High Transcript Level of FLT3 Associated with High Risk of Relapse in Pediatric Acute Myeloid Leukemia

Identification of prognostic factors and risk-based post-remission therapy was proposed to improve the outcomes of acute myeloid leukemia (AML) and a mutation of FLT3 has been reported to be a risk factor, especially for pediatric patients. Recently, FLT3 expression level was implicated to have prognostic significance in adults, but little is known for childhood AML. To define the prognostic significance, transcript level of FLT3 was analyzed in 52 pediatric AML patients. The median copy number of FLT3 was 4.6×10^3 ($40\text{-}5.9 \times 10^7$ copies)/1.0×10^6 GAPDH copy, and the relapse free survival of patients with high transcript level of FLT3 (>10^6 copy number) (0%) was significantly lower than that of the others (53.2%). High transcript level of FLT3 was associated with a markedly high risk of relapse. The development of new therapeutic scheme such as a frontline allogeneic stem cell transplantation or administration of FLT3 inhibitor is needed to improve outcomes.

Key Words : *FLT3; Transcript Level; Leukemia, Myeloid, Acute; Pediatric Age*

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INTRODUCTION

Dose-intensive treatment by induction chemotherapy and post-remission therapy has brought a lot of progress in childhood acute myeloid leukemia (AML) (1), and allogeneic stem cell transplantation (SCT) with a matched related donor (MRD) is regarded as an effective post-remission therapy in pediatric patients (2-4). But the majority of patients without an appropriate related donor, received continuous chemotherapy. Because relapse remains a major cause of failure and nearly a half of patients are destined for relapse and a poor outcome after complete remission, the identification of prognostic factors and risk-based post-remission therapy have been proposed (5, 6). Although alternative donor transplants such as SCT with a matched unrelated donor (MUD) are mainly applied to restricted categories of cases (AML in second complete remission [CR], third CR or early relapse), the high risk patients are regarded to have the advantage of receiving alternative donor transplants during the first remission (7). Recently, the FLT3 internal tandem duplication (FLT3/ITD) was found to have profound prognostic effects, especially in pediatric patients (8-15) and was proposed that a patient with

this mutation could be salvaged by allogeneic SCT, although there has been some controversy (6, 16-18). Transcript level of FLT3 was also proposed to have a prognostic significance in adults (19, 20), but little is known for childhood AML. In this study, transcript level of FLT3 was examined and analyzed with other prognostic factors in pediatric AML patients without very good prognostic factors such as t(15;17) and inv(16), who achieved complete remission and we proposed that high transcript level of FLT3 is associated with poor prognosis in pediatric AML.

MATERIAL AND METHODS

Patients and treatment

Transcript level of FLT3 was analyzed with the initial bone marrow sample in newly diagnosed pediatric patients (aged ≤18 yr) with AML who achieved the first complete remission. Patients with secondary AML or refractory anemia with excess blasts in transformation (RAEB-t) were included, but patients with very good prognostic factors such as t(15;17)

or inv(16) were excluded. The use of human material for scientific purposes in this study was approved by the Institutional Review Board of Seoul National University Hospital (0507-507-153).

All patients received the same treatment scheme as previously reported after informed consent (9, 21). Briefly, patients received N4-behenoyl-1-β-D-arabinofuranosyl-cytosine (BH-AC), idarubicin, 6-thioguanine (6-TG), and intrathecal cytosine arabinoside (Ara-C) as an induction therapy. Primary refractory patients received a second cycle of induction therapy, excluding the 6-TG from the first regimen. Once in remission, patients with a MRD received SCT after two courses of consolidation therapy. Patents without a donor received autologous peripheral blood stem cell transplantation (APBSCT) with BCVAC (BCNU, etoposide, Ara-C, and cyclophosphamide) conditioning regimen after four courses of consolidation therapy. Recently, patient with appropriate alternative donor received unrelated SCT or cord blood transplantation.

Analysis of FLT3 mutation and transcript level

Transcript level of FLT3 was analyzed with total RNA extracted from bone marrow samples by using EASY-BLUE total RNA extraction solution (InTron Inc., Seoul, Korea) according to the manufacturer's instruction. cDNA was synthesized from each RNA (RNA measured by nano drop spect auto calculation machine; error range $\pm$ 0.1-0.5%) by using a RT premix kit (InTron Inc.). The transcript level of FLT3 was measured by using a real-time fluorescence detection method on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The standard curves for FLT3 and GAPDH mRNA were generated using tenfold serial diluted FLT3 or GAPDH PCR products as template, and the copy number of FLT3 and GAPDH transcript was calculated from each standard curve. To compare the absolute expression level of each sample, the FLT3 mRNA was normalized using the expression of GAPDH gene, i.e. the number of FLT3 copies was expressed as the equivalent copy number per 10^6 copies of GAPDH. Each gene expression level was analyzed in triplicate, and the mean was subjected to analysis. The primer and probe sequences for RT-Q-PCR of FLT3 were sense primer, 5′-TTTCACAGGACTGGACA-GAGATTT -3′ antisense primer, 5′-GAGTCCGGGTGT-ATCTGAACTTCT-3′; and TaqMan probe, FAM-5′-CCAA-ATTCCAGCATGCCTGGTTCAA-3′-TAMRA. The housekeeping gene, GAPDH, served as a control for cDNA quality and normalization and the primer and probe sequences for real time quantitative PCR were sense primer, 5′ - TTTCA-CAGGACTTGGACAGAGATTT-3′ antisense primer, 5′ - GAGTCCGGGTGTATCTGAACTTCT-3′ ; and TaqMan probe, FAM-5′ -CAAGCTTCCCGTTCTCAGCC-3′ -TA-MRA. FLT3 mutations were analyzed as previously reported with genomic DNA isolated from bone marrow samples (9).

Statistics

Clinical and laboratory data were analyzed using standard statistical methods in SPSS version 12.0. Fisher's exact test was used to compare categorical variables among subgroups. Differences in means of continuous variables were analyzed with the Mann-Whitney U test or Kruskal-Wallis test. Relapse-free survival (RFS) was defined as the time from complete remission to relapse, and for survival analysis, Kaplan-Meier life tables were constructed and curves were compared using the log-rank test. For multivariate analysis of prognostic factors, a Cox proportional hazard regression model was used.

RESULTS

Patient characteristics

Fifty-two patients (33 males and 19 females) were analyzed in this study. The median age at diagnosis was 8 yr 5 months (4 months-17 yr 2 months). The clinical characteristics of the 52 patents are detailed in Table 1. All patients achieving CR and thirty-six patients received post-remission therapy including 16 APBSCT and 7 bone marrow transplantation (BMT) with a MRD. One patient continued to receive chemotherapy because of renal insufficiency compromising the transplant. Twelve recently diagnosed patients with appropriate alternative donors received unrelated SCT or cord blood transplantation.

Transcript level of FLT3

Transcript level of FLT3 was normalized with the 10^6 GAPDH copy number and the median level of 52 patients was 4.6×10^3 copies (40-5.9×10^7 copies). FAB subtype and risk factors of AML such as an initial high WBC count ($>100 \times 10^9$/L), monosomy 7, more than one course for complete remission, secondary AML, MDS characteristics (RAEB-t), and FLT3/ITD were not associated with the expression level of FLT3 (Table 1).

Clinical characteristics of the patients with high transcript level of FLT3

High transcript level of FLT3 ($>10^6$ copy number) was found in 5 patient (9.6%). Among them 2 patients had the FLT3/ITD mutation. High expression of FLT3 was not associated with FLT3/ITD. Four patients experienced disease recurrence before transplantation and one patient relapsed after autologous peripheral blood transplantation. The clinical data of these 5 patients are summarized in Table 2. A comparison of risk factors revealed that patients with high transcript level of FLT3 were not associated with any risk factors

including FLT3/ITD (Table 3).

Clinical outcome and prognostic significance of transcript level of FLT3

The overall survival (OS) ($\pm$ standard error [SE]) and RFS ($\pm$ SE) of the 52 patients were $51.0 \pm 7.9\%$ and $46.9 \pm 7.7\%$, respectively. High transcript level of FLT3 ($>10^6$ copy number) was found to be a significant prognostic factor for RFS. The RFS of patients with high expression level of FLT3 (0%) was poorer than that of the other patients ($53.2 \pm 8.0\%$) (Fig. 1), with statistical significance ($P<0.001$), and it was also a significant prognostic factor for RFS by multivariate analy-

sis ($P=0.042$, RR=4.80 [1.06-21.73]) (Table 4).

DISCUSSION

Currently, the remission rate of pediatric non-promyelocytic AML is about 70-90%, but the survival rate has been reported to be only 45-66% despite intensive post-remission therapy (22). After the great success of treatment in pediatric acute lymphoblastic leukemia (ALL) with risk based therapy, this kind of efforts are also introduced to AML (5, 6). But treatment of AML is more intensive than ALL, and there is

Fig. 1. The relapse free survival (RFS) of patients with high transcript level of FLT3 (0%) was lower than the RFS of the other patients ($53.2 \pm 8.0\%$) ($P<0.001$).

Table 1. Patient characteristics and FLT3 transcription level

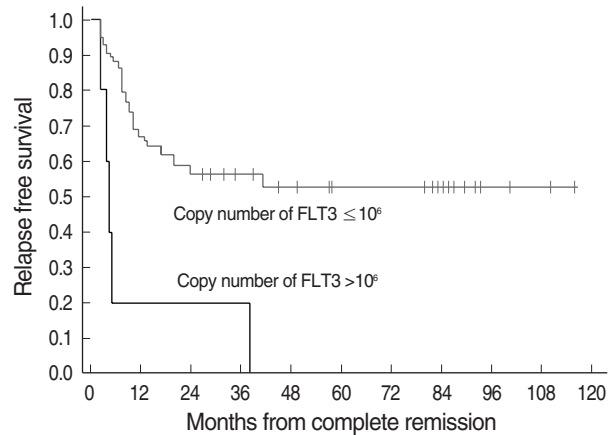| Parameters	Number	Transcript level of FLT3, median (range) (copies/1.0×10^6 GAPDH copy)
Total	52	4,673 (40-59,283,120)
FAB classification		
M1	5	5,025 (328-8,417,879)
M2	16	16,045 (122-59,283,120)
M4	12	3,548 (40-22,566,841)
M5	11	4,421 (81-2,882,400)
M6	1	3,018 (3,018-3,018)
M7	5	14,360 (1,209-475,389)
RAEB-t	2	348 (67-630)
Adverse prognostic factor		
≥ 2 courses of induction	4	240,207 (1,425-8,417,879)
High initial WBC count*	6	7,409 (4,421-8,417,879)
Secondary AML	3	49,650 (122-22,566,841)
Monosomy 7	1	81 (81-81)
Complex karyotype[†]	5	401,314 (14,360-59,283,120)
Favorable prognostic factor		
t(8;21)	14	5,569 (178-3,326,274)
FLT3/ITD		
Positive	7	8,964 (461-8,417,879)
Negative	45	4,421 (40-59,283,120)
FLT3/TK		
Positive	2	461 (178-744)
Negative	50	4,934 (40-59,283,120)

*WBC count of more than 100×10^9/L at diagnosis; [†]Presence of a clone with at least five unrelated cytogenetic abnormalities.

Table 3. Relation between high FLT3 level and other risk factors

Risk factors	FLT3 high level (n=5)	FLT3 low level (n=47)	P
≥ 2 courses of induction	1	3	0.34
High initial WBC count*	1	5	0.47
Secondary AML	1	2	0.27
Monosomy 7	0	1	1.00
Complex karyotype[†]	2	3	0.07
RAEB-t	0	2	1.00
FLT3/ITD	2	5	0.13

*WBC count of more than 100×10^9/L at diagnosis; [†]Presence of a clone with at least five unrelated cytogenetic abnormalities.

Table 2. Characteristics of patients with high FLT3 level ($>10^6$ copy number)

Patient number	Age (yr)/ Sex	FLT3 level ($\times 10^6$ copies)	FLT3/ ITD	FAB subtype	Karyotype	RFS (months)	Post-remission transplantation
7	1/M	2.89	–	M5	t(1;3)	5	*
8	12/M	3.33	+	M2	complex	4	*
9	9/M	59.28	–	M2	complex	38	APBSCT
24	2/F	22.57	–	M4	t(11;22)	4	*
42	12/M	8.42	+	M1	46,XY	3	*

*Relapse before post-remission transplantation.
APBSCT, autologous peripheral blood stem cell transplantation; RFS, relapse free survival.

Table 4. Multivariate analysis for the prognostic factors of RFS

	P value	RR	95% C.I.
≥2 courses of induction	0.264	2.18	0.57-8.52
High initial WBC count*	0.054	3.39	0.98-11.72
Secondary AML	0.190	2.89	0.59-14.15
Monosomy 7	0.173	4.25	0.53-33.92
Complex karyotype†	0.658	1.45	0.28-7.43
RAEB-t	0.144	4.99	0.58-42.91
High FLT3 level	0.042	4.80	1.06-21.73

*WBC count of more than 100×10^9/L at diagnosis; †Presence of a clone with at least five unrelated cytogenetic abnormalities.

not so much room for the stratification of treatment according to the prognostic factors in AML. In the view of urgency at diagnosis, it is somewhat hard to delay treatment in AML until cytogenetic results are confirmed. Thus, many centers start an induction regimen arranged beforehand. From this point, the prognostic factors after CR have tremendous importance.

BMT with matched sibling donor, introduction of intensive induction chemotherapy and post-remission therapy have done much to progress in the treatment of pediatric AML. Recent development of SCT with the increase of unrelated donor pool, the current proposal of risk based treatment in AML is focused on finding patients who would have a benefit from the allogeneic SCT. Some high risk factors have been established in pediatric AML. An initial high WBC count ($>100 \times 10^9$/L), monosomy 7, more than one course for complete remission, secondary AML, prior MDS, and extramedullary leukemia (non CNS) are well known classical adverse factors (23). Monosomy 7 as well as complex karyotype, monosomy 5, deletion 5q and abnormal 3q have been proposed as adverse cytogenetic risk factors (5, 6, 24).

FLT3 is a receptor tyrosine kinase involved in the survival of hematopoietic stem cells and expressed in most acute leukemia, including more than 90% cases of AML (2, 25, 26). Mutations of FLT3 have been reported to be of prognostic significance in many studies including our previous report (8-15). But the role of allogeneic SCT in FLT3/ITD positive AML was in debate (6, 16-18).

In our previous study, FLT3/ITD was a poor prognostic factor and no patient survived (9), and FLT3/ITD also a poor prognostic factor in present study but one patient with FLT3/ITD was salvaged after cord blood transplantation.

To find another biological and clinical importance of FLT3, overexpression of FLT3 has been studied and proposed to have a prognostic significance in two adult studies (19, 20). But the prognostic role of expression level of FLT3 was not known for pediatric patients.

In the present study, FLT3 transcript level was analyzed in pediatric AML patients and high expression of FLT3 was found to have prognostic significance. Our results simply imply that patients with very high expression of FLT3 or FLT3/ITD mutation have an extremely high risk of relapse,

and that this may justify the use of an alternative transplant early after CR.

FLT3 is a receptor tyrosine kinase and considered as a therapeutic target in AML. Many phase I and II clinical studies show a modest effect of small molecular compounds that directly inhibit FLT3 and preliminary reports show the combination of FLT3 inhibitors with chemotherapy was well-tolerated with encouraging rates of clinical response (27, 28). As with the promising results of incorporation of imatinib into HSCT in Philadelphia positive leukemia, this kind of agent would be incorporated into protocols for AML and hopefully improve the treatment results in high-risk patients with high expression of FLT3.

In conclusion, high transcript level of FLT3 was associated with a markedly high risk of relapse. The development of new therapeutic scheme such as a frontline allogeneic stem cell transplantation or administration of FLT3 inhibitor is needed to improve outcomes.

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