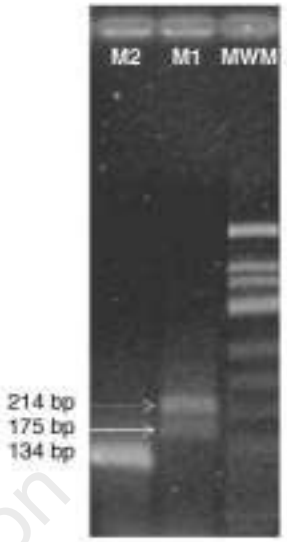

Figure 3. Ethidium bromide 2% agarose gel electrophoresis of the PCR products obtained with the M1 primer-set (TEL2+ AML1-R3) and the M2 primer-set (TEL2+X2AML) and the cDNA from *TEL-AML1*-positive samples. MWM = molecular weight marker (φX174 DNA / *Hinf*I).

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* C_T, 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* C_T, 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*

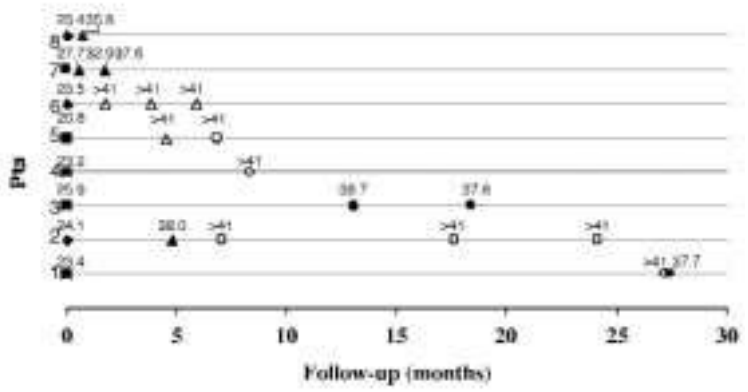

Figure 4. *TEL-AML1* transcript levels over the course of treatment of the patients (Pts) listed in Table 1.
Symbols: ■, at diagnosis; ◆, after relapse; ▲, during or soon after induction treatment; ●, during maintenance. Unfilled symbols (white) mean PCR-negative and filled in symbols (black) PCR-positive. The figures indicate the TEL-AML1 C_T of the corresponding samples.

transcript, we estimated for C_T 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 C_T ±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 C_T ±SD was 34.8±1.3 cycles (CV = 3.8%) and 823±470 copies *TEL-AML1*/μL cDNA *TEL-AML1* (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 C_T 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, 29×10^9 (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, 10×10^9 (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*/*AML* 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-AML1*/*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 (17,625–2,986,850) 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™ 7700[12,13] 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 HybProbes is clearly superior to the use of TaqMan™ in this equipment, since HybProbes 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 C_T 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 C_T and 7.0% for *TEL-AML1* transcripts are also equivalent to those reported by Ballerini *et al.*[12] This precision seem to be sufficient to monitor samples reliably from each patient. However, the between-assay CV of *TEL-AML1* concentration increases considerably in parallel with C_T, from a CV of 13% at C_T of 23 cycles to 50% at C_T 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 C_T limits the quantitative reliability of the method to samples with a C_T value lower than 30 cycles, being semi-quantitative for a C_T between 30 and 35 cycles, qualitative from 35 to 40 cycles and negative for C_T >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 *AML1*, 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 (C_T>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 *AML1* 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 β_2-microglubulin[13] since they have similar expression levels in leukemic and normal cells.

The results reported by Satake *et al.*[5] 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 who 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.

Contribution and Acknowledgments

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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Disclosures

Conflict of interest: none.

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References

1. Mauvieux L, Delabesse E, Macintyre E. Molecular genetics of acute leukemias. Rev Clin Exp Hematol 1997; 2:3-26.
2. Camitta BM, Pullen J, Murphy S. Biology and treatment of acute lymphocytic leukemia in children. Semin Oncol 1997; 24:83-91.
3. Sallan SE, Golub TR, Pui CH, et al. Acute leukemia. Acute lymphoblastic leukemia. American Society of Hematology. 39 th Annual Meeting, San Diego, California, USA. 1997; p. 103-19.
4. Nakao M, Yokota S, Horiike S, Taniwaki M, Kashima K, Sonoda Y, et al. Detection and quantification of TEL/AML1 fusion transcripts by polymerase chain reaction in childhood acute lymphoblastic leukemia. Leukemia 1996; 10: 1463-70.
5. Satake N, Kobayashi H, Tsunematsu Y, Kawasaki H, Horikoshi Y, Koizumi S, et al. Minimal residual disease with TEL-AML1 fusion transcript in childhood acute lymphoblastic leukaemia with t(12;21). Br J Haematol 1997; 97:607-11.
6. van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999; 13:1901-28.
7. Romana SP, Poirel H, Leconiat M, Flexor MA, Mauchauffe M, Jonveaux P, et al. High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia. Blood 1995; 86:4263-9.
8. Borkhardt A, Cazzaniga G, Viehmann S, Valsecchi MG, Ludwig WD, Burci L, et al. Incidence and clinical relevance of TEL/AML1 fusion genes in children with acute lymphoblastic leukemia enrolled in the German and Italian multicenter therapy trials. Associazione Italiana Ematologia Oncologia Pediatrica and the Berlin-Frankfurt-Munster Study Group. Blood 1997; 90:571-7.
9. Shurtleff SA, Buijs A, Behm FG, Rubnitz JE, Raimondi SC, Hancock ML, et al. TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with excellent prognosis. Leukemia 1995; 9:1985-9.
10. McLean TW, Ringold S, Neuberg D, Stegmaier K, Tantravahi R, Ritz J, et al. TEL/AML1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood 1996; 88:4252-8.
11. Harbott J, Viehmann S, Borkhardt A, Henze G, Lampert F. Incidence of TEL/AML1 fusion analyzed consecutively in children with acute lymphoblastic leukemia in relapse. Blood 1997; 90:4933-7.
12. Ballerini P, Landman Parker J, Laurendeau I, Olivi M, Vidaud M, Adam M, et al. Quantitative analysis of TEL/AML1 fusion transcripts by real-time RT-PCR assay in childhood acute lymphoblastic leukemia. Leukemia 2000; 14:1526-8.
13. Pallisgaard N, Clausen N, Schroder H, Hokland P. Rapid and sensitive minimal residual disease detection in acute leukemia by quantitative real-time RT-PCR exemplified by t(12;21) TEL-AML1 fusion transcript. Genes Chromosomes Cancer 1999; 26:355-65.
14. Henze G, Adams HP. Treatment of children with relapsed ALL: studies of the BFM Study Group. The ALL-REZ BFM Study Group. Ann Hematol 1997; Suppl 1:A57.
15. Henze G, Fengler R, Adams HP. Studie zur Behandlung von Kindern mit einer akuten lymphoblastischen Leukämie. ALL-REZ BFM 96. Berlin. Germany, Gesellschaft für Pädiatrische Onkologie und Hämatologie (GPOH), 1997.
16. Chomczynski P, Sacchi N. Single-step method of RNA

isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-9.
17. Kozu T, Miyoshi H, Shimizu K, Maseki N, Kaneko Y, Asou H, et al. Junctions of the AML1/MTG8(ETO) fusion are constant in t(8;21) acute myeloid leukemia detected by reverse transcription polymerase chain reaction. Blood 1993; 82:1270-6.
18. Barragan E, Bolufer P, Moreno I, Martin G, Nomdedeu J, Brunet S, et al. Quantitative detection of AML1-ETO rearrangement by real-time RT-PCR using fluorescently labeled probes. Leuk Lymphoma 2001; 42:747-56.
19. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996; 6:986-94.
20. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (NY) 1993: 11:1026-30.
21. Kreuzer KA, Lass U, Bohn A, Landt O, Schmidt CA. LightCycler technology for the quantitation of bcr/abl fusion transcripts. Cancer Res 1999; 59:3171-4.
22. Mensink E, van de Locht A, Schattenberg A, Linders E, Schaap N, Geurts van Kessel A, et al. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. Br J Haematol 1998; 102:768-74.
23. Bolufer P, Sanz GF, Barragan E, Sanz MA, Cervera J, Lerma E, et al. Rapid quantitative detection of BCR-ABL transcripts in chronic myeloid leukemia patients by real-time reverse transcriptase polymerase chain reaction using fluorescently labeled probes. Haematologica 2000; 85:1248-54.
24. Loh ML, Silverman LB, Young ML, Neuberg D, Golub TR, Sallan SE, et al. Incidence of TEL/AML1 fusion in children with relapsed acute lymphoblastic leukemia. Blood 1998; 92:4792-7.
25. Garcia-Sanz R, Alaejos I, Orfao A, Rasillo A, Chillon MC, Tabernero MD, et al. Low frequency of TEL/AML1 fusion gene in acute lymphoblastic leukaemia in Sapin. Br J Haematol 1999; 107:667-9.

Peer Review Outcomes

What is already known on this topic

TEL-AML1 is the most frequent genetic lesion associated with pediatric ALL and its detection identifies patients with a favorable prognostic outcome.

What this study adds

It defines the applicability of a novel quantitative PCR approach for better detection and quantitative measurement of this lesion.

Manuscript processing

This manuscript was peer reviewed by two external referees and by Dr. Francesco Lo Coco, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Lo Coco and the Editors. Manuscript received April 12, 2001; accepted October 6, 2001.

Potential implications for clinical practice

The technique described may provide more reliable and accurate estimates of a leukemic marker useful for better assessment of response to therapies and minimal residual disease in 20-25% pediatric ALLs.

Francesco Lo Coco, Associate Editor