European Review for Medical and Pharmacological Sciences 2015; 19: 2679-2688

Comparison of minimal residual disease (MRD) monitoring by WT1 quantification between childhood acute myeloid leukemia and acute lymphoblastic leukemia

R. ZHANG[1], J.Y. YANG[2], H.Q. SUN[3], H. JIA[1], J. LIAO[1], Y.J. SHI[3], G. LI[1]

[1]Department of Pediatrics Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu, Sichuan Province, China
[2]Sichuan Provincial Key laboratory for Human Disease Gene Study, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu, Sichuan Province, China
[3]Laboratory of Transplant Engineering and Immunology, West China Hospital, Sichuan University, Chengdu, Sichuan Province, China

Abstract. – OBJECTIVE: Wilms tumor gene 1 (WT1) has been identified as an independent risk prognostic factor in acute leukemia. However, there exists a controversy that WT1 as a marker for minimal residual disease (MRD) monitoring in acute leukemias. We detected WT1-RNA transcript level to estimate the diagnostic value of monitoring MRD in childhood acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).

PATIENTS AND METHODS: WT1 mRNA expression levels were detected by real-time quantitative reverse transcriptase PCR (qRT-PCR) in bone marrow (BM) samples from 107 childhood ALL and 35 childhood AML at diagnosis. MRD was consecutively performed after induction and consolidation (early intensification in ALL) chemotherapy. Receiver operating characteristics (ROC) analysis and the largest areas under the curve (AUC) were applied to define optimal threshold value of MRD level. Sensitivity, specificity, positive likelihood ratio (+LR) and negative likelihood ratio (-LR) were used to evaluate diagnostic power for MRD. Relapse free survival (RFS) was evaluated by the Kaplan-Meier statistical method.

RESULTS: The largest areas under the curve (AUC), specificity, +LR and –LR showed higher accuracy in childhood AML than ALL. Compared the diagnostic parameters, the post-induction time wasn't good enough to show the better time than post-consolidation time for MRD assessment in AML. The threshold was set at 150 WT1 copies/10^4 ABL copies as the optimal cut-off value of MRD level post induction in childhood AML. MRD+ (WT1>150) children had increased the risk of relapse with poor prognosis, showing lower RFS than MRD- group (p=0.01). However, the threshold 70 WT1 copies/10^4 ABL copies post induction in childhood ALL did not show clinical significance for predicting prognosis (p=0.056).

CONCLUSIONS: WT1 gene will be a useful marker for monitoring MRD to predict relapse in childhood AML. But it did not show good enough to monitor MRD in childhood ALL.

Key Words:
Wilms' tumor gene 1 (WT1), Minimal residual disease (MRD), Childhood AML, Childhood ALL, Real-time quantitative PCR (RQ-PCR).

Introduction

Despite high remission rate after chemotherapy, relapse always presents eventually in some children with leukemia. So, it is necessary to look for markers that could predict relapse. Monitoring MRD is of clinical value in the assessment of response to chemotherapy, predicting relapse, and guiding therapeutic intervention[1-6]. Some studies have shown that leukemia-specific targets such as fusion gene and genetic lesions proved to be useful markers in MRD monitoring[7-10]. However, these markers are limited to about 40-60% patients with a normal karyotype or normal cytogenetics lacking suitable specific fusion genes and molecular mutation for MRD monitoring[11-14]. Thus, there are pressing needs to develop alternative markers to detect MRD for a greater proportion of patients.

WT1 was originally found in Wilms tumor as tumor suppressor gene. WT1 gene is located on chromosome 11p13 and encodes a zinc-finger transcription factor as transcriptional activator or

Corresponding Author: R. Zhang, MD; e-mail: 2738988234@qq.com

repressor, which regulates cells growth, differentiation and apoptosis in different malignant diseases[15-17]. Studies in the recent ten years have shown that 70-80% of leukemia patients overexpress the WT1 gene, and it is regarded as universal leukemia marker[18-21]. Although the pathogenesis of WT1 in leukemia has not been completely revealed, the phenomenon that low expression of WT1 and high expression of WT1 are accompanied with clinical remission and relapse respectively shows that WT1 may be a potential prognostic factor, MRD marker and therapeutic target in acute leukemia.

However, WT1 as a molecular marker for MRD and prognostic factor remains controversial. Some researchers regard the WT1 transcript level as a reliable marker for MRD monitoring in acute leukemia[22-28], others argued that the WT1 transcript level was not suitable for MRD monitoring[29-32].

We detected WT1-RNA transcript by RQ-PCR after induction and consolidation (early intensification in ALL) chemotherapy and used ROC curve, the area under the curve and relapse-free survival (RFS) to better predict prognosis and response to chemotherapy.

Patients and Methods

One hundred and forty-two patients (107 ALL and 35 AML) with *de novo* acute leukemia children were randomly recruited for the research on detecting WT1 transcript expression for MRD monitoring at the department of Pediatric Hematology of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital in China from June 2005 to June 2014. Patients were analyzed at two-time points for MRD monitoring: post-induction therapy and post-consolidation therapy in AML and post-induction therapy and post-early intensification therapy in ALL. Major clinical characteristics of the patients are shown in Table I. The diagnosis and classification were based on morphologic, immunophenotypic, cytogenetic, molecular biologic criteria according to the revised French-American-British classification. The study was approved by the Ethical Committee, and informed consent was obtained from all patients' parents.

Treatment Regimens

The chemotherapy regimen was used according to 04 Protocol (suggested by the Pediatric Hematology Group of Chinese Medical Association in 2004)[33,34] and other acute lymphoblastic leukemia patients according to CCLG (the Chinese Children's Leukemia Group) ALL 08 Protocol[35].

Methods

Bone marrow (BM) was collected in sterile tubes with EDTA anticoagulant. BM mononucleated cells were separated by a Ficoll-Hypaque densing gradient centrifugation. The total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction within 24 hours after their collection. And the concentration of the extracted RNA was evaluated by spectrophotometry. The cDNAs were synthesized using one μg of RNA,

Table I. Children in AML and ALL characteristics.

Feature	ALL	AML
No. patients	107	35
Median age at diagnosis (range)	5.3 (0.3-13.7)	5.9 (0.9-14.2)
Male	46	18
Female	61	17
Molecular biologic group		
PML-RARa		5
AML1-ETO		11
Others		3
Normal		16
TEL-AML1	33	
E2A-PBX1	10	
BCR-ABL	7	
MLL-AF4	8	
Others	9	
Normal	40	
FAB classification		
L1	85	
L2	17	
L3	5	
M1		2
M2		21
M3		5
M4		3
M5		2
M6		1
M7		1
Blast lineage		
B cell	96	
T-cell	11	
Risk group		
Favorable	51	10
Intermediate	36	11
Poor	20	14
Induction	107	35
Consolidation/early intensify	102	31
Relapse	28	19

according to the recommendations from PrimeScript™ RT reagent kit (Takara, Otsu, Shiga, Japan). Then, the cDNAs were stored at -80°C for later processing.

Real time PCR was performed using 40 ng of cDNA. Both WT1 and ABL TaqMan primer/probe sets in Premix Ex Taq™ (Probe qPCR) kit (Takara). ABL was used to normalize all reactions. The following oligonucleotide primers and Taqman probe were used[36-37]: WT1 forward primer, 5'-ACAGGGTACGAGAGCGATAACCA-3' (exon 6, nt position: 1205-1227, NM_024426), WT1 reverse primer: 5'-CACACGTCGCACATCCTGAAT-3' (exon 6/7, nt position: 1289-1309, nM_024426). WT1 fluorescent probe, 5'-6-FAM-CAACGCCCATCCTCTGCG GAGCCCA-TAMRA-3' (exon 6, nt position: 1230–1254, NM_024426). ABL forward primer, 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3' (nt position: 228–258, NM_005157); ABL reverse primer: 5'-GATGTAGTTGCTTGGGACCCA-3' (nt position: 331-351, NM_005157). ABL fluorescent probe, 5'-6-FAM -CCATTTTTG-GTTTGGGCTTCACACCAT-TAMRA-3' (nt position: 297-323, NM_005157).

Real-time PCR was performed with ABI PRISM 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following thermocycling conditions: 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30s.

The WT1 copy number and WT1 normalized copy number were calculated according to the standard curve method. Standard curves were created using plasmid DNA calibrators – ABL and WT1 plasmid calibrators prepared in the lab (see below).

The total RNA contents were extracted from the K562 cell line, and cDNAs were reverse transcripted. WT1 were amplified by PCR kit. After purification, PCR production was connected with pMD 18-T Vector and transfected into *Escherichia coli* DH-5a. Then, we cultured the positive clones, and the plasmids were extracted and quantified using spectrophotometry.

The WT1 plasmid construct was serially diluted ranging from 10^3 to 10^7 copies/μl for the standard curve. ABL plasmid dilutions were prepared in the same way. With WT1 and ABL plasmid calibrators, the standard curve generated a slope of -3.310 and -3.346, as the correlation coefficient was ≥ 0.99.The results of WT1 expression are depicted as some WT1 copies/10^4 copies of the housekeeping gen e ABL.

Statistical Analysis

Results were analyzed by using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p<0.05$. Comparison between two groups of WT1-RNA level was conducted using the nonparametric Mann-Whitney U test. Survival analyzes were performed by Kaplan-Meier survival curves and the log-rank test for comparing the MRD positive and MRD negative groups. The ROC curve and the AUC were used for comparing the diagnostic value of MRD in two types of leukemia and the two-time points. Sensitivity and specificity were also calculated for diagnostic power. The diagnostic threshold was defined as cut off the value of the largest area under the curve.

Complete remission (CR) was defined as morphologically normal marrow with <5% blasts, the absence of extra medullary leukemia and recovery of hematologic parameters. Partial remission (PR) was characterized by the appearance of at least 5% and less of 20% BM blast cells. Relapse was considered when finding more than 5% blasts in the bone marrow or reappearance of the blasts in the peripheral blood or development of extra medullary leukemia. Relapse-free survival (RFS) was measured from the date of CR until the date of relapse or death or last follow-up date available.

Results

Thirty-five children with AML and 107 children with ALL were followed up for MRD monitoring after induction. Four children with AML and five with ALL gave up consolidation therapy by themselves, so there were 19 children with AML and 102 children with ALL left for consolidation therapy. Patients' characteristics were shown in Table I. Nineteen children with AML and 28 children with ALL relapse at the end of follow-up time. The median follow-up time was 23 months (range 2-84) and 28 months (range 2-96) for AML and ALL children respectively.

MRD monitoring and Parameter Analysis Compared Between AML and ALL Children

The median level of WT1-RNA in RQ-PCR at diagnosis was 2091.2 WT1 copies/10^4 ABL copies (range 25.2 to 35421.3), 652.3 (range 136.3 to 8947.4) after induction and 183.6 (range 2.3 to 4388.1) post-consolidation in childhood

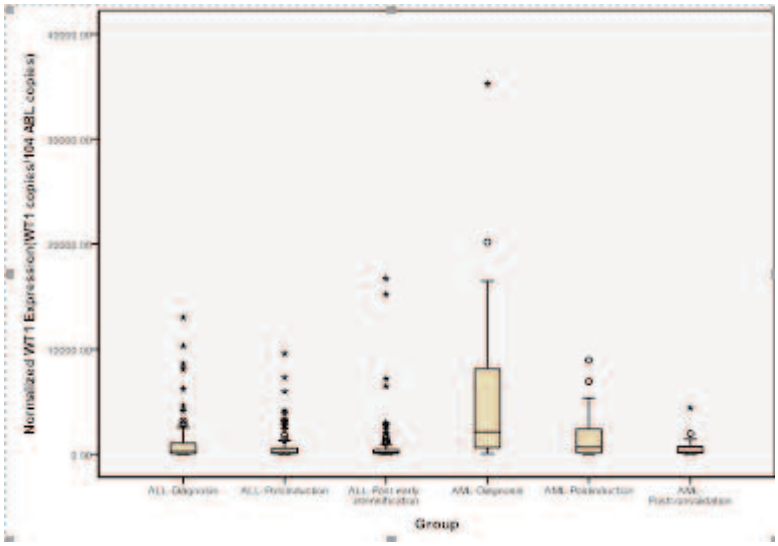

Figure 1. Comparision of WT1-RNA transcript expression between ALL and AML at diagnostic, post induction and post-consolidation time point. The median value corresponds to the black bold line. The box refers to the 25th and 75th percentile range. Differences between different time point in childhood ALL were not significant ($p>0.05$), but significant in childhood AML ($p<0.05$).

AML. The level of WT1-RNA after induction and consolidation showed lower level than that of diagnosis significantly ($p<0.05$) (Figure 1). The ALL patients showed relatively lower level than AML with 213.1 WT1 copies/10^4 ABL copies (range 1.1 to 12992.8) at diagnosis, 189.2 (range 1.33 to 9563.8) after induction and 135.0 (range 0.9 to 16692.0) post-early intensification. Although the level of WT1-RNA at CR time showed lower level than that of diagnosis, this did not reach significance from a statistical stand point ($p>0.05$) (Figure 1).

ROC Curves Analyzes for MRD Monitoring Compared Between AML and ALL Children

ROC curve can predict good reliability and validity of relapse in patients with the largest AUC. ROC curves analysis also determined the optimal threshold (cutoff point) separating patients into two groups with MRD positive and MRD negative. The AUCs were compared in order to find out which is the best diagnosis of the different type leukemia and time points. The largest AUC was 0.860 for post induction with cutoff value 150.0 WT1 copies/10^4 ABL copies (Figure 2A) and AUC 0.807 for post-consolidation (Figure 2B) with cutoff value 100.0 WT1 copies/10^4 ABL copies in AML patients, but the different between these two parameters was not significant ($p>0.05$). Similarly, the largest AUC was 0.676 for post induction with cutoff value 70.0WT1 copies/10^4 ABL copies (Figure 3A) and 0.659 for post-early intensification (Figure 3B) with cutoff value 50.0WT1 copies/ 10^4 ABL copies in ALL patients, but the different between these two parameters was not significant ($p>0.05$). The threshold value indicated 95% confidence intervals 0.738-0.983 and 0.656-0.958, optimal sensitivity 86.4% and 68.4%, specificity 76.9% and 83.3%, +LR 3.74 and 4.10, -LR 0.18 and 0.38 for post induction and post-consolidation in AML higher than 95% confidence intervals 0.564-0.789 and 0.547-0.771, optimal sensitivity 89.7% and 92.9% specificity 35.9% and 36.5%, +LR 1.40 and 1.46, -LR 0.29 and 0.20 post induction and post-early intensification in ALL respectively.

The AUC of post induction were higher than the post-consolidation but showed no significant in AML ($p>0.05$). Compared the AUC, specificity, +LR and –LR, the diagnostic value of WT1 in MRD monitoring in childhood AML was more efficient than ALL.

Relapse Free Survival Between AML and ALL Children

We used the MRD level of 150.0 WT1 copies/10^4 ABL copies as a threshold to discriminate MRD^- group from MRD^+ group at the post-

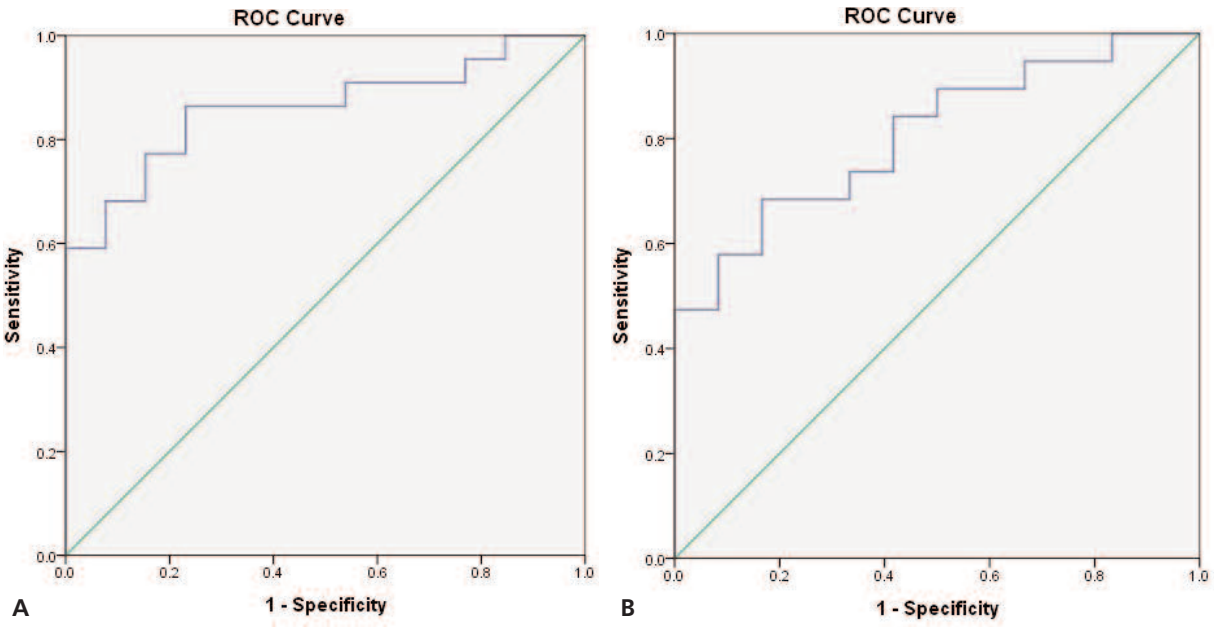

Figure 2. ***A,*** A ROC curve of WT1 mRNA monitoring MRD in AML children at the post induction time point with AUC 0.860 (95% confidence intervals 0.738 to 0.983). ***B,*** A ROC curve of WT1 mRNA monitoring MRD in AML children at the post consolidation time point with AUC 0.807 (95% confidence intervals 0.656 to 0.958).

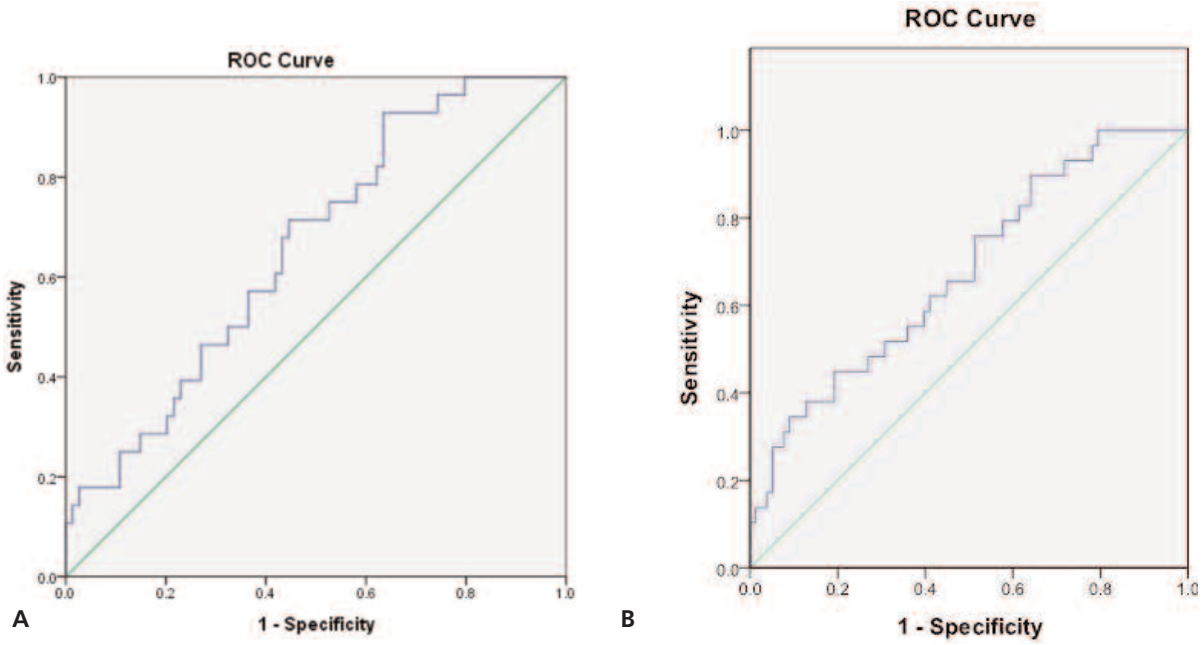

Figure 3. ***A,*** A ROC curve of WT1 mRNA monitoring MRD in ALL children at the post induction time point with AUC 0.676 (95% confidence intervals 0.564 to 0.789). ***B,*** A ROC curve of WT1 mRNA monitoring MRD in ALL children at the post-early intensification timepoint with AUC 0.659 (95% confidence intervals 0.547 to 0.771).

induction time point in childhood AML. 63% of the children were MRD^+, and 37% patients were MRD^-. 72.73 % children in the MRD^+ group were relapse and had disease relapse at a median time of 19 months (range 6 to 41). Whereas 23.08% children in the MRD^- group relapse and

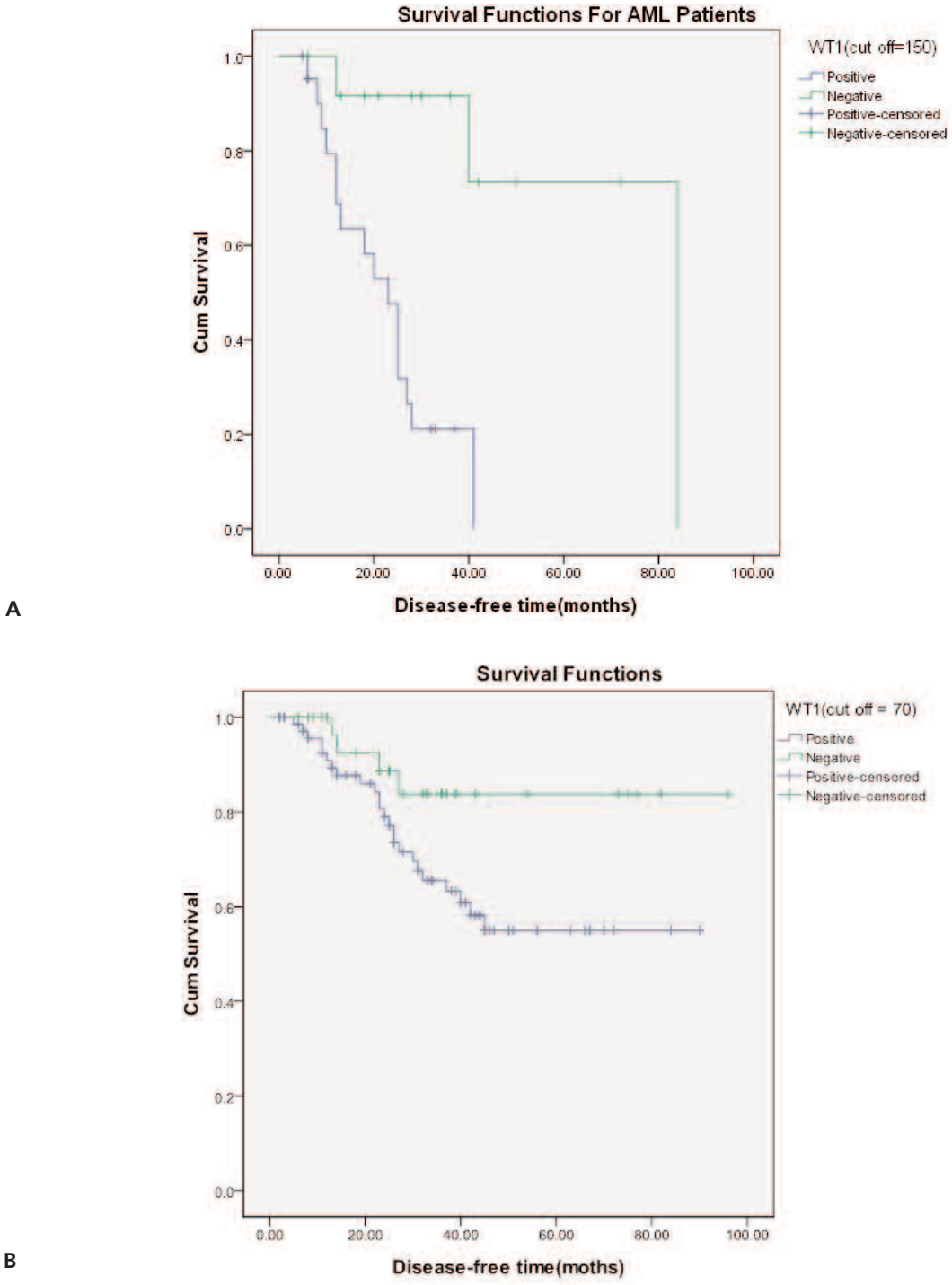

Figure 4. ***A,*** RFS of AML children according to MRD levels (cut off as 150 WT1 copies/10^4 ABL copies) after induction chemotherapy, who have positive MRD vs. negative MRD (p = 0.01). ***B***, RFS of ALL children according to MRD levels (cut off as 70 WT1 copies/10^4 ABL copies) after induction chemotherapy, who have positive MRD vs. negative MRD (p = 0.056).

had, disease relapse at a median time of 40 months (range 12 to 84). Relapse free survival of the two groups showed different significance (p=0.01) (Figure 4A).

Patients with WT1 >70.0 copies were categorized as MRD^+ group, whereas those with WT1 ≤70.0 copies were grouped as MRD^- post-induction chemotherapy in childhood ALL. A to-

tal of 27.1% of the children were MRD^+, and 72.9% patients were MRD^-. 82.8 % children in the MRD^+ group were relapse and had disease relapse at a median time of 23.5 months (range 5 to 45). Whereas 5.1% children in the MRD^- group relapse and had disease relapse at a median time of 18.5 months (range 13 to 27). Relapse free survival of the two groups showed different significance (p=0.056) (Figure 4B).

Discussion

MRD monitoring based on the specific marker can help us predict relapse of leukemia and determine the best-individualized treatment. Also for the antigen receptor rearrangements for immunoglobulins (Ig) or the T-cell receptors (TCR), genetic lesions, and fusion genes, WT1 is another potential target for MRD. The present methods suitable for MRD detection are the polymerase chain reaction and the flow cytometry. Real-time quantitative PCR (RQ-PCR) has been introduced over the last 10 years and this technology has striking features of high sensitivity with reaching 10^{-6}, which has enabled the detection of leukemic cells far beyond the threshold of cytomorphology. Numerous studies from various laboratories have demonstrated the reliability and potential clinical value for MRD study of RQ-PCR[38-39].

The WT1 mRNA level is regarded as a useful MRD parameter for predicting relapse of AML by most researches[22-27,40,41]. However, a few researchers claim that WT1 transcript level has not been testified as a good enough MRD marker in AML[30,31]. Similarly, the parameter is also inconsistent in MRD monitoring in childhood ALL[27-29,32].

We are using a real-time quantitative RT-PCR method for detecting WT1mRNA level to elucidate whether WT1 is a valuable marker for MRD monitoring in childhood AML and ALL. Our current data indicate that the WT1 mRNA levels at diagnosis in childhood ALL were lower than AML and showed a bit discrete. Some study group also found the median copy number of WT1 in ALL was lower than in AML[25,28]. Compared diagnosis, WT1mRNA level tended to be lower after induction and post-early intensification. But it was not high enough to reach statistical significance in childhood ALL. However, it showed that WT1 expression levels have significant discrimination between the diagnostic and remission time points in childhood AML. This proved that WT1 might be as tumor promoting factors in childhood acute myeloid leukemia and likely to be a good marker for AML than ALL.

We applied ROC curve to MRD investigation at two-time points including post induction, post-consolidation (post-early intensification in childhood ALL). ROC curve is a comprehensive index of sensitivity and specificity of continuous variables. The greater AUC is, the higher the accuracy of diagnosis gets. On the ROC curve, the coordinate of the upper left corner of the curve is close to the point of critical values with highest sensitivity and specificity. The largest AUC corresponding determined the threshold in our study to the cutoff value. Compared AUC and 95% confidence intervals between AML and ALL, WT1mRNA level was demonstrated more useful for MRD monitoring in childhood AML with larger AUC and better confidence level than ALL. Rossi et al[40] proved that WT1 was useful for MRD monitoring in childhood AML by ROC curve analysis using cutoff value 90 WT1 copies/10^4 ABL copies. This result is similar to ours.

Our study also showed that larger AUC, higher sensitivity post induction than post-consolidation in childhood AML, but with similar confidence intervals and smaller specificity at post induction. So differences concerning these parameters of the two-time point group varied no significantly. Therefore, it could not say that the post-induction time is the time point better than post-consolidation in our study. This result did not get in common with that of Rossi et al[40]. Maybe, it is related to the selected children patients different with the adult, besides the small number of patients involved in the study.

As there is no consistent threshold and no uniform optimal time point of WT1 assessment to guide clinical decisions at present. The threshold of WT1 for MRD were various, such as the cutoff value of WT1-RNA transcripts for MRD defined as 250 copies/10^4 ABL copies in BM and 50 WT1 copies/10^4 ABL copies in PB in AML[41], 90 WT1 copies/10^4 ABL copies as MRD cut off value in AML[40], and different time with different cut-off value such as bone marrow WT1 levels 170.5 copies post induction and 10 copies post-intensification[42]. This may be related to primers and probes designed and conditions used in the PCR reaction, and maybe to the different human groups, especially different ways decision to the cutoff value.

The cut-off values of our study was regarded as threshold resulted from the largest AUC of

ROC analysis. It divided patients into MRD positive and negative groups. Our data showed that patients with MRD positive at post induction time point had a worse RFS than negative group (p=0.01) in childhood AML. We could predict who will most likely relapse and take personalized treatment to the patients by MRD monitoring with detecting WT1 transcript expression in childhood AML. This is consistent with early other researches[40,41,43].

However, it did not reach statistical significance in ALL (p=0.056). So the cut off was not valid to evaluate prognosis in childhood ALL. Some studies also indicated that WT1 may be limited value for monitoring MRD in childhood ALL[29,32,44]. It may be relevant to part patients with WT1 expression too lower to MRD monitoring or no stratification for patients or small number of patients. We will expand the sample size and make risk stratification or other stratification research in the future. This further illustrated that WT1 mRNA level was more useful for MRD monitoring in childhood AML than ALL.

Conclusions

We have shown that WT1 mRNA quantification displayed a reliable and sensitive marker for MRD monitoring to identify high-risk patients with the residual disease most likely to relapse in childhood AML. But, on the other hand, WT1 mRNA was showed an unsatisfied marker for MRD monitoring in childhood ALL.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Sievers EL, Lange BJ, Alonzo TA, Gerbing RB, Bernstein ID, Smith FO, Arceci RJ, Woods WG, Loken MR. Immunophenotypic evidence of leukemia after induction therapy predicts relapse: results from a prospective children's cancer group study of 252 patients with acute myeloid leukemia. Blood 2003; 101: 3398-3406.

2) Bader P, Kreyenberg H, Henze GHR, Eckert C, Reising M, Willasch A, Barth A, Borkhardt A, Peters C, Handgretinger R, Sykora KW, Holter W, Kabisch H, Klingebiel T, von Stackelberg A. Prognostic value of minimal residual disease quantification before allogeneic stem-cell transplantation in relapsed childhood acute lymphoblastic leukemia: The ALL-REZ BFM Study Group. J Clin Oncol 2009; 27: 377-384.

3) Coustan-Smith E, Sancho J, Hancock ML, Boyett JM, Behm FG, Raimondi SC, Sandlund JT, Rivera GK, Rubnitz JE, Ribeiro RC, Pui CH, Campana D. Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. Blood 2000; 96: 2691-2696.

4) Logan AC, Vashi N, Faham M, Carlton V, Kong K, Buno I, Zheng J, Moorhead M, Klinger M, Zhang B, Waqar A, Zehnder JL, Miklos DB. Immunoglobulin and T cell receptor gene high-throughput sequencing quantifies minimal residual disease in acute lymphoblastic leukemia and predicts post-transplantation relapse and survival. Biol Blood Marrow Transplant 2014; 20: 1307-1313.

5) Sawada A, Sakata N, Kishimoto T, Higuchi B, Koyama M, Kondo O, Sato E, Okamura T, Yasui M, Inoue M, Yoshioka A, Kawa K. Significance of four MRD markers in MRD-based treatment strategy for childhood acute lymphoblastic leukemia. Leukemia Research 2009; 33: 1710-1713.

6) Rubnitz JE, Inaba H, Dahl G, Ribeiro RC, Bowman WP, Taub J, Pounds S, Razzouk BI, Lacayo NJ, Cao X, Meshinchi S, Degar B, Airewele G, Raimondi SC, Onciu M, Coustan-Smith E, Downing JR, Leung W, Pui CH, Campana D. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. Lancet Oncol 2010; 11: 543-552.

7) Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, Van Dongen, JJM. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia--a Europe against cancer program. Leukemia 2003; 17: 2318-2357.

8) Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, Loffler H, Sauerland CM, Serve H, Buchner T, Haferlach T, Hiddemann W. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood 2002; 100: 59-66.

9) Ross DM, Watkins DB, Hughes TP, Branford S. Reverse transcription with random pentadecamer primers improves the detection limit of a quantitative PCR assay for BCR-ABL transcripts in chronic myeloid leukemia: implications for defining sensitivity in minimal residual disease. Clin Chem 2008; 54: 1568-1571.

10) Zhang L, Cao Z, Ruan M, Zeng Q, Zhao L, Li Q, Zou Y, Wang J, Zhu X. Monitoring the AML1/ETO fusion transcript to predict outcome in childhood acute myeloid leukemia. Pediatr Blood Cancer 2014; 61: 1761-1766.

11) Themeli M, Petrikkos L, Waterhouse M, Bertz H, Lagadinou E, Zoumbos N, Finke J, Spyridonidis A. Standardized MRD quantification in European ALL trials: Proceedings of the Second Internation-

al Symposium on MRD assessment in Kiel, Germany, 18-20 September 2008. Leukemia 2010; 24: 536-543.

12) Mrozek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood 2007; 109: 431-448.

13) Willasch AM, Gruhn B, Coliva T, Kalinova M, Schneider G, Kreyenberg H, Steinbach D, Weber G, Hollink IH, Zwaan CM, Biondi A, van der Velden VH, Reinhardt D, Cazzaniga G, Bader P, Trka J. Standardization of WT1 MRNA quantitation for minimal residual disease monitoring in childhood AML and implications of WT1 gene mutations: a European Multicenter Study. Leukemia 2009; 23:1472-1479.

14) Lapillonne H, Renneville A, Auvrignon A, Flamant C, Blaise A, Perot C, Lai JL, Ballerini P, Mazingue F, Fasola S, Dehee A, Bellman F, Adam M, Labopin M, Douay L, Leverger G, Preudhomme C, Landman-Parker J. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. J Clin Oncol 2006; 24: 1507-1515.

15) Toska E, Roberts SG. Mechanisms of transcriptional regulation by WT1 (Wilms' tumour 1). Biochem J 2014; 461: 15-32.

16) Morrison AA, Viney RL, Ladomery MR. The post-transcriptional roles of WT1, a multifunctional zinc-finger protein. Biochim Biophys Acta 2008; 1785: 55-62.

17) Sugiyama H, Wilms' tumor gene WT1: its oncogenic function and clinical application. Int J Hematol 2001; 73:177-187.

18) Ostergaard M, Olesen LH, Hasle H, Kjeldsen E, Hokland P. WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients--results from a single-center study. Br J Haematol 2004; 125: 590-600.

19) Boublikova L, Kalinova M, Ryan J, Quinn F, O'Marcaigh A, Smith O, Browne P, Stary J, McCann SR, Trka J and Lawler M. Wilms' tumor gene 1 (WT1) expression in childhood acute lymphoblastic leukemia: a wide range of WT1 expression levels, its impact on prognosis and minimal residual disease monitoring leukemia. Leukemia 200; 20: 254-263.

20) Menssen HD, Siehl JM, Thiel E. Wilms tumor gene (WT1) expression as a panleukemic marker. Int J Hematol 2002; 76:103-109.

21) Cilloni D, Saglio G. WT1 as a universal marker for minimal residual disease detection and quantification in myeloid leukemias and in myelodysplastic syndrome. Acta Haematol 2004; 112: 79-84.

22) Kletzel M, Olzewski M, Huang W, Chou PM. Utility of WT1 as a reliable tool for the detection of minimal residual disease in children with leukemia. Pediatr Dev Pathol 2002; 5: 269-275.

23) Lapillonne H, Renneville A, Auvrignon A, Flamant C, Blaise A, Perot C, Lai JL, Ballerini P, Mazingue F, Fasola S, Dehée A, Bellman F, Adam M, Labopin M, Douay L, Leverger G, Preudhomme C, Landman-Parker J. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. J Clin Oncol 2006; 24: 1507-1515.

24) Weisser M, Kern W, Rauhut S, Schoch C, Hiddemann W, Haferlach T, Schnittger S. Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. Leukemia 2005; 19: 1416-1423.

25) Cilloni D, Gottardi E, De Micheli D, Serra A, Volpe G, Messa F, Rege-Cambrin G, Guerrasio A, Divona M, Lo Coco F, Saglio G. Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. Leukemia 2002; 16: 2115-2121.

26) Candoni A, Toffoletti E, Gallina R, Simeone E, Chiozzotto M, Volpetti S, Fanin R. Monitoring of minimal residual disease by quantitative WT1 gene expression following reduced intensity conditioning allogeneic stem cell transplantation in acute myeloid leukemia. Clin Transplant 2011; 25: 308-316.

27) Garg M, Moore H, Tobal K, Liu Yin JA. Prognostic significance of quantitative analysis of wt1 gene transcripts by competitive reverse transcription polymerase chain reaction in acute leukaemia. Br J Haematol 2003; 123: 49-59

28) Hu SY, Gu WY, Chen ZX, Wang XL, Cen JN, He HL, Chai YH, Chen CS. The significance of detecting WT1 expression in childhood acute leukemias. Pediatr Hematol Oncol 2010; 27: 581-591.

29) Boublikova L, Kalinova M, Ryan J, Quinn F, O'Marcaigh A, Smith O, Browne P, Stary J, McCann SR, Trka J, Lawler M. Wilms' tumor gene 1 (WT1) expression in childhood acute lymphoblastic leukemia: a wide range of WT1 expression levels, its impact on prognosis and minimal residual disease monitoring. Leukemia 2006; 20: 254-263.

30) Gaiger A, Schmid D, Heinze G, Linnerth B, Greinix H, Halhs P, Tisljar K, Priglinger S, Laczika K, Mitterbauer M, Novak M, Mitterbauer G, Mannhalter C, Haas OA, Lechner K, Jäger U. Detection of the WT1 transcript by RT-PCR in complete remission has no prognostic relevance in de novo acute myeloid leukemia. Leukemia 1998; 12: 1886-1894.

31) Elmaagacli AH, Beelen DW, Trenschel R, Schaefer UW. The detection of WT1 transcripts is not associated with an increased leukemic relapse rate in patients with acute leukemia after allogeneic bone marrow or peripheral blood stem cell transplantation. Bone Marrow Transplant 2000; 25: 91-96.

32) Chen JS, Hsiao CC, Sheen JM, Cheng CN. Comparison of minimal residual disease (mrd) estimated by flow cytometry and by real-time quantitative PCR of Wilms tumor gene 1 (WT1) transcript expression in children with acute lymphoblastic leukemia. Leukemia Research 2007; 31: 1351-1357.

33) Hematologic group of Pediatrics branch, Chinese Medical Association, Editorial Committee of Chinese Journal of Pediatrics. Recommendations for diagnosis and treatment in childhood acute myeloblastic leukemia. Chin J Pediatr 2006; 44: 877-878.

34) Hematologic group of Pediatrics branch, Chinese Medical Association, Editorial Committee of Chinese Journal of Pediatrics. Recommendations for diagnosis and treatment in childhood acute lymphoblastic leukemia (The third amendment). Chin J Pediatr 2006; 44: 392-395.

35) Hematologic group of Pediatrics branch, Chinese Medical Association, Editorial Committee of Chinese Journal of Pediatrics. Recommendations for diagnosis and treatment in childhood acute lymphoblastic leukemia (The third amendment). Chin J Pediatr 2014; 52: 641-644.

36) Kreuzer KA, Saborowski A, Lupberger J, Appelt C, Na IK, le Coutre P, Schmidt CA. Fluorescent 5'-exonuclease assay for the absolute quantification of Wilms' tumour gene (WT1) MRNA: implications for monitoring human leukaemias. Br J Haematol 2001; 114: 313-318.

37) Cilloni D, Gottardi E, Messa F, Fava M, Scaravaglio P, Bertini M, Girotto M, Marinone C, Ferrero D, Gallamini A, Levis A, Saglio G; Piedmont Study Group on Myleodysplastic Syndromes. Significant correlation between the degree of WT1 expression and the International Prognostic Scoring System Score in patients with myelodysplastic syndromes. J Clin Oncol 2003; 21: 1988-1995.

38) Thörn I, Forestier E, Botling J, Thuresson B, Wasslavik C, Björklund E, Li A, Lindström-Eriksson E, Malec M, Grönlund E, Torikka K, Heldrup J, Abrahamsson J, Behrendtz M, Söderhäll S, Jacobsson S, Olofsson T, Porwit A, Lönnerholm G, Rosenquist R, Sundström C. Minimal residual disease assessment in childhood acute lymphoblastic leukaemia: a Swedish multi-centre study comparing real-time polymerase chain reaction and multicolour flow cytometry. Br J Haematol 2011; 152: 743-753.

39) Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, van Dongen JJ, Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – A Europe Against Cancer Program. Leukemia 2003; 17: 2318-2357.

40) Rossi G, Minervini MM, Carella AM, de Waure C, di Nardo F, Melillo L, D'Arena G, Zini G, Cascavilla N, Comparison between multiparameter flow cytometry and WT1-RNA quantification in monitoring minimal residual disease in acute myeloid leukemia without specific molecular targets. Leukemia Research 2012; 36: 401-406.

41) Cilloni D, Renneville A, Hermitte F, Hills RK, Daly S, Jovanovic JV, Gottardi E, Fava M, Schnittger S, Weiss T, Izzo B, Nomdedeu J, van der Heijden A, van der Reijden BA, Jansen JH, van der Velden VH, Ommen H, Preudhomme C, Saglio G, Grimwade D. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized wt1 assay to enhance risk stratification in acute myeloid leukemia: a European Leukemia Net Study. J Clin Oncol 2009; 27: 5195-5201.

42) Nomdedéu JF, Hoyos M, Carricondo M, Bussaglia E, Estivill C, Esteve J, Tormo M, Duarte R, Salamero O, de Llano MP, García A, Bargay J, Heras I, Martí-Tutusaus JM, Llorente A, Ribera JM, Gallardo D, Aventin A, Brunet S, Sierra J; CETLAM Group. Bone marrow WT1 levels at diagnosis, post-induction and post-intensification in adult de novo AML. Leukemia 2013; 27: 2157-2164.

43) Lapillonne H, Renneville A, Auvrignon A, Flamant C, Blaise A, Perot C, Lai JL, Ballerini P, Mazingue F, Fasola S, Dehee A, Bellman F, Adam M, Labopin M, Douay L, Leverger G, Preudhomme C, Landman-Parker J. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. J Clin Oncol 2006; 24: 1507-1515.

44) Sawada A, Sakata N, Kishimoto T, Higuchi B, Koyama M, Kondo O, Sato E, Okamura T, Yasui M, Inoue M, Yoshioka A, Kawa K. Significance of four mrd markers in MRD-based treatment strategy for childhood acute lymphoblastic leukemia. Leukemia Research 2009; 33: 1710-1713.