Quantification of *AML1-ETO* fusion transcript as a prognostic indicator in acute myeloid leukemia

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Background and Objectives. In spite of the high complete remission rate that chemotherapy achieves in acute myeloid leukemia with *AML1-ETO* gene rearrangement, relapse is a major cause of treatment failure in this condition. We aimed to determine a predictor of relapse with the real-time quantitative reverse transcription polymerase chain reaction (RQ-PCR) of *AML1-ETO* chimeric mRNA.

Design and Methods. We serially monitored *AML1-ETO* fusion transcripts using RQ-PCR in 113 bone marrow or peripheral blood samples from 21 patients with *AML1-ETO*-positive acute meyloid leukemia and analyzed the prognostic relevance of the results.

Results. Higher transcript levels at diagnosis were associated with a higher probability of relapse (p=0.038 in all patients and p=0.001 in adult patients). A decrease of less than 3-log at the time of achieving complete remission was also associated with a higher risk of relapse (p=0.035 in all patients and p=0.011 in adult patients). RQ-PCR detected the reappearance of *AML1-ETO* fusion transcripts in both peripheral blood and bone marrow during apparent complete remission. Detection of the transcripts preceded hematologic relapse by one to three months. The transcript levels in peripheral blood correlated with those in bone marrow at the same time point.

Interpretation and Conclusions. Our findings indicate that regular monitoring of *AML1-ETO* chimeric transcript levels by RQ-PCR on bone marrow or peripheral blood samples could be extremely useful for the selection of high-risk patients and be an early predictor of relapse.

Key words: AML1-ETO gene rearrangement, acute myeloid leukemia, quantitative real-time RT-PCR, minimal residual disease.

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The translocation t(8;21)(q22;q22) resulting in the *AML1-ETO* (*RUNX1-MTG8*) fusion gene is the most common chromosomal abnormality in acute myeloid leukemia, being present in 5-12% of patients.[1] The *AML1-ETO* fusion gene is generally considered a good prognostic feature.[2,3] However, relapse occurs in about 35% of patients, especially in the first two years of remission.[3] Furthermore, the relapse rate in older patients is up to 84%.[4] There are limitations to the identification of risk factors for relapse using morphology, immunophenotyping, and cytogenetics.[5-9] Polymerase chain reaction (PCR) permits specific and sensitive detection of t(8;21)-positive cells through the amplification of the *AML1-ETO* fusion transcripts. Given its high sensitivity, this assay is a powerful tool for the detection of subclinical minimal residual disease; however, its clinical usefulness is hampered by difficulties in quantification and persistently positive results during remission.[10-13]

The development of real-time quantitative reverse transcription PCR (RQ-PCR) has recently made identification and quantification of small amounts of residual leukemic cells possible. Several groups have used RQ-PCR to detect *AML1-ETO* transcripts; however, the available reports evaluating the quantification of *AML1-ETO* fusion transcripts for prognostic purposes are weakened by limited numbers of cases or samples.[14-21] Recently, a few studies with large numbers of patients presented a critical prognostic score.[22-24] However, they evaluated other groups of good prognosis acute myeloid leukemia altogether and could not show the longitudinal monitoring of minimal residual disease during the clinical course in each patient.[22,23] Moreover, the indices are different in each of the studies and need to be confirmed.[24]

We collected diagnostic and follow-up samples from 21 patients with the *AML1-ETO* fusion gene who were treated in one institution. We tracked the minimal resid-

Table 1. Demographics, hematologic features and clinical findings of AML patients with *AML1-ETO* gene rearrangement.

Pt. No.	*Age (years)*	*Sex*	*Diagnosis (FAB criteria)*	*WBC ($10^3/\mu L$)*	*Bone marrow blasts (%)*	*Karyotype*	*Duration of first CR*	*Last follow-up (months)*
1	19	M	M1	6,100	89.6	45,X,Y,t(8;21)(q22;q22)[10]/46,XY[2]	12.8	Alive (15) in 1st CR
2	53	F	M2	6,900	64.2	46,XX,t(2;21;8)(q37;q22;q22)[17]/46,XX[3]	26.7	Alive (28) in 1st CR
3	44	M	M2	5,100	46.0	45,X,-Y,t(8;21)(q22;q22)[15]/47,XY,+8, t(8;21)(q22;q22)[2]/46,XY[3]	27.9	Alive (29) in 1st CR
4	47	M	M2	6,100	59.4	45,X,-Y,t(8;21)(q22;q22)[19]/45,X,-Y[1]	16.7	Alive (18) in 1st CR
5	44	F	M2	7,400	25.0	45,X,-X,t(8;21)(q22;q22)[20]	16.2	Alive (17) in 1st CR
6	56	F	M2	22,700	75.8	46,XX,t(8;21)(q22;q22)[20]	13.7	Dead (33) in 3rd relapse
7	47	F	M2	198,700	20.8	46,XX,t(8;21)(q22;q22)[6]/45,X,-X,t(8;21)(q22;q22)[14]	16.6	Dead (19) in 1st relapse
8	66	M	M2	5,600	35.6	46,XY,t(8;21)(q22;q22),del(9)(q22q34)[17]/46,XY[3]	18.9	Alive (23) in 2nd CR
9	45	F	M2 with mastocytosis	12,900	57.2	46,XX,t(8;21)(q22;q22),add(11)(p11.2)[20]	14.8	Alive (17) in 2nd CR
10	44	F	M2	7,600	62.4	46,XX,t(8;21)(q22;q22)[14]/45,idem,-X[4]/46,XX[2]	8.6	Alive (20) in 2nd CR
11	18	M	M2	16,800	46.8	45,X,-Y,t(8;21)(q22;q22)[20]	4.9	Alive (26) in 2nd CR
12	42	M	M2	29,200	90.0	45,X,-Y,t(8;21)(q22;q22)[17]/46,XY[3]	15.6	Alive (37) in 4th CR
13	61	M	M2	6,700	20.0	45,X,-Y,t(8;10;21)(q22;q22;q22)[23]/46,XY[2]	8.6	Dead (15) in 2nd relapse
14	30	F	M1 with mastocytosis	1,700	81.0	46,XX,t(8;21)(q22;q22)[20]/46,XX[5]	9.0	Dead (25) in 2nd relapse
15	7	M	M2 with dysplasia	10,300	21.6	45,X,-Y,t(8;21)(q22;q22)[16]/46,XY[4]	22.9	Alive (24) in 1st CR
16	9	F	M2	2,100	62.8	45,X,-X,t(3;6)(p13;p25),t(8;21)(q22;q22)[19]/46,XX[1]	19.2	Alive (20) in 1st CR
17	2	M	M2 with dysplasia	12,000	37.6	46,XY,t(8;21)(q22;q22)[1]/45,idem,-Y[8]/470, idem,+4[4]/46,XY[7]	21.2	Alive (22) in 1st CR
18	7	M	M2	16,000	46.8	45,X,-Y,add(7)(q32),t(8;21)(q22;q22)[13]/46,XY[7]	12.0	Alive (20) in 2nd CR
19	10	F	M4	17,400	61.0	46,XX,t(8;21)(q22;q22)[4]/47,idem,+4[13]/46,XX[3]	7.7	Alive (20) in 2nd CR
20	7	M	M2	16,300	52.8	45,X,-Y,t(8;21)(q22;q22)[15]/46,XY[5]	19.4	Dead (22) in 1st relapse
21	12	F	M2	49,000	70.8	46,XX,t(8;21)(q22;q22)[6]/46,XX[14]	0.0	Alive (16) with persistent disease
Median	42		M1, 2; M2, 18; M4, 1	11,150	58.3		14.8	

CR: complete remission.

ual disease levels at different time points and correlated the results with the hematologic and clinical findings, focusing particularly on the relationships between the transcript levels during hematologic complete remission and the risk of relapse. We also evaluated the usefulness of peripheral blood samples in monitoring minimal residual disease.

Design and Methods

Patients and samples

From January 2001 to March 2004, 276 acute myeloid leukemia patients were diagnosed and treated in a Korean institution, the Asan Medical Center and College of Medicine, University of Ulsan. Thirty-eight patients (13.8%) had the *AML1-ETO* gene rearrangement, as determined by conventional karyotyping with unstimulated short-term culture and qualitative nested RT-PCR. A total of 21 patients (11 males and 10 females) whose bone marrow or peripheral blood samples were collected at diagnosis and at two or more time points during the follow-up periods were included. The main characteristics of these patients are shown in Table 1. Their median age at diagnosis was 42 years (range, 2 to 66 years). There were 14 adult patients (patients 1 to 14, 18 to 66 years) and 7 pediatric patients under 16 years old (patients 15-21, 2 to 12

years). A total of 113 samples (100 bone marrow and 13 peripheral blood) were analyzed by RQ-PCR. The median number of samples analyzed per patient was 4 (range, 3 to 9). t(8;21)-positive Kasumi-1 and t(8;21)-negative HL-60 cell lines were used in this study as positive and negative controls.

Treatment

Induction chemotherapy for adult patients was cytarabine 200 mg/m^2 for 7 days plus daunorubicin 45 mg/m^2/day for 3 days (the AD regimen). In the case of persistent diasease, the patients were given a second cycle of induction chemotherapy. In the case of complete remission, the patient received consolidation chemotherapy consisting of four cycles of high-dose cytarabine 3,000mg/m^2/day on days 1, 3, and 5 and two cycles of the AD regimen. Allogeneic bone marrow transplantation was considered for adult patients who relapsed and was actually performed in three of them. Induction therapy in the pediatric patients was cytarabine 100 mg/m^2 for 7 days plus idarubicin 12 mg/m^2/day for 3 days whereas consolidation therapy included cytarabine 1,000 mg/m^2/12 hours for 4 days plus idarubicin 12 mg/m^2/day for 3 days. For pediatric patients, allogeneic bone marrow transplantation was considered in first remission state and was actually performed in four patients who had HLA-matched donors.

RNA extraction and qualitative nested RT-PCR

Bone marrow mononuclear cells were separated by Ficoll-Hypaque density centrifugation. RNA was extracted using the guanidinium-thiocyanate, phenol-chloroform procedure of Chomczynski and Sacchi and stored at -70°C.[25] The reverse transcription reaction was performed with a commercial kit (1st Strand cDNA Synthesis Kit for RT-PCR kit, Roche Diagnostics, Mannheim, Germany) using random hexamers and AMV reverse transcriptase according to the manufacturer's protocol. The reaction mixture was incubated at 25°C for 10 minutes and then 42°C for 60 minutes and finally heated at 99°C for 5 minutes.

The qualitative nested RT-PCR reaction for *AML1-ETO* fusion gene was carried out using a previously described method with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the control gene.[26]

RQ-PCR for AML1-ETO

Each *AML1-ETO* fusion transcript level was normalized against the expression of *GAPDH*. Real-time PCR using the LightCycler™ (Roche Diagnostics, Mannheim, Germany) was performed to quantify the fusion gene. The primers, probes, and standard cDNA for *AML1-ETO* and *GAPDH* were designed and synthesized by Nihon Gene Research Lab's Inc. (Sendai, Japan). For *AML1-ETO*, the forward primer was positioned at *AML1* exon 4, whereas the reverse primer and the hybridization probes were positioned at exon 3 of the *ETO* gene. The amplicon size was 183 bp for *AML1-ETO* and 307 bp for *GAPDH*. The fluorogenic 3' hybridization probe labeled with fluorescein and the 5' probe labeled with LC Red640 were designed to hybridize to target sequences in a head-to-tail configuration. Each RQ-PCR was carried out according to the manufacturer's protocol using FastStart LightCycler DNA Master Hybridization Probes (Roche Molecular Biochemicals, IN, USA). The absolute copy numbers of *AML-ETO* and *GAPDH* transcripts in the samples were calculated using fluorescence curves obtained from serially diluted standard cDNA. The results were expressed as the *AML1-ETO/GAPDH* ratio. Samples with fewer than 2,000 copies of *GAPDH* (corresponding to a C_T value of 26.1-27.0) were not evaluated.

Clinical end-points and statistical analysis

The follow-up of the patients was updated on June 20, 2005. The median follow-up was 20.3 months (range, 14.7-37.1 months). Hematologic relapse was defined as more than 5% blasts in a bone marrow sample. Kaplan-Meier analyses and log-rank tests were used for the survival analysis. Overall survival was calculated from the first day of chemotherapy to death. Event-free survival was calculated from the day of achieving complete remission until hematologic or extramedullary relapse. Pearson's correlation test was used to assess the correlations between *AML1-ETO/GAPDH* ratios in bone marrow and peripheral blood samples taken at the same time point. All descriptive analyses were performed using the SPSS 11.5 software package (SPSS, Chicago, IL, USA). Results with a p value of less than 0.05 were considered statistically significant.

Results

Evaluation of nested RT-PCR and RQ-PCR

The regression coefficients obtained for *AML1-ETO* and *GAPDH* standard curves were all greater than 0.99. The median slopes were –3.44 (range -3.21 to -3.67) for *GAPDH* and -3.45 (range -3.25 to -3.58) for *AML1-ETO*. The sensitivity, tested using 10-fold serial dilutions of the Kasumi-1 cells in the HL-60 cells, was 10-fold higher for RQ-PCR (10^{-4}) than for conventional RT-PCR (10^{-3}). The inter-assay coefficient of variation for the absolute copy numbers of each *GAPDH* was 0.5% (40883.3±196.0 copies), 3.3% (3943.3±131.6), and 4.3% (41.2±1.8) for high, intermediate, and low copy numbers, respectively. The corresponding inter-assay coefficient of variation for the *AML1-ETO* copy numbers were 10.5% (42583.3±4459.6 copies), 9.1% (3806.3±347.4), and 11.3% (42.9±4.8). Non-specific amplification of *AML1-ETO* and *GAPDH* was not observed in

the reaction without cDNA. Also, there was no amplification of the *AML1-ETO* fusion gene in the reaction of HL-60 cells. Analysis of *AML-ETO* transcripts in contemporaneously obtained bone marrow and peripheral blood samples (n=6) showed a good correlation (R=0.917, p=0.01, Pearson's correlation).

AML1-ETO mRNA levels at presentation

Figure 1 shows the hematologic findings and RQ-PCR results at the variable time points and the patients' clinical outcomes. At diagnosis, the ratios of *AML1-ETO/GAPDH* copies ranged between 2.74×10^{-3} and 9.79×10^{0} with a median of 9.38×10^{-2}.

To analyze the prognostic impact of the *AML1-ETO* expression at diagnosis, the patients were divided into two groups according to the median initial transcript ratio. The overall survival was not different between the two groups (p=0.575). Moreover, the two groups showed no differences in parameters known to be poor prognostic factors, such as age, peripheral white cell count, the percentage of blasts in peripheral blood and bone marrow, CD56 expression, and additional karyotypic abnormalities (*data not shown*). The groups with low expression levels at diagnosis (less than the 50th percentile) had a better event-free survival (p=0.038) (Figure 2-A). Among the adult patients, five (patients 10-14) had high levels of transcripts initially and all of them relapsed later (duration of complete remission: median, 8.6 months; range, 4.6 to 15.6 months), whereas nine (patients #1-9) had low levels of transcripts initially and only four of them (patients #6-9, 44.4%) relapsed (duration of complete remission: median, 18.9 months; range, 12.8 to 27.9 months). Kaplan-Meier analyses indicated the significant difference in event-free survival between the two groups (p=0.001) (Figure 2B). Among the pediatric patients, only one (patient #20) had a low level of transcripts initially and this patient relapsed after bone marrow transplantation. The difference in event-free survival was not significant in pediatric patients. At hematologic relapse, 13 bone marrow specimens and five simultaneous peripheral blood specimens were analyzed. The median of *AML1-ETO/GAPDH* was similar to that at diagnosis (3.84×10^{-3}); however, the range below the median was wider (range, 3.00×10^{-6} to 2.14×10^{-2}). There were two cases of chloroma without hematologic relapse after bone marrow transplantation. RQ-PCR detected the transcripts from bone marrow with a ratio of 6.70×10^{-4} in one case (patient #14), and the transcripts from peripheral blood with a ratio of 7.90×10^{-4} in the other case (patient #17).

Reduction of AML1-ETO mRNA after chemotherapy

Seventy-one follow-up samples (64 bone marrow and 7 peripheral blood) were analyzed after chemotherapy or bone marrow transplantation. Twenty of the 21 patients (95.2%) achieved complete remission following induction chemotherapy: 17 patients after one cycle and three patients after two cycles of the induction regimens. We quantified the transcript levels at the time of initial complete remission in 17 patients (all patients who achieved complete remission except patients #14, #16, and #20) and evaluated the prognostic impact. Thirteen patients showed a decrease of transcripts of 3-log or more, compared to the transcript level at diagnosis. Seven of them (53.8%) later relapsed (duration of complete remission: median, 18.9 months; range 7.7 to 27.9 months). Four patients showed a decrease less than 3-log (patients 9, 11-13) and all of them relapsed (duration of complete remission: median 8.6 months, range 4.9 to 15.6 months). A decrease of more than 3-log in transcript levels in first complete remission was correlated with better event-free survival (p=0.035) (Figure 3A). For adult patients, the Kaplan-Meier analysis showed more significant differences in event-free survival (p=0.011) (Figure 3-B). The four available pediatric patients showed reductions of more than 3-log; however, three of them relapsed after bone marrow transplantation. The overall survival was not different between the two groups (p=0.897).

The combination of two parameters, high initial transcript levels or reduction less than 3-log at achievement of first complete remission, was also a significant predictor of relapse (Figure 4).

AML1-ETO fusion transcripts were quantified in seven patients with hypocellular marrow states, around the 14th day after induction chemotherapy (day 11-14). Two patients (patients 4# and #5) showed negative results and they had no relapse or other events for 17 and 18 months under chemotherapy. The remaining five patients showed positive results with variable degrees of reduction; three of these patients later relapsed. However, this difference did not have a significant effect on event-free survival (p=0.343).

Among the patients who had serial samples taken until a time point after the second cycle of chemotherapy, first cycle of consolidation or second induction chemotherapy; day 55-74), only three patients (patients #11, #12, and #21) showed the persistence of fusion transcripts and all of them had unfavorable clinical courses. Patient #21 was refractory to several cycles of different chemotherapy regimens. Patients #11 and #12 showed persistence of fusion transcripts in spite of hematologic complete remission, and they had relapse of disease 4 and 14 months later, respectively.

Reduction of AML1-ETO mRNA after allogeneic bone marrow transplantation

Seven patients underwent allogeneic bone marrow transplantation; three adults in the second or third complete remission after relapse and four children in the first complete remission. All six patients who had follow-up specimens taken in the fourth or fifth week

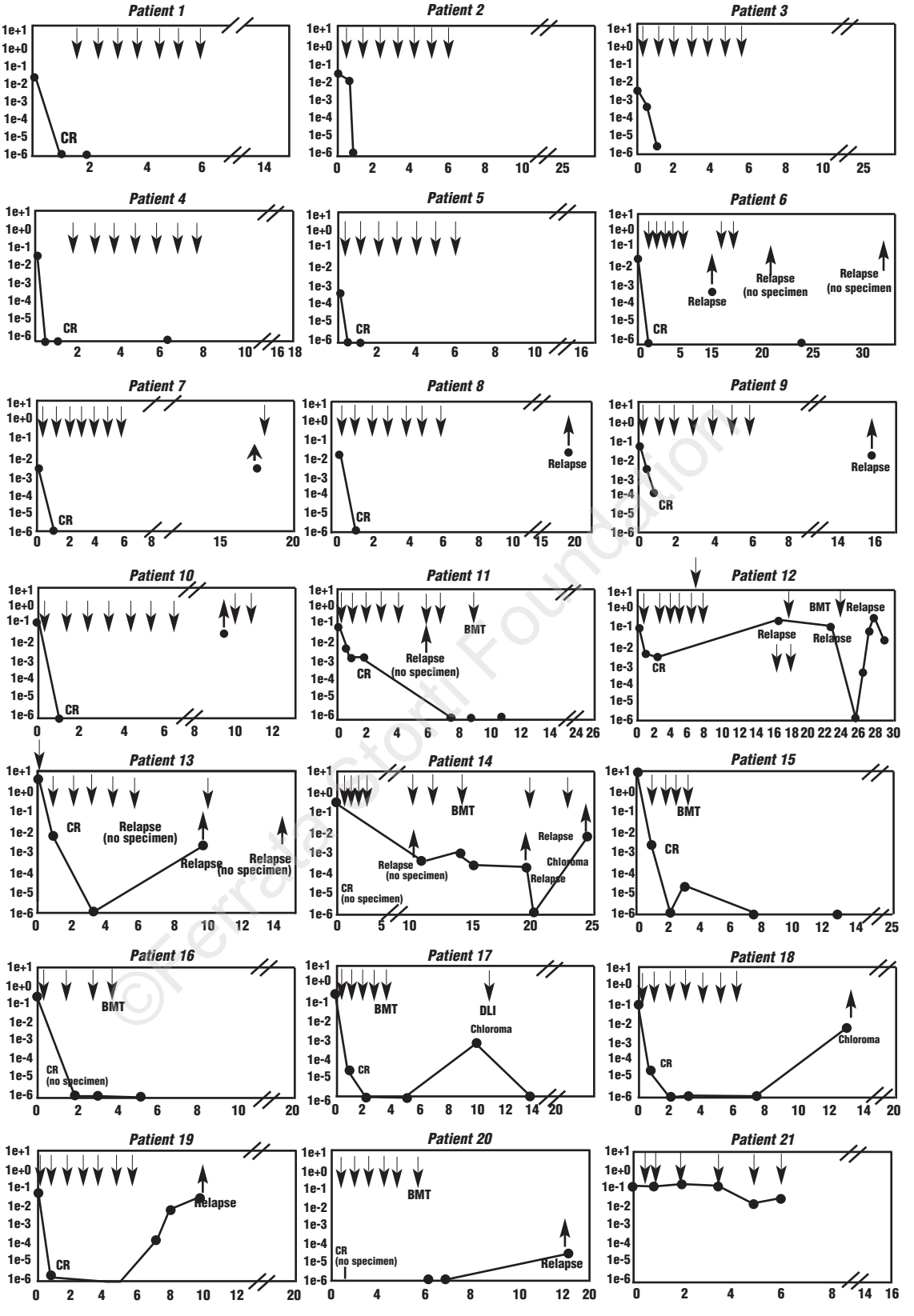

Figure 1. Serial quantification of *AML1-ETO* fusion transcripts and hematologic findings in 21 patients. The X axis represents the follow-up duration (in months) and the Y axis the *AML1-ETO/GAPDH* ratio.

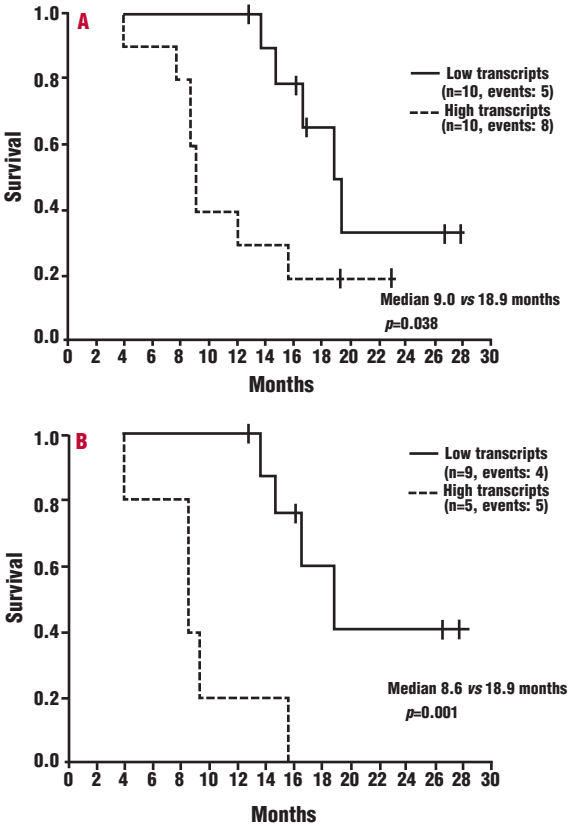

Figure 2. Event-free survival of the patients according to whether the transcript level at diagnosis was above (high) or below (low) the cut-off of the 50th percentile value in all available patients, except patient 21 who did not achieve complete remission (A) and in 14 adult patients (B).

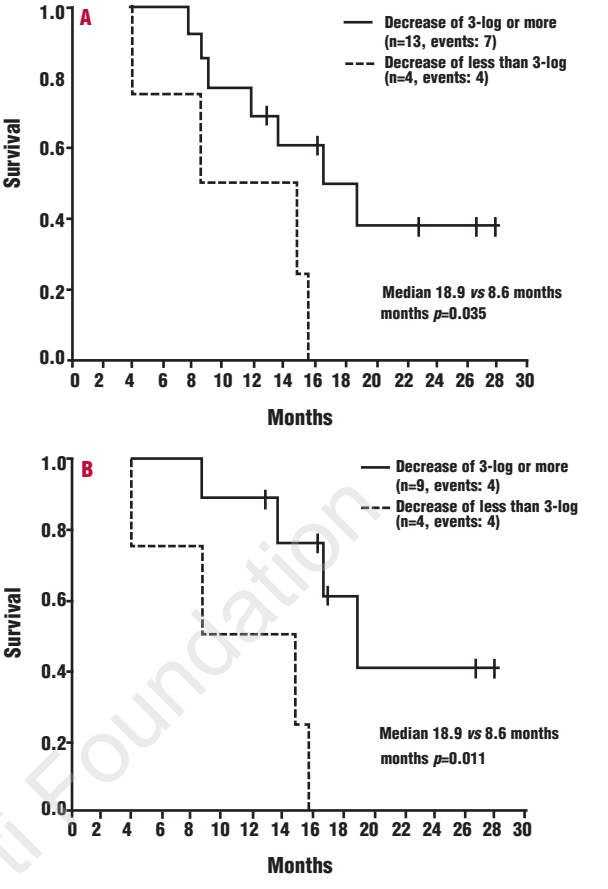

Figure 3. Event-free survival according to the decrease of *AML1-ETO* fusion transcript levels (more or less than 3-log) at the time of initial hematologic complete remission in all available patients (A) and in 13 adult patients (B).

after bone marrow transplantation showed negative conversion of *AML1-ETO* fusion transcripts at that time point. Patient #15 had positive conversion at day 90 after chemotherapy. He received the conditioning regimen immediately afterwards and underwent allogeneic bone marrow transplantation two weeks later. He showed negative results in week 18 after the transplant without previous specimens being available and was in continuous complete remission for 21 months.

Early detection of relapses

Overall 13 patients relapsed after chemotherapy or bone marrow transplantation. Eleven patients relapsed after chemotherapy and the first relapse of each patient occurred within 5-20 months (median, 10). Three patients (patients #9, #11, and #12) showed persistence of fusion transcripts before relapse in spite of hematologic complete remission. Seven patients (patients #6, #7, #8, #10, #13, #18, and #19) relapsed after negative conversion of transcripts (no specimen was available for patient 14 during complete remission). The level of *AML1-ETO* fusion transcripts increased in patient #19 in the seventh month in hematologic complete remission and with a negative RT-PCR result. The *AML1-ETO/GAPDH* ratio was 2.42×10^{-4} in this patient's bone marrow and 6.75×10^{-5} in her peripheral blood. One month later, still in hematologic complete remission, a 2-log increase of *AML1-ETO* fusion transcripts was observed with positive conversion of RT-PCR. The patient had a hematologic relapse in the tenth month. This demonstrates that the RQ-PCR detected *AML1-ETO* fusion transcripts 3 months before clinical relapse. Among seven patients who underwent bone marrow transplantation, four (patients #12, #14, #17, and #20) had one or two hematologic or extramedullary relapses. They relapsed within 2-11 months (median, 5) after bone marrow transplantation with the reappearance of fusion transcripts. Patient #12 showed negative conversion on day 28 post-transplantation. However, the *AML1-ETO/GAPDH* ratio rose to 3.18×10^{-4} in peripheral blood on day 61 post-transplantation without the appearance of blasts or positive conversion of RT-PCR. On day 97 post-transplantation, this patient had a hematologic relapse with a 2-log increase of *AML1-*

ETO fusion transcripts. Patients #14 and #17 showed chloroma in the absence of hematologic relapse on day 108 and 276 post-transplantation, respectively. In both patients, *AML1-ETO* fusion transcripts rose without hematologic or cytogenetic evidence of relapse in bone marrow. Patient #17 had donor lymphocyte infusions and patient #14 showed hematologic relapse six weeks later. Unfortunately, the remaining patients had no available specimens from 4 to 17 months prior to relapse.

Discussion

In our group of 21 patients with *AML1-ETO*-positive acute myeloid leukemia, serial quantification of *AML1-ETO*-positive fusion transcripts showed that high levels at diagnosis and reductions of less than 3-log at initial achievements of complete remission were significant indicators of poor event-free survival.

A high level of fusion genes at diagnosis was proposed as a new score for predicting prognosis by Schnittger *et al.*[23] Although they suggested the level of the 75th percentile as a cut-off value, we did not find any significance using that value as a cut-off. In our study, the expression ratios at diagnosis had a significant impact on event-free survival but not on overall survival using a cut-off of the 50th percentile. Moreover, these values showed no correlation with other prognostic parameters, suggesting that transcript levels at diagnosis could be a significant independent prognostic factor.

In addition, examining levels of minimal residual disease after chemotherapy, we found that a decrease in transcripts of more than 3-log in first complete remission was a useful marker with a strong impact on event-free survival. Leroy *et al.* also suggested that a 3-log decrease was a significant predictor of the absence of relapse in patients uniformly treated in a single institution.[24] We studied fewer patients and included both adults and children, so there were some differences in therapeutic regimens. In spite of the heterogeneity of the mixed cohort the results were consistent with those of previous reports. This finding suggests that RQ-PCR can identify patients with an unfavorable prognosis independently of age and treatment and that the parameters have statistical significance. The 3-log decrease would serve as a useful discriminator for risk-based stratification and decision-making concerning additional treatment for patients in first hematologic complete remission. Moreover, monitoring minimal residual disease at this time point should not be difficult in clinical practice since a bone marrow examination is routinely carried out at this time.

In many previous reports on acute myeloid leukemia with *AML1-ETO* gene rearrangements, children were shown to have a poorer prognosis than adult patients.

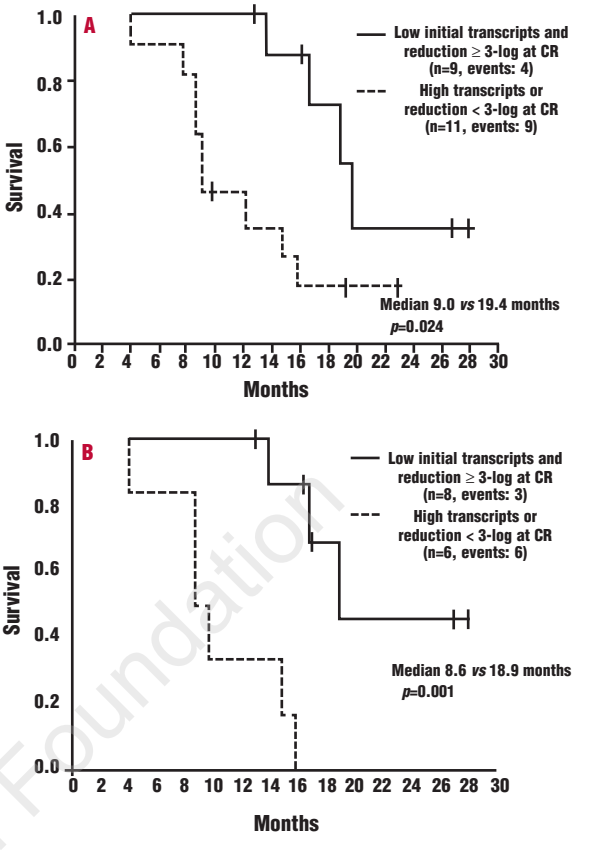

Figure 4. The differences in event-free survival between two groups by quantification of *AML1-ETO* fusion transcripts; high initial levels or a decrease less than 3-log at initial complete remission versus initial low levels and a decrease more than 3-log at complete remission in all available patients (A) and in 14 adult patients (B).

Among our seven pediatric patients, four relapsed and one was resistant to chemotherapy. Although bone marrow transplantation was performed in first complete remission in four patients, two of them relapsed. RQ-PCR of *AML1-ETO* fusion transcripts demonstrated that most of the children (6 out of 7) had high transcript levels, predicting poor event-free survival. For monitoring at the achievement of initial complete remission, although all four available patients showed reductions of more than 3-log, two of them later relapsed. Further separate studies of pediatric patients are needed. In our study, the prognostic value of initial transcript levels and minimal residual disease at complete remission were more significant in adult patients (Figures 2A, 3A). The implication is that closer follow-up and more intensive care, such as early bone marrow transplantation in the first complete remission, would be needed for the patients who had higher initial transcript levels or less than 3-log reductions at achievement of complete remission. The combination of the two parameters would be an excellent predictor of

relapse of disease, as shown in Figure 4.

In hypocellular marrow states, about 14 days after a cycle of induction chemotherapy, early negative conversion suggested the possibility of a new prognostic index. At the time point after two cycles of chemotherapy (second induction or first consolidation regimens), just three patients of our series had detectable *AML1-ETO* mRNA. This persistence of fusion transcripts suggested the possibility of non-responsiveness or the presence of a remnant clone that could relapse later. This was consistent with the results of a study by Viehmann *et al.*, in which four out of seven children relapsed, and two of them had a decrease of less than 2-log before starting consolidation (after two cycles of induction) around 42-56 days after diagnosis.[21] Molecular eradication at this time point seemed to be a prerequisite for long-term remission, but did not guarantee long-term disease-free survival. The limited case numbers with hypocellular marrow states or after two cycles of therapy necessitate a study with more patients for longer periods to verify the prognostic values.

Some studies described that the *AML1-ETO* mRNA levels decreased, but remained at a detectable level in spite of hematologic remission for as long as 2 years. Positive quantitative results near the detection levels did not have a significant impact on the prediction of clinical courses in previous reports.[17,21] Unlike previous studies, in our series most patients showed a reduction of transcript levels below the detection limit during chemotherapy. The discrepancy could be due to differences in the sensitivity of the assays or to other unknown factors such as the different intensities of the therapeutic regimens used in the various institutes and ethnic variations of response to chemotherapeutic agents. Unfortunately, there are no available reports presenting quantitative data for *AML1-ETO* fusion transcripts in Korean patients. Recently Buonamici *et al.* reported similar findings in seven patients.[27] They suggested that once a patient had achieved RQ-PCR negativity, the patient might have intrinsically manageable levels of residual disease for at least several years without the need for further maintenance chemotherapy. In net contrast, many patients of our series relapsed after molecular eradication. These findings indicate that regular follow-up is mandatory even after conversion to RQ-PCR negativity.

In our study, the sequential analysis of the expression of *AML1-ETO* mRNA with peripheral blood or bone marrow specimens was able to detect positive conversion of fusion transcripts during hematologic complete remission; this conversion was a sensitive and specific predictor of relapse. Furthermore, RQ-PCR detected conversion preceded the conversion as detected by a nested RT-PCR method. In the patient 19, an increase of the *AML1-ETO* fusion transcript became obvious three months prior to hematologic relapse in both bone marrow and peripheral blood. Once the level of *AML1-ETO* transcripts reached the detection limit under chemotherapy, an interval of less than three months seems to be reasonable, as suggested by Krauter *et al.*[22] At least 2 years of follow-up after chemotherapy seems to be essential given that all nine patients in our series who relapsed after chemotherapy had their first relapse within 20 months. Patient 12 showed positive conversion of *AML1-ETO* fusion transcripts in peripheral blood the second month after bone marrow transplantation, and this forecasted the reappearance of leukemic blasts a month later. Our preliminary data suggest that serial quantification of *AML1-ETO* mRNA at shorter intervals might be helpful during the early post-transplantation period. In practice, the interval of the obligate bone marrow aspiration after consolidation or bone marrow transplantation is more than three months. This problem could be solved by minimal residual disease analyses of peripheral blood samples. We confirmed the correlation of quantification results between peripheral blood and bone marrow. Identifying patients at a high risk of relapse by *AML1-ETO* quantification may be helpful for offering a window of opportunity between one and three months, during which pre-emptive therapy such as stem cell transplantation or donor lymphocyte infusions could be administered. However, more uniformly treated patients and consecutively assembled follow-up data are needed to draw conclusions for the precise recommendable time interval.

Around day 28 post-transplantation, all monitored patients showed a decrease in the level of *AML1-ETO* mRNA below the detection limits. At the same time post-chemotherapy, five had persistence of transcripts but then conversion to negativity later. Sugimoto *et al.* reported similar findings.[17] The greater reduction in the *AML1-ETO* mRNA following the different regimens might only indicate a greater myeloablative activity of a given regimen, and does not always mean a good prognosis for the patients as shown by the similar relapse rates after the two regimens in our series. In spite of post-transplantation relapses, allogeneic bone marrow transplantation was still the best option for salvage therapy. For example, patient 15 had positive conversion in the third month after chemotherapy. A prompt conditioning regimen and allogeneic bone marrow transplantation kept the patient under control without any event for 21 months. Considering the poor response to the salvage chemotherapy in relapsed patients with *AML1-ETO* gene rearrangements, bone marrow transplantation at the appropriate time would help patients.

The therapeutic options for extramedullary relapse after bone marrow transplantation are still controversial. Two patients (patients 14 and 17) developed chloroma without hematologic relapse and positive

conversion of *AML1-ETO* fusion transcripts at that time point. These findings suggest that the occurrence of chloroma indicates impending hematologic relapse, and we urge systemic therapy. As a matter of fact, patient 14 did undergo hematologic relapse six weeks after developing chloroma. Minimal residual disease monitoring with RQ-PCR would be a useful method for predicting chloroma and the subsequent hematologic relapse. The preliminary data from our institution are promising and provide the basis for further evaluation with RQ-PCR in prospective clinical trials. Precise information on minimal residual disease at appropriate time points during treatment may provide the biological basis for therapeutic decision-making to allow risk-directed therapy.

SJY and HSC designed the study, were responsible for the laboratory studies, and drafted the manuscript. SJY and EJ-S were responsible for collecting the clinical data and collaborated in the statistical analysis. JJS, J-HL, and H-SP analyzed and interpreted the results and critically revised the manuscript. CJP contributed to conducting the work and interpreting the results.

The primary responsibility for the paper rests with SJY and HSC, who contributed equally to the paper. The authors declare that they have no potential conflicts of interest.

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