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ORIGINAL ARTICLE

High *WT1* mRNA expression after induction chemotherapy and *FLT3*-ITD have prognostic impact in pediatric acute myeloid leukemia: a study of the Japanese Childhood AML Cooperative Study Group

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Abstract The prognostic value of *WT1* mRNA expression in pediatric acute myeloid leukemia (AML) remains controversial. A sample of newly diagnosed ($n = 158$) AML patients from the Japanese Childhood AML Cooperative Treatment Protocol, AML 99, were simultaneously analyzed for *WT1* expression, cytogenetic abnormalities and gene alterations (*FLT3*, *KIT*, *MLL*, and *RAS*). *WT1* expression (including more than 2,500 copies/μgRNA) was detected in 122 of the 158 (77.8 %) initial diagnostic AML bone marrow samples (median 45,500 copies/μgRNA). Higher *WT1* expression was detected in French American British (FAB)-M0, M3, M7 and lower expression in M4 and M5. Higher *WT1* expression was detected in AML with inv(16), t(15;17) and Down syndrome and lower in AML with 11q23 abnormalities. Multivariate analyses demonstrated that *FLT3*-internal tandem duplication (ITD), *KIT* mutation, *MLL*-partial tandem duplication were correlated with poor prognosis; however, higher *WT1* expression was not. *FLT3*-ITD was correlated with *WT1* expression and prognosis. Furthermore, 74 *WT1* expression after induction

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chemotherapy was analyzed. Higher *WT1* expression after induction chemotherapy was significantly correlated with M1 or M2/M3 marrow, *FLT3*-ITD and poor prognosis. Multivariate analyses in 74 AML patients revealed that *FLT3*-ITD, *MLL*-PTD, and *KIT* mutations were associated with poor prognosis; however, *NRAS* Mutation, *KRAS* mutation and high *WT1* expression (>10,000 copies/μgRNA) did not show poor prognosis. Our findings suggest that higher *WT1* expression at diagnosis does not correlate with poor prognosis, but that *WT1* expression after induction chemotherapy is considered to be a useful predictor of clinical outcome in pediatric AML.

Keywords *WT1* · *FLT3* · AML · Pediatric

Introduction

The risk classification for acute myeloid leukemia (AML) patients according to cytogenetic abnormalities has been widely accepted in pediatric and adult AML [1–4]. AML patients with t(8;21), inv(16) and t(15;17) have been classified into the low risk (LR) group, while those with 5q- and -7 have been categorized into the high risk (HR) group, and others into the intermediate risk (IR) group [2–4]. Detecting chimeric transcripts such as, *PML-RARA* appears to be a good method for detecting minimal residual disease (MRD); however, approximately half of AML patients lack a suitable leukemia-specific marker; thus, there has been considerable interest in developing alternative approaches for MRD detection. One strategy involves the use of flow cytometer to identify and monitor leukemia-associated aberrant phenotypes [5]. Another approach is to detect Wilms tumor 1 (*WT1*) mRNA, which is highly expressed in AML blasts relative to normal PB and BM using a quantitative reverse transcribed-polymerase chain reaction (qRT-PCR) [6–12]. Several researchers have suggested that *WT1* expression is a good prognostic marker; thus, monitoring of the *WT1* mRNA expression is useful for MRD [7–12]. However, the prognostic relevance of *WT1* expression in AML at diagnosis is still controversial. Previous research has suggested that high *WT1* expression at diagnosis correlated with poor prognosis [6–9], while some others did not [10, 11, 15]. Recent work suggests that high *WT1* expression after chemotherapy correlates with poor prognosis [13–15]. This may depend on the uncertain mechanism of *WT1* mRNA expression in AML blasts due to the fact that *WT1* has a role not only for the suppression of tumors, but also for oncogenic potential [16, 17]. Recently, *FLT3*-internal tandem duplication (ITD) [18–21], *MLL*-partial tandem duplication (PTD) [20, 21] and *KIT* gene mutations [22, 23] have been strongly associated with a poor prognosis of adult and pediatric AML. A recent study suggested that *NRAS* gene alterations were associated with poor prognosis, while others did not find the same results [24]. High *WT1* expression was suggested to correlate with *FLT3*-ITD [25], however, the association between *WT1* expression and several gene alterations remains unknown in adult and pediatric AML. Recently, *WT1* gene mutations were frequently found in AML patients with normal karyotypes who showed poor clinical outcome [26, 27].

Thus, the present study aimed to simultaneously investigate *WT1* mRNA expression and examine the association of *WT1* mRNA expression with several gene alterations (*FLT3*, *KIT*, *MLL*, and *RAS*) in a sample of 158 newly diagnosed AML patients. We found that *FLT3*-ITD patients showed higher *WT1* mRNA expression at time of diagnosis as well as after the induction of chemotherapy; however, the prognostic significance of *WT1* mRNA expression was found only after induction chemotherapy. We discuss the usefulness of *WT1* mRNA expression associated with several gene alterations in pediatric AML patients.

Patients and methods

Patients

Patients included 318 newly diagnosed pediatric AML patients [0–15 years old, (240 de novo AML patients, 32 t(15;17)-AML, and 46 Down syndrome related myeloid leukemia (DS-ML)] from the Japanese Childhood AML Cooperative Treatment Protocol, AML 99, recruited between January 2000 and December 2002 [23, 28, 29]. Diagnosis of AML was made according to French–American–British (FAB) classification. Cytogenetic analysis was performed using the G-banding method. Among them, 158 samples at diagnosis were available for molecular analysis including, 13 patients with FAB-M3 and 10 patients with Down syndrome (DS) who were treated with different treatment protocols (see Table 1). Excluding FAB-M3 and DS, there were no significant differences between the analyzed 135 patients and the unanalyzed 105 patients regarding age (median, 6 years [range: 0–15 years] versus 6 years [range: 0–15 years], respectively) and initial WBC count (median, 24.8 $\times$ 10^9/L [range: 1.65–621.0 $\times$ 10^9/L] versus 13.8 $\times$ 10^9/L [range: 1.0–489.0 $\times$ 10^9/L, p = .0764], respectively). Also, no significant difference between the analyzed and not-analyzed patients in FAB-M3 and DS was found. The treatment protocol and risk classification were previously reported and described elsewhere [23, 28, 29]. We analyzed *WT1* expression in 85 paired bone marrow patient samples after 1st induction chemotherapy and also tested the blast counts for M1 (blast counts <5 %), M2 (5 % ≤ blast counts <25 %), M3 marrow (>25 %) at 22–74 days (median, 44 days) after the

Table 1 Genetic alterations and *WT1* mRNA expression in AML subgroup according to FAB classification

FAB classification	No. of patients	*FLT3*-ITD	*FLT3*-D835Mt	*MLL*-PTD	*KIT* -Mt	*NRAS* -Mt	*KRAS* -Mt	*WT1* expression (median) (copies/μgRNA)*	
M0	6	1	0	1	0	2	0	64,750	(2,400–190,000)
M1	24	4	2	7	0	1	2	23,000	(0–390,000)
M2	46	4	2	5	8	4	4	23,000	(85–680,000)
M3	13	3	3	0	0	0	0	140,000	(450–290,000)
M4	22	1	1	4	3	2	3	9,200	(50–150,000)
M5	25	5	3	3	0	2	5	9,600	(0–440,000)
M6	1	0	0	0	0	0	0	670	–
M7	9	1	0	1	0	0	0	36,000	(0–980,000)
M7 with DS	10	0	0	0	1	0	1	63,500	(140–630,000)
Unclassified	2	1	0	0	0	0	0	27,000	(14,000–40,000)
Total	158	20	11	21	12	11	15	23,500	(0–980,000)
	(%)	12.7	7.0	13.3	7.6	7.0	9.5		

ITD internal tandem duplication, *PTD* partial tandem duplication, *Mt* mutation, *DS* Down syndrome

* The difference of median *WT1* mRNA expression in each subgroups according to FAB classification was statistically significant ($p = 0.0011$)

initiation of induction chemotherapy. The evaluation for complete remission (CR) was conducted after two consecutive induction chemotherapy cycles. We also analyzed *WT1* expression at the time point after three consecutive chemotherapy cycles ($n = 56$) and the completion of therapy ($n = 47$).

Next, the 28 bone marrow samples from the healthy individuals who were the donors for bone marrow transplantation aged 7–52 years old from Nagoya University Hospital were also analyzed. Informed consent was obtained from the patients or their care-givers, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center and Nagoya University Hospital approved the present study.

Detection of *WT1* mRNA expression by qRT-PCR

Total RNA extracted from the bone marrow samples was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *WT1* expression was measured using real time PCR system (ABI 7700, Applied Biosystems). A set of primers used in the present study were as follows, *WT1* forward primer located in exon 6 (5′-GAT AAC CAC ACA ACG CCC ATC-3′) and reverse primer located in exon 6 and 7 (5′-CAC ACG TCG CAC ATC CTG AAT-3′) [12]. The TaqMan probe (5′-ACA CCG TGC GTG TGT ATT CTG TAT TGG-3′) was designed to hybridize at the sense strand of exon 6 and labeled with FAM as reporter dye at the 5′ end and with the quencher dye carboxy-teramethyl-rhodamin (TAMRA) at the 3′ end terminus. As endogenous mRNA control ubiquitous gene *GAPDH* mRNA was simultaneously quantified using a set of primers [forward primer (5′-GAA GGT GAA GGT CGG AGT C-3′), reverse primer (5′-GAA GAT GGT GAT GGG ATT TC-3′)] and TaqMan probe (5′-CAA GCT TCC CGT TCT CAG CC-3′). The *WT1* expression was corrected by the each *GAPDH* mRNA expression and multiplied by 2.7×10^7 (copies/μgRNA), because 1.0 μg RNA contains 2.7×10^7 GAPDH. *WT1* mRNA expression was examined in 158 individuals from the diagnostic bone marrow samples, 85 paired patient samples after the 1st induction chemotherapy, 56 samples after three consecutive chemotherapy sessions, and 47 samples during the finalization of therapy.

Analyses of *FLT3*, *KIT*, *MLL* and *RAS* genes

Mutational analysis for internal tandem duplication (ITD) within the JM domain and D835 mutation (D835Mt) within the TK2 domain of *FLT3* were performed as previously described [30, 31]. The mutation analysis of kinase domain, extracellular domain and transmembrane domain for the *KIT* gene was performed with RT-PCR followed by direct sequencing as previously reported [23]. *MLL*-partial tandem duplication (PTD) was examined by simple first round RT-PCR with 35 cycles using the primer pair 6.1 (located on exon 9) and E3AS (located on exon 4) as previously described [21, 32, 33]. The amplified products were purified and directly sequenced to confirm the *MLL*-PTD.

Exons 2 and 3 of the *NRAS* and *KRAS* genes were amplified by RT-PCR and directly sequenced using primer pairs for *NRAS* as previously reported. [34]

Statistical analysis

Estimates of the survival distributions were performed using the Kaplan–Meier method. Differences were

compared using a log-rank test. Overall survival (OS) was defined as the time from diagnosis to death (owing to any cause), or to the last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to the date of relapse and death. Patients with Down syndrome ($n = 10$) or FAB-M3 ($n = 13$) were treated using different protocols, thus, we analyzed the 5-year OS and EFS in 135 patients except for patients with DS and FAB-M3. Mann–Whitney *U* tests were performed to detect the statistical differences between two groups and the Krusukal–Wallis test was performed to detect differences among several groups. The prognostic significance of the clinical variables was assessed using a Cox proportional hazards model. These statistical analyses were performed with the statistical software StatView (Abacus Concepts, Inc.). For all analyses, the *p* values were two-tailed, and a *p* of less than 0.05 ($p < .05$) was considered to be statistically significant.

Results

WT1 mRNA expression in normal bone marrow samples

We analyzed *WT1* mRNA expression in 28 RNA samples extracted from mononuclear cells of normal bone marrow samples and found that it ranges from 123 to 3,725 copies/μgRNA (median, 1,200 copies/μgRNA, see supplemental Figure 1). We determined the cut-off value to be 2,500 copies/μgRNA, because the value for the 90th percentile was 2,519 copies/ugRNA. Alternatively, the cut-off value for peripheral blood was 50 copies/μgRNA while using the same method [12]. Additionally, we set the *WT1* mRNA expression for bone marrow samples more than 2,500 copies/μgRNA as *WT1* expression positive.

WT1 mRNA expression at diagnosis, gene alterations and clinical outcomes

The *WT1* expression at time of diagnosis in 122 (77.8 %) of the 158 AML patients demonstrated to have more than the cut-off value (2,500 copies/ugRNA, see supplemental Figure 1) and was markedly elevated (median, 45,500 copies/μgRNA). No association was found between *WT1* expression and sex, age or initial WBC count. Complete remission (CR) was obtained in 149 of the 158 patients (94.3 %) after two consecutive induction chemotherapy cycles; however, the median *WT1* expression at time of diagnosis was not statistically different in patients with or without CR. The *WT1* expression in each FAB subgroup was statistically different (p=0.0011), (see Table 1). High *WT1* mRNA expression was found in FAB-M0, M3, M7, whereas low levels of expression were found in M4 and M5 ($p < 0.0001$). The median *WT1* mRNA expression in each karyotypic subgroup is presented in Table 2. Higher *WT1* mRNA expression was found in t(15;17), inv(16) and in patients with Down syndrome, whereas lower expression of *WT1* was found in AML patients with an 11q23 abnormality. In particular, more than 90 % of t(15;17), inv(16) and Down syndrome samples presented with a *WT1*-positive (>2,500 copies/μgRNA); whereas more than half (55 %) of AML patients with 11q23 abnormalities were *WT1* negative.

WT1 expression and prognosis were not different between patients with and without *FLT3*-ITD in 13 t(15;17) AML patients. In FAB-M7, there was no difference in *WT1* expression in DS and non-DS patients.

Excluding 13 FAB-M3 and 10 DS patients, 102 out of 135 patients (75.6 %) were *WT1* positive at the time of diagnosis. Multivariate analyses in 135 AML patients revealed that *FLT3*-ITD, *MLL*-PTD and *KIT* mutations were associated with poor prognosis, however, *FLT3*-D835 mutation (Mt), *N-RAS* Mt, *K-RAS* Mt and high *WT1* expression (>100,000 copies/μgRNA) did not show poor prognosis in patients in 5-year OS and EFS (see Tables 3, 4). Specifically, *FLT3*-ITD was found in 20 (12.7 %) and *FLT3*-D835Mt was found in 11 (7.0 %) of the 158 patients. The median *WT1* mRNA expression of patients with *FLT3*-ITD, D835Mt and wild type *FLT3* was 89,500 copies/μgRNA (330–330,000 copies/μgRNA), 85,000 copies/μgRNA (0–250,000 copies/μgRNA) and 18,500 copies/μgRNA (0-980,000 copies/μgRNA), respectively. This difference was statistically significant ($p = 0.0025$); however, our data suggested that only *FLT3*-ITD indicated poor prognosis [5-year overall survival for *FLT3*-ITD, D835Mt and wild type were 35.3, 100 and 84.3 %, respectively ($p < 0.0000001$)] [21]. For additional genetic alterations, *MLL*-PTD was found in 21 (13.3 %) out of 158 patients, the median *WT1* expression was not different in patients with (median, 21,000 copies/μgRNA) or without *MLL*-PTD (median, 28,000 copies/μgRNA), however, the prognosis was quite different within both subgroups [5y OS 56.3% in *MLL*-PTD (+) and 83.2 % in *MLL*-PTD(-)] [21]. *KIT* gene mutation (Mt) was found in 12 (7.6 %) out of 158 patients. The median *WT1* expression was not different in patients with (13,500 copies/μgRNA) or without *KIT*-Mt (32,500 copies/μgRNA). Interestingly, the median *WT1* expression in t(8;21)-AML patients with or without *KIT*-Mt was statistically different [3,300 copies/μgRNA (85-55,000 copies/μgRNA) when compared with 30,000 copies/μgRNA (200-680,000 copies/μgRNA)] ($p = 0.020$). Mutation in codons 12, 13 or 61 of the *NRAS* gene was found in 11 (7.0 %) out of 158 patients, the median *WT1* expression was not different in patients with (36,000 copies/μgRNA) or without *NRAS*-Mt (23,000 copies/μgRNA). Mutation in codons 12, 13 or 61 of the *KRAS* gene in 15 (9.5 %) out of

Table 2 Genetic alterations and *WT1* mRNA expression in AML subgroup according to karyotypic abnormalities

Karyotypic abnormalities	Number of patients	*FLT3*-ITD	*FLT3*-D835Mt	*MLL*-PTD	*KIT*	*NRAS*	*KRAS*	*WT1* mRNA expression (copies/μgRNA)	
Normal	33	9	2	8	2	2	3	18,000	(73–280,000)
t(8;21)	46	2	1	4	8	4	5	23,500	(85–680,000)
inv(16)	7	0	2	0	1	2	0	56,000	(8,700–220,000)
11q23 abnormalities	20	0	1	5	0	1	4	720	(0–150,000)
t(15;17)	13	3	3	0	0	0	0	140,000	(450–290,000)
Down syndrome	10	0	0	0	1	0	1	63,500	(140–360,000)
Others[a]	29	6	2	4	0	2	2	23,000	(0–980,000)
Total	158	20	11	21	12	11	15	32,000	(0–980,000)
	(%)	12.7	7.0	13.3	7.6	7.0	9.5		

The median value of *WT1* mRNA expression is shown and the difference was statistically significant ($p = 0.0003$)

[a] The subgroup of other karyotypic abnormalities included 2 patients with unknown karyotype

Table 3 Prognostic factors for overall survival in 135 AML patients except for FAB-M3 and Down syndrome

Variable	*P* value	Odds ratio	95% CI
FLT3-ITD	<0.0001	6.767	2.844–16.101
FLT3-D835Mt	n.d.	n.d.	n.d.
MLL-PTD	0.0443	2.229	1.021–4.870
KIT-Mt	0.0148	3.661	1.290–10.395
NRAS-Mt	0.3538	1.789	0.523–6.122
KRAS-Mt	0.1313	2.407	0.769–7.537
WT1>100,000 copies/μgRNA	0.8733	0.931	0.386–2.244

Table 4 Prognostic factors for Event Free Survival in 135 AML patients except for FAB-M3 or Down syndrome

Variable	*P* value	Odds ratio	95% CI
FLT3-ITD	0.0015	3.455	1.607–7.430
FLT3-D835Mt	0.3770	0.404	0.054–3.019
MLL-PTD	0.0213	2.177	1.123–4.221
KIT-Mt	0.0013	3.816	1.686–8.635
NRAS-Mt	0.7613	0.832	0.254–2.726
KRAS-Mt	0.1849	1.852	0.745–4.607
WT1>100,000 copies/μgRNA	0.9169	0.960	0.447–2.063

158 patients, the median *WT1* expression was not different with (23,000 copies/μgRNA) or without *KRAS*-Mt (24,000 copies/μgRNA).

In patients with normal karyotype, the median *WT1* expression in patients with and without *FLT3*-ITD were 65,000 copies/μgRNA (73–280,000 copies/μgRNA 9 *FLT3*-ITD and 2 *FLT3*-D835Mt) and 7,150 copies/μgRNA (330–240,000 copies/μgRNA, 22 *FLT3* wild type), respectively, which was a statistically significant ($p = 0.023$).

In AML with 11q23 abnormalities, the median *WT1* expression was quite low (median, 720 copies/μgRNA) and more than half (55 %) of AML patients with 11q23 abnormalities were *WT1* negative. There were significant differences among each of the subgroups with t(9;11) ($n = 8$; median = 35 copies/μgRNA); t(6;11) ($n = 3$; 81,000 copies/μgRNA); and other variants ($n = 9$; 780 copies/μgRNA) ($p = 0.0139$). Furthermore, there were significant differences in their clinical outcomes, with 5-year OS rates nearly to 100 % in t(9;11), 0 % in t(6;11) and 77.8 % in other variants, respectively.

WT1 mRNA expression after 1st induction therapy

We also evaluated the *WT1* expression and blast counts in 85 bone marrow samples including 5 t(15;17) and 6 DS patients after 1st induction chemotherapy. There were no significant differences between the analyzed 85 patients and unanalyzed 73 patients regarding age (median 6 vs. 7-years old, respectively), WBC count (median 20,200 vs. 21,400/μl, respectively), or initial *WT1* expression level (median 21,000 vs. 32,000 copies/μg RNA, respectively).

In 5 t(15;17)-AML patients examined, *WT1* expression decreased by a log of 1–3 after induction chemotherapy except for one patient who showed 32,000 copies/μgRNA after induction chemotherapy. This patient died after relapse. In all six DS patients analyzed, *WT1* expression decreased by a log of 2–3 and all were cut-off value after induction chemotherapy. All these patients were alive without relapse.

Excluding 5 t(15;17) and 6 DS patients, 58 out of 74 patients (78.4 %) were *WT1* positive at the time of diagnosis (median, 18,000 copies/μgRNA) and 11 out of 74 patients (14.9 %) remained *WT1* positive after induction chemotherapy (median, 215 copies/μgRNA) (see

supplemental Figure 2). Specifically, *WT1* expression decreased after the 1st round of chemotherapy at a rate of 1–4 log(s) in 53 out of 57 *WT1*-positive patients at the time of diagnosis (93.0 %) [1 log reduction in 15 of 57 (26.3 %), 2 log reduction in 22 of 57 (38.6 %), 3 log reduction in 12 of 57 (21.0 %) and 4 log reduction in 4 of 57 (7.0 %)]. Furthermore, 10 of 57 (17.5 %) patients still showed *WT1* positive after the 1st round of chemotherapy. A total of 16 out of 17 patients with *WT1* negative at the time of diagnosis remained *WT1* negative after induction chemotherapy, and only one patient whose *WT1* expression increased at a rate of 1 log could not achieve complete remission and died. There was a statistically difference in 5-year OS between the *WT1*-positive subgroup (54.5 %, $n = 11$) and the *WT1* negative subgroup (79.4 %, $n = 63$) ($p = 0.036$) (see Fig. 1). The difference between the subgroup with *WT1* ≥10,000 copies/μgRNA (25 %, $n = 4$), and *WT1* <10,000 copies/μgRNA (77.1 %, $n = 70$) after induction chemotherapy was significant ($p = 0.0018$).

There was also a statistical difference in 5-year OS between M1 marrow (79.1 %, $n = 67$) and M2/M3 marrow (42.9 %, $n = 7$) ($p = 0.013$). The *WT1* expression was also quite different between M1 marrow (median, 200 copies/μgRNA, $n = 67$) versus the M2/M3 marrow (median, 14,000 copies/μgRNA, $n = 7$) ($p < 0.0001$). There was a moderate to strong correlation between *WT1* positivity and M1/M2/M3 marrow. Finally, three out of seven M2/M3 marrow patients did not achieve CR after two consecutive induction chemotherapy cycles.

FLT3-ITD was found in 8 patients, *FLT3*-D835Mt in 5, *KIT*-Mt in 7, *MLL*-PTD in 8, *RAS*-Mt in 6 and *KRAS*-Mt in 8 patients out of the 74 diagnostic samples. Eight patients with *FLT3*-ITD showed higher *WT1* expression at the time of diagnosis (median, 60,000 copies/μgRNA) and after the initiation of chemotherapy (median, 1,850 copies/μgRNA) compared with those without *FLT3*-ITD (at diagnosis 15,500 copies/μgRNA, after induction chemotherapy 200 copies/μgRNA, $n = 66$). Three out of 4 patients with *WT1* positive after induction chemotherapy relapsed and died.

Interestingly, a total of 11 *WT1* positive patients after induction chemotherapy included 5 *FLT3*-ITD, 2 *MLL*-PTD, 1 *FLT3*-D835Mt, and 1 *KIT*-Mt. In particular, three out of four patients with *WT1* expression with more than 10,000 copies/μgRNA had the *FLT3*-ITD.

Multivariate analyses in 74 AML patients revealed that *FLT3*-ITD, *MLL*-PTD and *KIT* mutations were associated with poor prognosis; however, *NRAS* Mt, *KRAS* Mt and high *WT1* expression (>10,000 copies/μgRNA) did not show poor prognosis in patients with 5-year OS and EFS (see Supplemental Tables 1 and 2).

WT1 mRNA expression at the subsequent time point

Only 2 of the 56 (3.6 %) patients presented with *WT1* positive after three consecutive courses of chemotherapy. Unfortunately, both patients relapsed and died. Four of the 47 (8.5 %) patients at the finalization of therapy showed an expression of *WT1* positive and 3 patients relapsed (at 8, 9 and 16 months after the initiation of induction chemotherapy) and died. We found *MLL*-PTD in one patient.

Discussion

The present study aimed to investigate *WT1* mRNA expression and examined association between *WT1* expression and five gene alterations including *FLT3*, *KIT*, *MLL*, *NRAS* and *KRAS* in de novo 158 pediatric AML bone marrow samples. High *WT1* expression at diagnosis was reported to be associated with a poor prognosis in adult AML studies [6–9]; however, others have suggested the inverse results [10, 11, 15]. Consistent with previous works, our results also suggested that *WT1* expression was significantly different in subgroups according to FAB classification and karyotypic abnormalities [10, 11, 13–15].

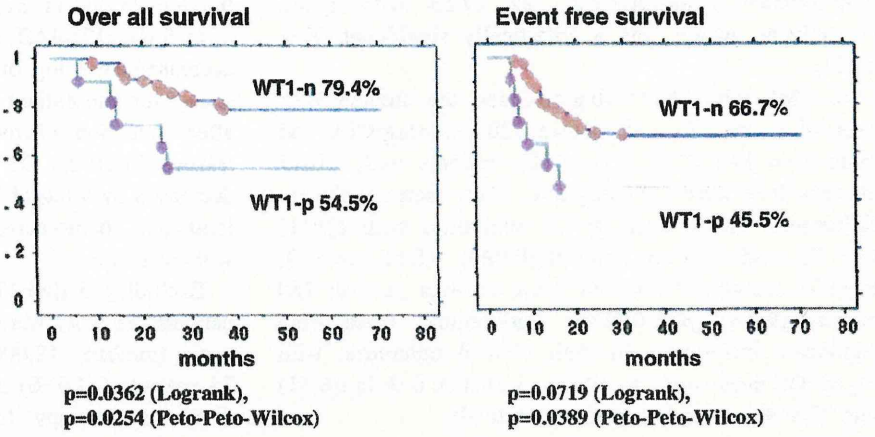

Fig. 1 The 5-year overall survival and event free survival rates for 74 AML patients according to *WT1* expression after 1st induction chemotherapy. *WT1-p WT1* mRNA expression had more than 2,500copies/μRNA, *WT1*-n; *WT1* mRNA expression had less than 2,500copies/μRNA. Overall survival is quite different according to *WT1*-p/n status

WT1 expression was higher in FAB-M0, M3, M7 and lower in M4 or M5. This may be partially explained by the fact that *WT1* expression was down-regulated with differentiation [35]. *WT1* expression in AML patients with 11q23 abnormalities was significantly lower, and thus, resulted in low *WT1* expression of FAB-M4 or M5, as previously reported [10, 13]. Conversely, t(8;21), inv(16) and t(15;17) displayed higher *WT1* expression and good prognosis as in previous studies [10, 11, 13]. Our results demonstrated that *WT1* expression of t(8;21) with *KIT* mutations (3,300 copies/μgRNA) was statistically lower than that without *KIT* mutations (30,000 copies/μgRNA) ($p = 0.020$). The reason for this finding remains unclear; however, a recent study suggested that *RUNX1-RUNX1T1* (AML1-ETO) rapidly induces AML in conjunction with *WT1* expression using a mouse model [36]. Furthermore, *FLT3*-ITD and D835Mt were strongly associated with higher expression of *WT1* mRNA in a total of 158 AML patients, which is consistent with previous adult AML [9, 10]. Patients with *FLT3*-ITD or D835Mt showed higher *WT1* expression, however, the prognosis was quite different between patients with *FLT3*-ITD and D835Mt in our study. Moreover, *WT1* expression in about 20 % of AML patients was lower than the specified cut-off value. The majority of AML patients with 11q23 abnormalities except for t(6;11)(q27;q23) showed lower *WT1* expression. We concluded that patients presenting with high *WT1* expression were comprised of several subgroups with heterogeneous clinical outcomes. Recently, a study suggested that *WT1* played a significant role in both tumor suppression and oncogenic potential [16, 17, 37]. There still remains an unexplained difference for higher *WT1* mRNA expression in clinically diverse prognostic subgroups (including those with good and poor outcomes). We could not discriminate *WT1* isoform in both status in this method. Further studies are needed to resolve this issue [38, 39].

From the analysis of paired 85 AML samples, *WT1* mRNA expression decreased to be below the cut-off value for the majority of patients (83.8 %), and these patients showed good prognosis (5-year OS: 79.4 %). Among the M1, M2, and M3 marrow, there was a significant correlation between *WT1* expression and M1 or M2/M3 marrow after 1st induction chemotherapy. *WT1* expression was considered to effectively reflect the MRD status after chemotherapy. The majority of AML patients with higher *WT1* expression after induction chemotherapy had *FLT3*-ITD and showed poor prognosis in the study. Thus, examining the *WT1* expression after induction therapy may predict patients' clinical outcomes.

The majority of the patients continuously presented with an expression of *WT1*-negative after the three course of chemotherapy; however, re-elevation of *WT1* expression was observed in some patients before haematological relapse was found and thus, showed poor prognoses. However, we also observed re-elevation of *WT1* expression after stem cell transplantation preceded the haematological relapse. We must await furtherer study to investigate which *WT1* expression in bone marrow or peripheral blood is more sensitive for haematological relapse.

We concluded that *WT1* mRNA expression was different in each karyotypic subgroup. *WT1* expression at diagnostic sample does not have any prognostic value; however, *WT1* expression after 1st induction chemotherapy would be associated with minimal residual disease or *FLT3*-ITD. Thus, it could be a good prognostic marker for assessing pediatric AML patients.

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ORIGINAL ARTICLE

RAS mutations are frequent in FAB type M4 and M5 of acute myeloid leukemia, and related to late relapse: a study of the Japanese Childhood AML Cooperative Study Group

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Abstract Mutations in *RAS* are frequent in acute myeloid leukemia (AML), and are thought to contribute to leukemogenesis in a subset of patients; however, their prognostic significance has not been firmly established. One hundred and fifty-seven pediatric patients with AML were analyzed for *NRAS* and *KRAS* mutations around hot spots at codons 12, 13, and 61. Twenty-nine patients (18.5%) had an activating mutation of *RAS*. We found *KRAS* mutations to be more frequent than *NRAS* mutations (18/29, 62.1% of patients with *RAS* mutation), in contrast to previous reports (18–40%). The frequency of *RAS* mutation was higher in French-American-British types M4 and M5 than other types ($P = 0.02$). There were no significant differences in other clinical manifestations or distribution in cytogenetic subgroups, or aberrations of other genes, including *KIT* mutation, *FLT3*-ITD, and *MLL*-PTD, between patients with and without *RAS* mutations. No significant differences were observed in the 3-year overall survival and disease-free survival; however, the presence of *RAS* mutation was related to late relapse. The occurrence of clinical events at relatively late period should be monitored for in AML patients with mutations in *RAS*.

Keywords Acute myeloid leukemia · *RAS* · Late relapse · Prognosis · FAB

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