ORIGINAL ARTICLE

Quantitative monitoring of minimal residual disease in childhood acute lymphoblastic leukemia using *TEL-AML1* fusion transcript as a marker

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ABSTRACT

Importance: By demonstrating with *TEL-AML1*, this study indicated that mRNAs transcribed from fusion genes are ideal targets for minimal residual disease (MRD) monitoring in childhood acute lymphoblastic leukemia, and that different thresholds are needed to apply them into the risk stratification.

Objective: *TEL-AML1* expression was measured at three time points to 1) determine cut-off values for predicting acute lymphoblastic leukemia (ALL) relapse; 2) investigate the prognostic value of this method and how well the results at these time points correlated; 3) determine the correlation between MRD levels assessed using this marker and that determined by immunoglobulin/T-cell receptor (Ig/TCR) rearrangement detection.

Methods: *TEL-AML1* expression in 62 children with ALL was quantitated by real-time quantitative PCR at day 15, day 33, and month 3. The relationship between patient outcome and *TEL-AML1* level was analyzed at each time point. The correlation between the MRD levels determined by *TEL-AML1* or Ig/TCR rearrangements was also analyzed.

Results: For day 33, 6.68 *TEL-AML1* copies/10^4 *ABL* copies was determined to be the best cut-off value. Higher levels were correlated with relapse ($P = 0.001$). For day 15 and month 3, the best cut-off values were 336.5 and 0.85 copies/10^4 *ABL* copies respectively; patients with higher expression levels had lower RFSs (day 15: $P = 0.027$; month 3: $P = 0.023$). For days 15 and 33, MRD levels assessed using *TEL-AML1* or Ig/TCR rearrangements were strongly correlated [Spearman rank correlation coefficient (ρ) = 0.729 (day 15), 0.719 (day 33); $P < 0.001$ (both)], and both methods were equally effective at predicting relapse. At month 3, there was moderate correlation between the results derived from the two markers ($\rho = 0.418$, $P = 0.003$); however, receiver operating characteristic curve analysis showed that *TEL-AML1* was a better prognostic marker.

Interpretation: *TEL-AML1* is an effective marker for MRD assessment and relapse prediction in children with ALL.

KEYWORDS
Childhood leukemia, Fusion transcript, Minimal residual disease

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INTRODUCTION

Monitoring minimal residual disease (MRD) is critical for the risk stratification in patients with childhood acute lymphoblastic leukemia (ALL). Currently, the primary methods used for MRD monitoring include detection of cluster of differentiation (CD) markers expression by flow cytometry and quantitation of fusion transcripts and immunoglobulin/T-cell receptor (Ig/TCR) gene rearrangements by real-time quantitative polymerase chain reaction (RQ-PCR).

In principle, mRNAs transcribed from fusion genes are ideal targets for MRD monitoring. Assessing MRD via these fusion gene mRNAs has many advantages, such as high sensitivity, excellent specificity, and technical simplicity. The RQ-PCR-based fusion transcripts detection method proposed by the Europe Against Cancer program has greatly expedited the clinical adoption of this technique.[1,2]

BCR-ABL1 has been widely reported to be an effective marker for MRD detection and ALL prognostic predicting. However, the usefulness of other fusion genes that are commonly observed in childhood ALL, such as *TEL-AML1*, in predicting outcomes is still unclear. As opposed to PCR detection of Ig/TCR gene rearrangements and flow cytometric identification of CD markers, fusion transcripts' expression is typically expressed as the number of transcripts. However, no previous study has established criteria that relate fusion transcript copy numbers to risk stratification, or has compared the risk level predicted by copy number to that determined by Ig/TCR gene rearrangement detection, which has been the obstacle of engaging fusion transcript copy numbers into the risk stratifying procedure built on DNA or cell based methods. Therefore, the present study investigated the association between *TEL-AML1* expression and ALL prognosis, and studied its relationship with MRD prediction based on Ig/TCR gene rearrangement detection.

METHODS

Patients and samples

Sixty two children (female: 18, male: 44; age: 1–10 years, median: 4 years) diagnosed as ALL and admitted to Beijing Children's Hospital (Beijing, China) from April 2008 to July 2010 were included in the study. All children were confirmed to have the *TEL-AML1* fusion, and were treated following the ALL-2008 protocol issued by the Chinese Children's Leukemia Group.[3-4] Bone marrow samples were collected from each patient at three key time points: day 15 (i.e., during remission induction therapy), day 33 (i.e., at the completion of remission induction therapy) and month 3 (i.e., before consolidation therapy began). All samples were assessed for MRD. Follow-up continued till 31th Aug 2017, median follow-up time was 2970 days (90 to 3938 days). This study was approved by the hospital's Research Ethics Committee.

MRD assessment by fusion transcript detection

Bone marrow mononuclear cells were isolated with Ficoll-Paque (MD Pacific, Tianjin, China; density: 1.077 g/mL) and stored at –70 °C. Total cellular RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Two micrograms of the collected RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega, Madison, WI, USA). The cDNA segment covering the *TEL-AML1* fusion site was amplified by PCR, cloned, and inserted into plasmids (pEASY-T5 Zero Cloning Kit, TransGen Biotech, Beijing, China). The resulting recombinant plasmids were linearized and serially diluted to generate the standard samples. Standard plasmid samples containing the *ABL1* gene (i.e., as an internal reference) were prepared in a similar manner.

Standard plasmid samples containing 10^2–10^6 copies/μL were amplified by RQ-PCR (RQ-PCR; ABI 7000, Applied Biosystems, Foster City, CA, USA)[1,2] to establish a calibration curve. Samples to be analyzed were amplified simultaneously, and the *TEL-AML1* mRNA copy number was calculated from the measured Ct value. During the PCR procedure, three parallel wells were used for the *TEL-AML1* gene quantification and two for the internal reference. The MRD was calculated by determining the ratio between the *TEL-AML1* mRNA and *ABL1* copy numbers (mean values for both), and was expressed as the number of *TEL-AML1* transcripts per 10 000 copies of *ABL1*. The quantitative threshold for *TEL-AML1* detection was determined based on the quality control design protocol from a report published in 2011.[5] Results were defined quantifiable if at least 2/3 wells in RQ-PCR detection had Ct ≤ 40. Positive results under the threshold and negative results were regarded as negative.

MRD detection based on Ig/TCR gene rearrangements

Genomic DNA was extracted (Blood DNA Kit, U-gene Biotechnology, Jixi, Anhui, China) from bone marrow mononuclear cells. DNA containing Ig/TCR gene rearrangements was amplified by multiplex PCR (BIOMED-2 protocol).[6] The PCR products were sequenced (Shanghai Sangon Biological Engineering Technology, Shanghai, China) and aligned using IMGT (http://www.imgt.cines.fr) or IgBLAST (www.ncbi.nlm.nih.gov/igblast/) databases. Ig/TCR gene arrangements were found in 56 patients. Allele-specific oligonucleotide (ASO) primers were designed based on the V-(D)-J junction sequences in these patients, and individual annealing temperatures were determined for the PCR reactions. Standard DNA samples collected at the first clinical visit were serially diluted with DNA from a single healthy subject to 10^{-5}–10^{-1} copies and then amplified by

RQ-PCR, with *N-ras* as the internal reference.[7] Detection was performed following guidelines established by the European Study Group on MRD Detection in ALL (ESG-MRD-ALL)[8] and by our previous study.[9] Detection results were defined as quantifiable, positive but unquantifiable, and negative respectively according to this guideline.[8]

Patients' stratification based on MRD

The details of ALL-2008 protocol had been described previously.[4] The patients were stratified into standard risk (SR), intermediate risk (IR) or high risk (HR) group and treated accordingly. MRD at day 15, day 33, and month 3 detected by RQ-PCR based on Ig/TCR gene rearrangements and/or flow cytometry were used in patients' risk stratification in present research. Patients with MRD $\geq 10^{-2}$ at day 33 or $> 10^{-3}$ at month 3, or other HR factors were classified into HR group, whereas patients with MRD $< 10^{-4}$ at day 33, without other HR factors were stratified into SR group. Other patients were in IR group.

Statistical analyses

The prognostic value of *TEL-AML1* quantitation was analyzed (SPSS 16.0, SPSS, Chicago, IL, USA) using receiver operating characteristic (ROC) curves. Relapse-free survival (RFS) was defined as the interval from complete remission to the first relapse, or to the latest follow-up. Event-free survival (EFS) was defined as the interval from complete remission to the first relapse, secondary malignancy, death from any cause, or the latest follow-up. RFS and EFS were analyzed by the Kaplan-Meier method, and inter-group comparisons were performed by log-rank tests. Spearman correlation analysis was applied to study the relationship among quantification of *TEL-AML1* fusion transcript at the three time points, and with MRD based on Ig/TCR rearrangements, for the whole detection results and only the quantifiable results respectively. Chi-square test was used to analyze difference in patients' distribution in Low- and high-MRD groups at the time points. $P < 0.05$ was considered statistically significant.

RESULTS

Long-term prognostic value of MRD level as quantified by *TEL-AML1* expression on day 33

Among all 62 patients included, relapse-free survival (RFS) and event-free survival (EFS) at 7 years from diagnosis was 88.4% ± 4.1% and 87.0% ± 4.3%, respectively.

As shown in Figure 1, analyses of the ROC curves indicated that the MRD assessment as determined by *TEL-AML1* quantitation on day 33 was closely related to subsequent relapse [Table 1; area under curve (AUC) = 0.794, $P = 0.012$]. The best cut-off value selected based on these findings was 6.68 *TEL-AML1* copies/10^4 *ABL* copies, which yielded a sensitivity of 71.4% and a specificity of 83.3%. Using this cut-off value, the patients were divided into a low-MRD group (≤ 6.68 *TEL-AML1* copies/10^4 *ABL* copies; $n = 47$) and a high-MRD group (> 6.68 copies/10^4 *ABL* copies; $n = 14$). The low-MRD group had a significantly higher RFS (95.7% ± 3.0% vs. 63.5% ± 13.1%, $P = 0.001$) and EFS (93.6% ± 3.6% vs. 63.5% ± 13.1%, $P = 0.004$) than the high-MRD group (Figure 2). These findings suggest that, on day 33, children with > 6.68 *TEL-AML1* copies/10^4 *ABL* copies had a poorer prognosis, thus require more intensive chemotherapy.

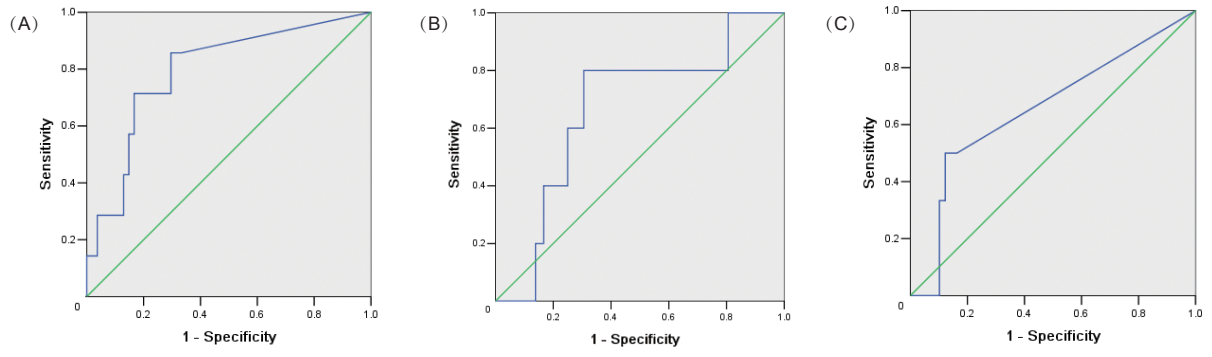

FIGURE 1 Receiver operating characteristic curves on relapse predicting values of *TEL-AML1* detection at day 33 (A), day 15 (B), month 3 (C) of acute lymphoblastic leukemia treatment.

TABLE 1 Prognostic values of MRD assessment using *TEL-AML1* as a marker (relapse-predicting values determined from ROC curves)

Time points	Number of patients	AUC	*P*	Best cut-off value (copies/10^4 *ABL* copies)	Sensitivity (%)	Specificity (%)
d15	41	0.667	0.232	336.50	80.0	69.4
d33	61	0.794	0.012	6.68	71.4	83.3
m3	55	0.655	0.219	0.85	50.0	87.8

MRD, minimal residual disease; ROC, receiver operating characteristic; AUC, area under curve.

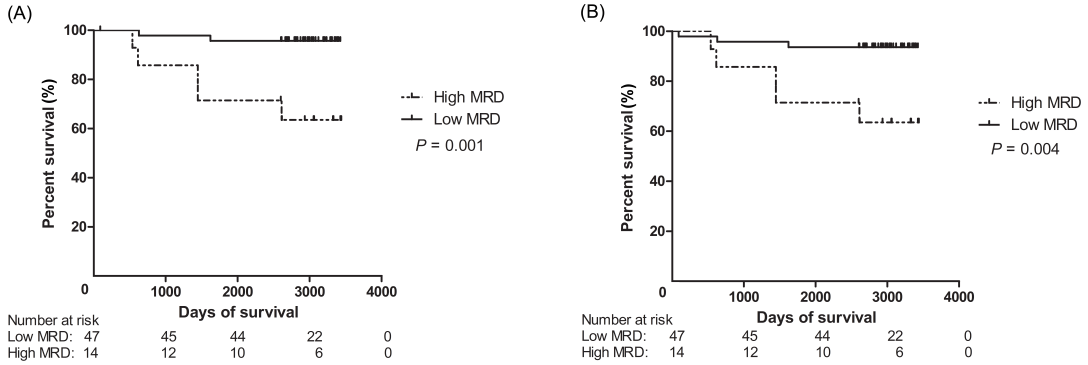

FIGURE 2 Relationship between outcome and minimal residual disease (MRD) assessment at day 33 using *TEL-AML1* expression as a marker. (A) Relapse-free survival (RFS). (B) Event-free survival (EFS). High MRD: >6.68 *TEL-AML1* copies/10^4 *ABL* copies; Low MRD: ≤ 6.68 *TEL-AML1* copies/10^4 *ABL* copies.

TABLE 2 Correlation between minimal residual disease (MRD) assessments at different time points using *TEL-AML1* or Ig/TCR gene rearrangement as a marker

(2A) Correlation analysis on all results

Time point	Number of patients	Spearman ρ	*P*
d15	34	0.729	<0.001
d33	56	0.719	<0.001
m3	47	0.418	0.003

(2B) Correlation analysis on quantifiable results

Time point	Number of patients	Spearman ρ	*P*
d15	30	0.720	<0.001
d33	25	0.314	0.126
m3	10	0.026	0.943

Relationship between relapse and MRD levels as quantified by *TEL-AML1* expression on day 15 and at month 3

Similarly, based on ROC curves (Figure 1), 336.5 *TEL-AML1* copies/10^4 *ABL* copies and 0.85 *TEL-AML1* copies/10^4 *ABL* copies were selected as the predictive cut-off values for day 15 and month 3, respectively (Table 1). For each time point, the patients were also divided into a high-MRD group and a low-MRD group.

On day 15 (Figure 3), compared with the low-MRD group (≤ 336.5 *TEL-AML1* copies/10^4 *ABL* copies; n = 26), the high-MRD group (> 336.5 *TEL-AML1* copies/10^4 *ABL* copies; n = 15) had a significantly lower RFS (71.1% ± 12.4% vs. 96.0% ± 3.9%, P = 0.027), although no statistically significant effect on EFS (71.1% ± 12.4% vs. 92.3% ± 5.2%, P = 0.090). At month 3 (Figure 4), the high-MRD group (> 0.85 *TEL-AML1* copies/10^4 *ABL* copies; n = 8) had significantly lower RFS and EFS rates (both: 66.7% ± 15.7% vs. 93.5% ± 3.6%,

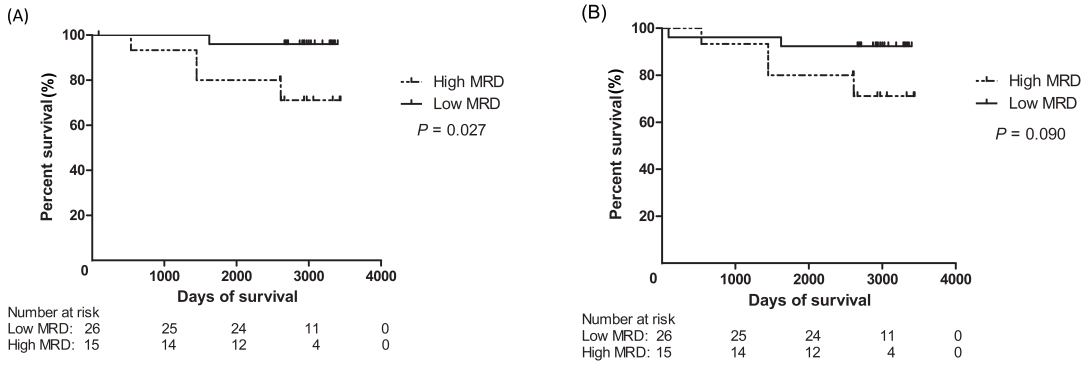

FIGURE 3 Relationship between outcome and minimal residual disease (MRD) assessment at day 15 using *TEL-AML1* expression as a marker. (A) Relapse-freesurvival (RFS). (B) Event-free survival (EFS). High MRD: > 336.5 *TEL-AML1* copies/10^4 *ABL* copies; Low MRD: ≤ 336.5 copies/10^4 *ABL* copies.

P = 0.023) than the low-MRD group (≤ 0.85 *TEL-AML1* copies/10^4 *ABL* copies; n = 46).

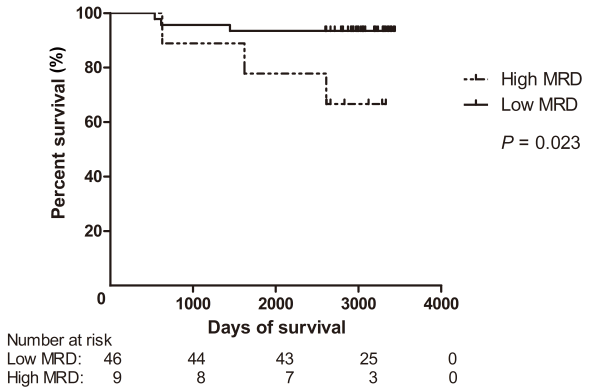

FIGURE 4 Relationship between outcome and minimal residual disease (MRD) assessment at 3 months into chemotherapy using *TEL-AML1* as a marker. High MRD: > 0.85 copies/10^4 *ABL*; Low MRD: ≤ 0.85 copies/10^4 *ABL* copies.

Correlation between MRD levels as quantified by *TEL-AML1* expression at three time points

Patients were divided into low-MRD and high-MRD groups at each time point and further analyzed by χ^2 tests. The patient distribution across the two groups on day 15 was significantly correlated with the distribution on day 33 (P < 0.001). MRD levels at all three time points showed skewed distributions (all P < 0.001). Therefore, there was significant correlation between MRD at day 15 and day 33 (Spearman correlation test, ρ = 0.617, P < 0.001). However, no correlation was found between MRD at day 15 or day 33 and MRD at month 3 (both P > 0.05).

As quantifiable level of *TEL-AML1* fusion transcript at day 15, day 33, month 3 were obtained in 34 out of 41, 24 out of 61, and 11 out of 54 patients respectively, we further analyzed the correlation of MRD levels among these patients. The MRD levels at day 15 and day 33 were significantly correlated (n = 18, ρ = 0.562, P = 0.015. No statistically significant correlation was found between the MRD levels at day 15 or day 33 and MRD at month 3 (both P > 0.05).

Correlation between MRD levels quantified by *TEL-AML1* expression and by Ig/TCR gene rearrangements

Of the 62 patients studied, 34 were analyzed for both Ig/TCR rearrangement and *TEL-AML1* expression on day 15, and 56 were analyzed on day 33, and 47 were analyzed at month 3. At any one of the three time points, significant correlations were found between the results of the two tests (Table 2A, Figure 5).

Excluding both negative results in the two tests and positive but unquantifiable results, MRD levels in 30, 25, and 10 patients at the three time points were analyzed for correlation respectively. Correlation was found significantly at day 15, however, MRD levels were not associated between the two tests at day 33 and month 3 (Table 2B), indicating difference in determination of low level of MRD by the two methods.

To further understand the clinical implications of these correlations and their difference, the prognostic value of Ig/TCR detection was also analyzed using ROC curves. For day 15 and month 3, the prognostic values of Ig/TCR detection were lower than the corresponding values provided by *TEL-AML1* expression quantification (Table 3). For day 33, the two markers offered nearly identical prognostic values. Notably, the MRD level could not be assessed by Ig/TCR marker analysis for 5 (8.1%) of the 62 patients because of the lack of detectable rearrangements, but they were satisfactorily assessed via *TEL-AML1*, suggesting that the latter marker may offer better clinical potential for this patient population.

TABLE 3 Prognostic values of MRD assessment using Ig/TCR gene rearrangement as a marker (relapse-predicting values determined from ROC curves)

Time point	Number of patients	AUC	*P*
d15	45	0.600	0.470
d33	57	0.793	0.013
m3	50	0.483	0.893

MRD, minimal residual disease; ROC, receiver operating characteristic; AUC, area under curve.

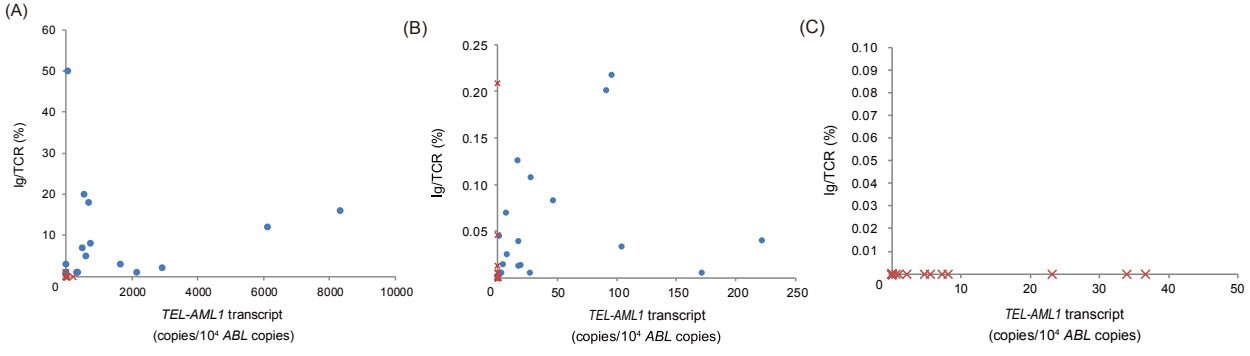

FIGURE 5 Dot plots showing correlations between detection of *TEL-AML1* and Ig/TCR rearrangements. (A) Results at day 15 (n = 34). (B) Results at day 33 (n = 56). (C) Results at month 3 (n = 47). X axis showed *TEL-AML1* results (copies/10^4 *ABL* copies), Y axis showed Ig/TCR results (%). Quantifiable results were represented with dots; unquantifiable results and negative results were shown with crosses.

DISCUSSION

In childhood ALL, *TEL-AML1* fusion gene is related to favorable outcome. Studies on *TEL-AML1* positive groups in different treatment protocols have shown EFS around 90%, at 5 to 10 years during follow-up.[10,11] In the present study (n = 62), EFS at 7 years from diagnosis was 87.0% ± 4.3%, which was close to the results of Enshaei et al (81%).[12]

MRD levels assessed at three time points during management of childhood ALL are closely related to relapse and have important prognostic and treatment guidance value. These levels are assessed in the middle of remission induction therapy (day 15), at completion of remission induction therapy (day 33), and before consolidation therapy begins (3 months). However, fusion transcript quantification has two major disadvantages compared with MRD monitoring based on Ig/TCR rearrangement or CD marker expression. First, it does not directly quantitate the proportion of leukemic cells to nucleated cells. Moreover, quantitative criteria guiding risk stratification are lacking. These disadvantages have limited the use of fusion transcripts as prognostic markers for future relapse.[13,14] To address this problem, we selected *TEL-AML1*, the most commonly observed fusion transcript in childhood ALL, as a marker for MRD, and used this marker to assess MRD level at the three critical time points outlined above. Our objective was to investigate the prognostic value of this marker in ALL management. Our results showed that, at day 33, patients with > 6.68 *TEL-AML1* copies/10^4 *ABL* copies had significantly lower RFS and EFS rates compared with patients with ≤ 6.68 *TEL-AML1* copies/10^4 *ABL* copies (Figure 2), indicating that *TEL-AML1* mRNA copy number was an effective predictor of relapse for this time point. Although > 336.5 *TEL-AML1* copies/10^4 *ABL* copies and > 0.85 *TEL-AML1* copies/10^4 *ABL* copies were associated with decreased RFS and EFS at day 15 and month 3, respectively (Figures 3, 4), MRD levels at these two time points were not effective predictors of subsequent relapse (Table 1), most likely due to the relatively small patient group evaluated in this study. Therefore, additional studies including more subjects are required to further determine the prognostic value of *TEL-AML1* expression, particularly for day 15 and month 3. Moreover, prospective studies should be performed to establish accurate stratification standards that enable effective classification and management of children with ALL who carry the *TEL-AML1* fusion.

Currently, RQ-PCR detection of Ig/TCR rearrangements and flow cytometry detection of abnormal CD markers expression are the primary methods used to assess MRD. Alm et al[15] detected MRD with flow cytometry and RT-qPCR of *TEL-AML1* fusion transcript. They found strong correlation between the results of the two methods, and showed a concordant results in patients' stratification at day 29 and day 78. Similar to Alm and his colleagues' results, the current study found that Ig/TCR detection and *TEL-AML1* expression quantitation assessments were significantly correlated for the three time points, although were only relatively weakly correlated for month 3 (Table 2A). It was noteworthy that significant correlation was observed only at day 15, but not at day 33 and month 3, when only quantifiable results were considered (Table 2B). This emphasized the distinctiveness of *TEL-AML1* expression quantization, and supported the necessity of an evaluation system of fusion transcripts detection in risk stratification of ALL. ROC curve analysis showed that, for days 15 and 33, the two markers had similar prognostic values. Considering that 5 patients (8.1%) failed to show detectable Ig/TCR rearrangements but all had *TEL-AML1* transcripts, fusion transcript quantification appears to be a simple and viable technique with better efficiency for ALL that could be an effective diagnostic tool for guiding the management of children with *TEL-AML1*-positive ALL.

Interestingly, the MRD levels measured by *TEL-AML1* on days 15 and 33 were significantly correlated, whether they were analyzed as numerical or categorical variables. In contrast, no correlation was found between the MRD level measured at month 3 and that measured on day 15 or 33. Moreover, at month 3, the prognostic value of *TEL-AML1* expression was significantly higher than Ig/TCR gene rearrangement detection (AUC: 0.655 vs. 0.483), primarily because three of the patients who experienced relapse were Ig/TCR rearrangement-negative but *TEL-AML1*-positive (0.98–5.49 *TEL-AML1* copies/10^4 *ABL* copies). This indicates that, when assessing MRD for *TEL-AML1*-positive patients at month 3, fusion transcript detection is likely a more effective marker than Ig/TCR gene rearrangements. Other studies[16,17] have reported that there are various genetic events secondary to *TEL-AML1*, which can lead to late relapse in up to 20% of *TEL-AML1* positive ALL, although this cohort responded well in treatment. This explained the positivity of RQ-PCR results at month 3 of treatment, and supported the importance of fusion transcript detection in *TEL-AML1* positive cohort.

In summary, analyzing fusion transcript expression by RQ-PCR specifically quantitates the MRD level in children with ALL. This technique overcomes a potential limitation of Ig/TCR rearrangement detection (i.e., lack of markers) while offering improved sensitivity at multiple time points (e.g., month 3 in chemotherapy). Combined use of the two markers may allow better MRD assessment and risk stratification for children with *TEL-AML1*-positive ALL, enabling more accurate diagnosis and management of these patients.

CONFLICT OF INTEREST

There is no conflicts of interest.

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