2.3 Statistical methods

OS, PFS, and EFS were estimated by using the Kaplan–Meier method. Efficacy endpoints were compared by patient age (≥60 years and <60 years), and by average daily dose of imatinib (i.e., ≥350 mg/day, 250 to <350 mg/day and <250 mg/day). The Wilcoxon two-sample test was used to compare the average daily dose between the age groups. In patients who had achieved CCyR, CCyR duration was compared by average daily dose of imatinib after achieving CCyR. Average daily dose was calculated as cumulative dosage divided by the total days on study.

3 Results

3.1 Patients

A total of 107 patients were enrolled in the study between November 2002 and June 2004, and administered imatinib. All patients were evaluable for efficacy and safety, and the median duration of imatinib exposure was 1091 days (range, 82–1156 days). Among these patients, 83 completed 3 years of study treatment, whereas 24 discontinued the study due to adverse events ($n = 7$), withdrawal of consent ($n = 5$), insufficient efficacy ($n = 4$), allogeneic bone marrow transplantation ($n = 4$), and other reasons ($n = 4$). Demographic characteristics of patients in the full analysis set are summarized in Table 1. The median age was 47 years (range, 16–74 years), with 71 males and 36 females. Prior therapies for CML included hydroxycarbamide ($n = 7$), and leukapheresis ($n = 1$). The median time from diagnosis of CML to initiation of imatinib was 8.0 days (range, 1–1526 days).

The initial dose of imatinib was 400 mg/day for all patients. The mean (±standard deviation) dose administered during the study was 343 (±90) mg/day. Dose modification was required in 70.1% of patients mainly due to adverse events. Details of dose modification are summarized in Table 2. There were no patients in whom IFN was added to imatinib. Average daily doses were ≥350 mg, 250 to <350 mg, and <250 mg in 68 (63.6%), 21 (19.6%) and 18 patients (16.8%), respectively. As shown in Table 3, the percentage of patients who continued imatinib at 400 mg/day without any dose modification was 48.6% during week 1–13, 57.5% during week 14–26, and was around 60% thereafter.

3.2 Treatment results

The cumulative rate of CHR was 99.1% at 1 year, and the cumulative rates of MCyR and CCyR were 90.9 and 90.2% at 3 years, respectively (Fig. 1). The median time to CHR

Table 1 Patient characteristics

Characteristics	Category	Number of patients
Total number of subjects		107
Sex	Male	71 (66.4)
	Female	36 (33.6)
Age	10s	4 (3.7)
	20s	10 (9.3)
	30s	24 (22.4)
	40s	18 (16.8)
	50s	26 (24.3)
	60s	19 (17.8)
	70s	6 (5.6)
	Mean ± SD	47.1 ± 14.7
	Minimum–maximum	16–74
	Median	47.0
Body weight	40 to <50 kg	13 (12.1)
	50 to <60 kg	34 (31.8)
	60 to <70 kg	40 (37.4)
	70 to <80 kg	13 (12.1)
	80 to <90 kg	5 (4.7)
	≥90 kg	2 (1.9)
	Mean ± SD	61.66 ± 10.88
	Minimum–maximum	43.0–103.0
	Median	61.50
Body surface area	1.2 to <1.4 m^2	5 (4.7)
	1.4 to <1.6 m^2	31 (29.0)
	1.6 to <1.8 m^2	53 (49.5)
	1.8 to <2.0 m^2	15 (14.0)
	≥2.0 m^2	3 (2.8)
	Mean ± SD	1.6705 ± 0.1670
	Minimum–maximum	1.307–2.151
	Median	1.6800
Previous CML therapy	No	99 (92.5)
	Yes	8 (7.5)
	Hydroxycarbamide	7 (6.5)
	Leukapheresis	1 (0.9)
ECOG performance status	0	95 (88.8)
	1	10 (9.3)
	2	2 (1.9)
Time elapsed from the first day of CML diagnosis to the start of study treatment	<4 weeks	92 (86.0)
	4 to <13 weeks	14 (13.1)
	≥52 weeks	1 (0.9)
	Mean ± SD	27.0 ± 146.9
	Minimum–maximum	1–1526
	Median	8.0

Values within parenthesis are given in percentage

SD standard deviation, *CML* chronic myeloid leukemia, *ECOG* Eastern Cooperative Oncology Group

and CCyR were 92.5 days (range, 75–207 days) and 179.5 days (range, 70–589 days), respectively. In 92 patients who had achieved CCyR, 77 patients remained in CCyR until the end of 3 years of imatinib treatment. All of the 15 patients who hadn't achieved CCyR discontinued the study. Among them, 4 patients progressed to AP or BC, and 5 patients proceeded to hematopoietic stem cell transplantation.

Of 107 patients, progression to AP or BC and death occurred in nine and seven patients, respectively. One death, which was because of pneumonia, was reported during the study and the remaining six deaths were reported after patients discontinued the study. The probabilities of OS, PFS and EFS at 3 years were 93.2% [95% confidence interval (CI) 88.3–98.1%), 91.4% (95% CI 86.1–96.8%), and 81.9% (95% CI 74.6–89.3%), respectively (Fig. 2).

3.3 Response and survival by average daily dose

Next, we evaluated cumulative CCyR rate, OS, PFS, and EFS according to the average daily dose of imatinib (≥350 mg/day, 250 to <350 mg/day, and <250 mg/day). As shown in Figs. 3, 4, CCyR and EFS were significantly associated with the average daily dose ($p < 0.001$, respectively). In particular, patients with the average daily dose <250 mg had low rates of CCyR and EFS. CCyR duration was also significantly different according to the average daily dose ($p < 0.001$, Fig. 5). OS and PFS seemed lower in those with lower average daily dose, although the differences did not reach statistical significance.

The average daily doses were significantly different by age group, with 360 (±81) mg in patients aged <60, and 287 (±97) mg in patients aged ≥60 years ($p < 0.001$). Patients aged <60 had statistically non-significant better EFS than those aged ≥60 years (85.3 vs. 70.6% at 3 years, $p = 0.101$). In terms of OS or PFS, there were no significant differences between the age groups.

Table 2 Summary of dose modification

	n	%
No dose change	32	29.9
Dose change	75	70.1
Reduction only	7	6.5
Reduction and interruption	43	40.2
Reduction and increase	1	0.9
Increase only	1	0.9
Increase and interruption	4	3.7
Interruption only	19	17.8
Total	107	100.0

3.4 Adverse events

Adverse events were reported in all of the 107 patients. Serious adverse events which developed in ≥2 patients included neutropenia ($n = 4$), blast crisis ($n = 3$), anemia, intestinal obstruction, gastric antral vascular ectasia, appendicitis, herpes zoster, thrombocytopenia, and leukocytopenia ($n = 2$, each). Grade ≥3 adverse events were reported in 31 patients (29.0%, 47 episodes). As listed in Table 4, grade ≥3 adverse events reported in >5% of patients were neutropenia (31.8%), leukocytopenia (19.6%), lymphocytopenia (17.8%), thrombocytopenia (14.0%), and rash (8.4%). When frequencies of adverse events were compared between this study and the IRIS study [3], nasopharyngitis, rash, upper respiratory tract infection, pyrexia, and grade ≥3 neutropenia seemed more frequent, while nausea, muscle cramp, joint pain seemed less frequent in our study.

4 Discussion

Although it is widely accepted that imatinib is the standard treatment for CP-CML, published experiences of imatinib

Table 3 Average daily dose of imatinib over time

Week:	1–13		14–26		27–39		40–52		53–78		79–104		105–130		131–156	
No. of patients (*n*):	107		106		102		95		92		90		88		88	
Average daily dose (mg)	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
<200	3	2.8	12	11.3	9	8.8	7	7.4	6	6.5	4	4.4	3	3.4	1	1.1
200 to <300	24	22.4	16	15.1	11	10.8	7	7.4	9	9.8	9	10.0	9	10.2	14	15.9
300 to <350	13	12.1	12	11.3	14	13.7	18	18.9	12	13.0	12	13.3	16	18.2	12	13.6
350 to <400	15	14.0	4	3.8	4	3.9	0	0.0	5	5.4	5	5.6	4	4.5	6	6.8
400	52	48.6	61	57.5	61	59.8	61	64.2	58	63.0	58	64.4	54	61.4	52	59.1
>400	0	0.0	1	0.9	3	2.9	2	2.1	2	2.2	2	2.2	2	2.3	3	3.4

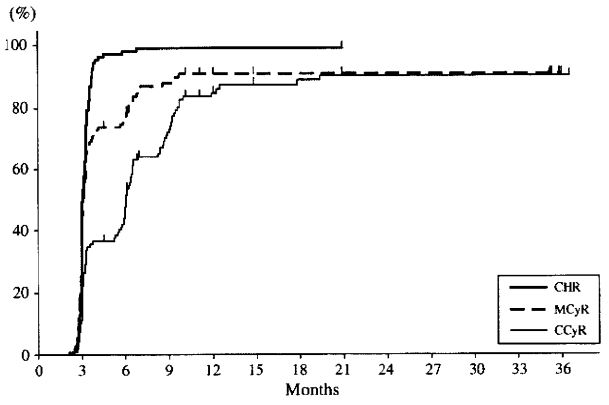
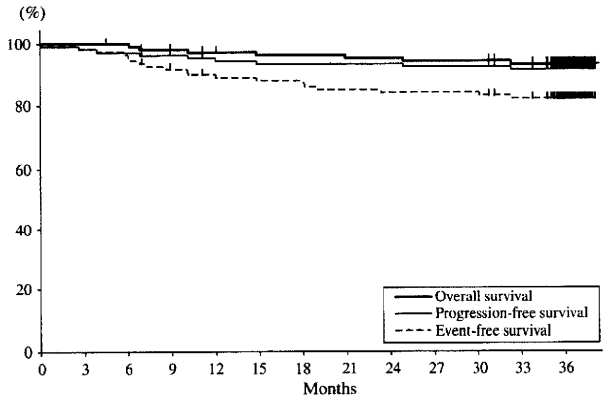


Fig. 1 Kaplan–Meier curves of cumulative rates of complete hematologic response (*CHR*), major cytogenetic response (*MCyR*) and complete cytogenetic response (*CCyR*)



	No. of Events	Estimated 3-year rate (%)
OS	7	93.2
PFS	9	91.4
EFS	19	81.9

Fig. 2 Kaplan–Meier curves of overall survival, progression-free survival and event-free survival of all patients

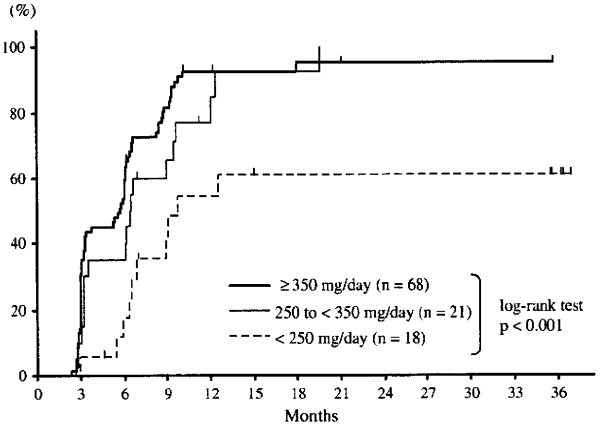


Fig. 3 Kaplan–Meier curves of cumulative rates of complete cytogenetic response (CCyR) by average daily dose of imatinib

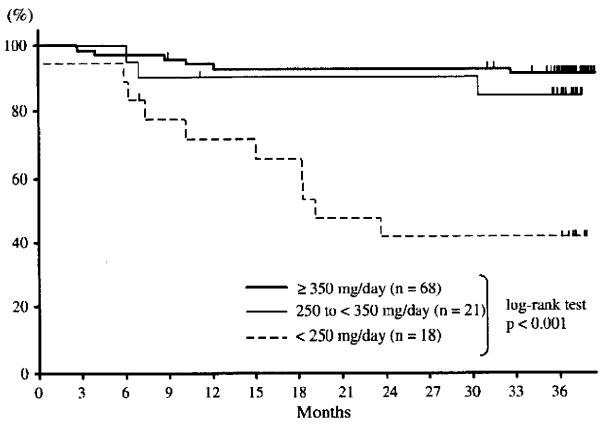


Fig. 4 Kaplan–Meier curves of event-free survival by average daily dose of imatinib

in Japanese patients are limited [8, 10–16]. Under such circumstances, a nationwide registration system for CML has been established by the Japanese Society of Hematology since 2003, and early results were published [15]. To further clarify the clinical utility of imatinib among Japanese patients, we conducted a prospective study of imatinib in 109 patients with newly diagnosed CP-CML. MCyR and CCyR rates at 12 months were 90.9 and 84.8%, which were comparable or even superior to those in the IRIS study (85 and 69%, respectively) [9]. Likewise, long-term outcomes were not different between both studies, because the OS rate in our study was 93.2% at 3 years, whereas, in the IRIS study, it was reported to be 97.2% at 18 months and 89% at 5 years [3, 9]. The safety profile observed in our study was almost comparable with that of the IRIS study, although grade ≥3 neutropenia occurred relatively frequently in our study than in the IRIS study (31.8 vs. 14.3%), while the incidences of neutropenia of all grades were not different (53.3% in our study versus 60.8% in the IRIS study). In both studies, imatinib was initiated at a daily dose of 400 mg and interrupted in the event of grade ≥3 neutropenia or thrombocytopenia until the toxicity resolved to grade <2. The reason for this observation was not clear; however, the finding that only seven of our patients discontinued the study due to adverse events showed feasibility of the treatment. Some non-hematological adverse events like nausea, muscle cramp, and joint pain were less frequent in Japanese than in Caucasians. These efficacy and safety results, taken together, confirmed the clinical utility of imatinib in Japanese patients with newly diagnosed CP-CML.

Based upon observations in a relatively small number of Japanese patients, some authors have suggested the possibility that the daily dose of imatinib could be reduced to less than 400 mg without significant disadvantage, partly

due to smaller body size as compared with Caucasians [12, 13]. Analyses of cumulative rate of CCyR and EFS by average daily dose in our study showed that patients given higher average daily doses of imatinib (≥350 mg) not only achieved higher CCyR rate but also had longer CCyR duration than those given lower average daily doses. EFS was also superior among patients who were treated with higher average daily doses of imatinib. Matsuo et al. [10] reported similar findings of a clear dose–response relationship between imatinib daily dose and treatment results. In that study, CCyR rate at 30 months was higher in patients receiving daily dose of imatinib >300 mg than in those receiving 250–300 mg, or <250 mg. Sugita et al. [16] also reported that mean daily doses of ≥300 mg led to higher CCyR rate, longer CCyR duration, and improved OS as compared to 200–300 mg. These results, taken together, suggest detrimental effect of low average daily dose on treatment results. Our observation that EFS was relatively lower in patients aged ≥60 years than in those aged <60 years might be explained partly by the difference in the average daily dose. To achieve and maintain better response, it would be beneficial to avoid excessive dose

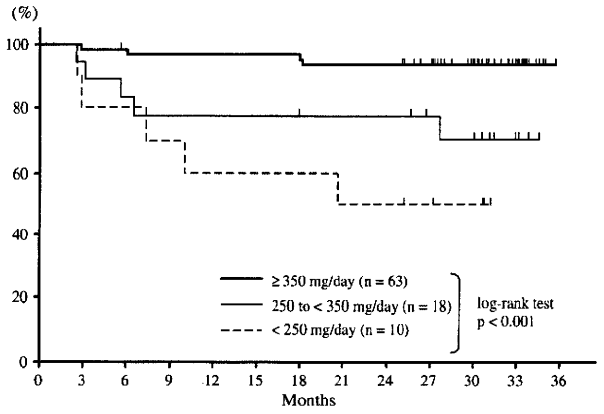


Fig. 5 Kaplan–Meier curves of duration of complete cytogenetic response (CCyR)

Table 4 Comparison of adverse events between this study and the IRIS study

	This study (n = 107)				IRIS study (n = 533) [3]			
	All grades		Grade 3/4		All grades		Grade 3/4	
	n	%	n	%	n	%	n	%
Hematological								
Neutropenia	57	53.3	34	31.8	324	60.8	76	14.3
Leukocytopenia	51	47.7	21	19.6	NR	NR	NR	NR
Lymphocytopenia	48	44.9	19	17.8	NR	NR	NR	NR
Thrombocytopenia	44	41.1	15	14.0	302	56.6	42	7.8
Anemia	33	30.8	3	2.8	238	44.6	17	3.1
Nonhematological								
Surficial edema	71	66.4	0	0.0	296	55.5	5	0.9
Nasopharyngitis	70	65.4	0	0.0	117	22.0	0	0.0
Rash	64	59.8	9	8.4	181	33.9	11	2.0
Diarrhea	44	41.1	3	2.8	175	32.8	10	1.8
Gastroenteritis	37	34.6	3	2.8	NR	NR	NR	NR
Nausea	35	32.7	0	0.0	233	43.7	4	0.7
Malaise	29	27.1	0	0.0	184	34.5	6	1.1
Myalgia	27	25.2	2	1.9	114	21.4	8	1.5
Upper respiratory tract infection	27	25.2	0	0.0	77	14.5	1	0.2
Muscle cramps	26	24.3	0	0.0	204	38.3	7	1.3
Pyrexia	26	24.3	0	0.0	70	13.1	4	0.7
Headache	23	21.5	0	0.0	166	31.2	2	0.4
Dizziness	17	15.9	0	0.0	77	14.5	5	0.9
Vomiting	16	15.0	0	0.0	90	16.9	8	1.5
Joint pain	14	13.1	0	0.0	151	28.3	13	2.4
Cough	13	12.1	0	0.0	77	14.5	1	0.2
Anorexia	11	10.3	0	0.0	28	5.3	0	0.0
Pruritus	11	10.3	0	0.0	39	7.3	1	0.2

NR not reported

reduction and interruption with careful monitoring of safety in individual patients. A similar concept was advocated by a study reported by Kanda et al. [14].

In summary, this prospective study confirmed remarkable efficacy and safety of imatinib in Japanese patients with newly diagnosed CP-CML. It also suggested a clear relationship between higher daily doses of imatinib (i.e., $\geq$350 mg) and better treatment results.

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ORIGINAL ARTICLE: CLINICAL

Prognostic potential of detection of WT1 mRNA level in peripheral blood in adult acute myeloid leukemia

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Abstract

We retrospectively analyzed the potential of Wilms' tumor gene 1 (WT1) mRNA levels in peripheral blood for predicting the prognosis of 50 patients with AML. After achieving complete remission (CR), 34 patients (69.4%) were determined to be positive and 15 (30.6%) were negative for WT1. The relapse rate of the positive and negative patients was 73.5% and 40.0% ($p = 0.02$), respectively. After consolidation therapy, only 15 patients (32.6%) were positive and 31 (67.4%) were negative for WT1. Although the relapse rate of the positive and negative patients was 80.0% and 54.8% ($p = 0.10$), respectively, the rate of relapse within 1 year was 73.3% in positive patients and only 33.3% in negative patients ($p = 0.01$), respectively. The disease-free survival (DFS) rate at 3 years was 20.0% for positive patients and 50.0% for negative patients ($p = 0.01$). The overall survival (OS) rate at 3 years was 42.8% in positive patients and 69.8% in negative patients ($p = 0.04$), respectively. WT1 mRNA levels in the peripheral blood can predict relapse after CR, and its levels after consolidation therapy are closely correlated with DFS, OS, and early relapse.

Keywords: *AML, WT1, prognosis, real-time RT-PCR, minimal residual disease*

Introduction

Approximately 70–80% of newly diagnosed patients with adult acute myeloid leukemia (AML) achieve a complete remission (CR) when treated with anthracyclines such as daunorubicin (DNR) or idarubicin (IDR), and cytosine arabinoside (Ara-C); however, relapse is common, and only about one-third of these patients remain free of disease for more than 5 years [1–5]. If left untreated, almost all patients in CR will suffer relapse and die [6]. The leukemic clone may regrow on account of persisting leukemic cells and cause relapse after CR. Therefore, minimal residual disease (MRD) is the most reliable marker for prognosis. If a subset of patients with a high risk of relapse are identified, these patients can be treated with intensified chemotherapy or stem cell transplant (SCT). However, MRD cannot be detected by standard methods, and therefore more sensitive methods such as polymerase chain reaction (PCR) or multi-dimensional flow cytometry are needed. Since half of AML cases do not contain any leukemia-specific genetic alteration, samples from these patients cannot be subjected to PCR. Recent reports suggest that adult AML is characterized by high expression of Wilms' tumor gene 1 (WT1), and that the monitoring of WT1 expression might be an appropriate method to detect MRD [7].

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The prognostic importance and the strong correlation of WT1 mRNA levels with the incidence of relapse and survival have been reported recently [8–12]. In most of these studies, WT1 mRNA level in the bone marrow, which contains normal CD34-positive cells expressing WT1 mRNA, was evaluated. Therefore, the background level of normal CD34-positive cells in remission limits the accurate assessment of WT1 mRNA levels in bone marrow cells. Therefore, we evaluated the WT1 mRNA levels in peripheral blood, which rarely contains normal CD34-positive cells and expresses low or undetectable levels of WT1.

In this study, we focused on the evaluation of WT1 expression in peripheral blood and its clinical significance for determining the relapse and survival of adult patients with AML by using a WT1 mRNA Assay Kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), which produces uniform results across hospitals.

Patients and methods

Patients

From 1 June 2001 to 30 October 2003, the clinical utility of the WT1 mRNA Assay Kit was determined for the early detection of relapse in 191 patients with AML [13]. In this study, we selected subjects who achieved a CR, and also had their WT1 expression analyzed at presentation, after induction (before consolidation), and after the final consolidation therapy. Patients of M3 type or who had undergone SCT before relapse were excluded. Table I shows the characteristics of these patients.

All patients received induction, consolidation, and maintenance therapies according to each institutional standard. Induction therapy consisted of cytarabine or behenoyl cytarabine and anthracyclines (idarubicin or daunorubicin); however, two patients received a separate induction therapy consisting of mitoxantrone or aclarubicin. All patients received three or four courses of consolidation therapy. Consolidation therapy consisted of the Japan Adult Leukemia Study Group (JALSG) post-remission regimen for AML (27/50) or high-dose cytarabine therapy (23/50) [14]. The definition of relapse was established when more than 5% blasts were observed in the marrow, or when blasts were detected in peripheral blood. The study was approved by the ethics committees of the participating institutions. Informed consent was obtained from the patients or their families prior to initiation of the study.

Table I. Patient characteristics.

Selected patients	50
Male/female	28/22
Age, median (range)	56 (22–86 years)
FAB	
M0	0
M1	9
M2	21
M4	12
M5	6
M6	2
M7	0
Karyotype	
Favorable	14
Intermediate	28
Adverse	7
Unknown	1

FAB, French–American–British classification.

Quantification of WT1 mRNA levels

Total RNA was extracted from peripheral blood using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). WT1 mRNA levels were determined using the WT1 mRNA Assay Kit (Otsuka). An outline of the estimation method described in the kit is as follows. One microgram of total RNA was converted into cDNA containing random hexamer using reverse transcriptase, RNase inhibitor, and dNTPs. Quantitative PCR was performed by the TaqMan method according to the previously described procedure [15]. The TaqMan probe was labeled with 6-carboxy-fluorescein phosphoramide (FAM) as the reporter dye at the 5′-end and with carboxy-tetramethyl-rhodamine (TAMRA) as the quencher dye at the 3′-end. For PCR analysis of WT1, 8 μL of cDNA mixture (corresponding to 400 ng of RNA) was used, and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression analysis, 1 μL of mixture (corresponding to 50 ng of RNA) was used. Known copy numbers of WT1 cDNA standard (5×10^3, 5×10^5, and 5×10^7 copies/mL) and GAPDH cDNA standard (5×10^5, 5×10^7, and 5×10^9 copies/mL) were simultaneously amplified by PCR, and standard curves for measuring WT1 and GAPDH mRNA levels in the samples were obtained. The WT1 mRNA level was adjusted according to that of GAPDH.

Procedures for assessing WT1 mRNA expression levels

The WT1 mRNA level was normalized with GAPDH mRNA level as follows. The ratio of WT1 transcripts to GAPDH transcripts was calculated, and the value of (WT1/GAPDH) $\times$ (2.7×10^7) (mean copy number/μg RNA of GAPDH in 94 normal human volunteers) was defined as copies/μg of WT1 RNA expression level in the samples. Next, 1 μg of RNA was dissolved in 20 μL of the reaction

solution and used for reverse transcriptase reactions, as described in the kit. Therefore, the WT1 mRNA expression level was 50 copies/μg RNA at the lower limit of detection (2500 copies/mL). This value was regarded as the 'cut-off' value for assessment of the presence or absence of WT1 mRNA expression. In other words, patients were negative for WT1 if the mRNA levels were less than 50 copies/μg RNA, and were positive if the mRNA levels were equal to or higher than 50 copies/μg RNA.

Statistical analysis

Overall survival (OS) was defined from the date of diagnosis to the date of death. Disease-free survival (DFS) for patients who had achieved CR was defined from the date of CR to the date of the first event (either relapse or death). The Kaplan–Meier method was used to estimate OS and DFS. The log-rank test was used for the comparison of OS and DFS. The χ^2 test was used for ordinal data, such as the detection of WT1 and relapse. For the comparison of time to relapse, the Wilcoxon/Kruskal–Wallis test was used. Statistical analyses were conducted using JMP software (SAS Institute, Inc., Cary, NC).

Results

WT1 mRNA levels according to FAB subtype

WT1 mRNA was detected in 107 of 114 patients (93.9%). The percentage of detection of WT1 mRNA was low in M5 (6/9) and M7 (0/1) types; however, the percentage in other types was high, and ranged from 85.7 to 100%. In 92 of the 94 healthy controls (97.9%), the expression of WT1 mRNA was undetectable.

WT1 mRNA levels during the course of chemotherapy

The median follow-up period was 39 months (range 34–46 months). The median WT1 mRNA level at diagnosis was 48 327 copies/μg RNA (range 137–329 185). After achieving CR, the WT1 mRNA level ranged from <50 copies/μg to 30 732 copies/μg RNA. Thirty-four (69%) patients were positive and 15 (31%) were negative for WT1. After consolidation therapy, the WT1 mRNA level ranged from <50 copies/μg RNA to 49 174 copies/μg RNA. Only 15 patients (33%) were WT1 positive and 31 (67%) were negative.

Prognosis and expression level of WT1 mRNA at diagnosis

The WT1 mRNA levels did not correlate with the relapse rate (Table II) or with DFS [Figure 1(A)] and OS [Figure 1(B)].

Prognostic relevance of detection of WT1 mRNA after CR

Six of the 15 negative patients relapsed after a median 7.6 months (range 3.7–11.7 months). In contrast, 25 of the 34 positive patients relapsed after a median 10.0 months (range 2.6–37.4 months). No significant differences were observed with regard to time to relapse between the negative and positive patients ($p = 0.21$). The relapse rate of positive patients and that of negative patients was 74% and 40% ($p = 0.02$; sensitivity = 81%; specificity = 50%), respectively (Table II). The DFS rate at 3 years was 30% in positive patients and 60% in negative patients ($p = 0.09$) [Figure 2(A)]. The OS rate at 3 years was 53% in positive patients and 79% in negative patients ($p = 0.12$) [Figure 2(B)].

Prognostic relevance of WT1 mRNA detection after consolidation

Seventeen of the 31 negative patients relapsed after a median 10.2 months (range 2.6–37.4 months). In contrast, 12 of the 15 positive patients relapsed after a median 6.3 months (range 3.7–27.0 months). There were significant differences in

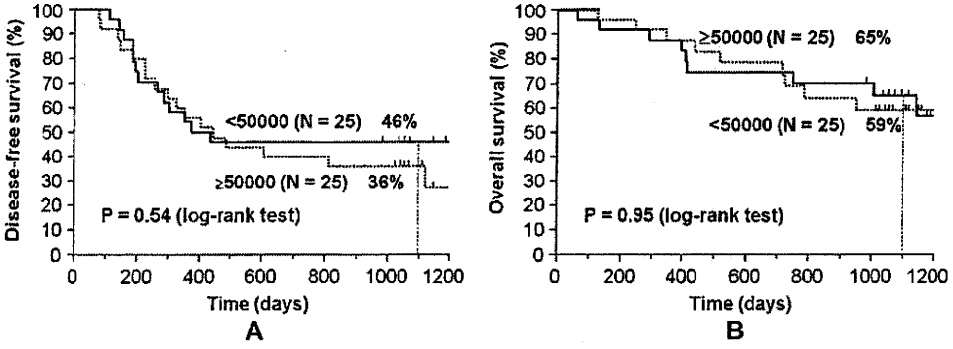

Figure 1. (A) Disease-free survival and (B) overall survival according to the expression level of WT1 mRNA at diagnosis.

time to relapse between the negative and positive patients ($p=0.04$).

The relapse rate of positive patients and that of negative patients was 80% and 55% ($p=0.10$), respectively (Table II).

The DFS rate at 3 years was 20% in positive patients and 50% in negative patients ($p=0.01$) [Figure 3(A)]. The rate of relapse within 1 year was 73% in positive patients and only 33% in negative patients ($p=0.01$). The OS rate at 3 years was 43% in positive patients and 70% in negative patients ($p=0.04$) [Figure 3(B)].

Table II. Relapse and expression level of WT1 mRNA.

Status	WT1 level	Relapse rate	*p*-Value
At diagnosis	<50 000 copies/μg RNA	14/25 (56%)	0.38
	≥50 000 copies/μg RNA	17/25 (68%)	
After CR	<50 copies/μg RNA	6/15 (40%)	0.02
	≥50 copies/μg RNA	25/34 (74%)	
After consolidation	<50 copies/μg RNA	17/31 (55%)	0.10
	≥50 copies/μg RNA	12/15 (80%)	

CR, complete remission.

Discussion

MRD in AML is one of the most important prognostic factors, and the evaluation of MRD by reverse transcriptase (RT)-PCR amplification of chromosome translocations is the most sensitive and reliable method. However, more than 50% of patients with AML lack a known chromosomal abnormality or genetic lesions suitable for MRD determination. Thus, multi-dimensional flow cytometry has been used for MRD measurement. However, this method is not popular because of technical complexity, non-uniform values, and changes in expression of surface markers.

In the light of this situation, WT1 mRNA would be an efficient marker for MRD. In this study, we evaluated WT1 mRNA levels in peripheral blood using the WT1 mRNA Assay Kit (Otsuka). Previously, we reported that 107 of 114 untreated patients with AML were positive for WT1 mRNA in the peripheral blood; levels of WT1 mRNA were reduced to fewer than 50 copies/μg RNA ('negative')

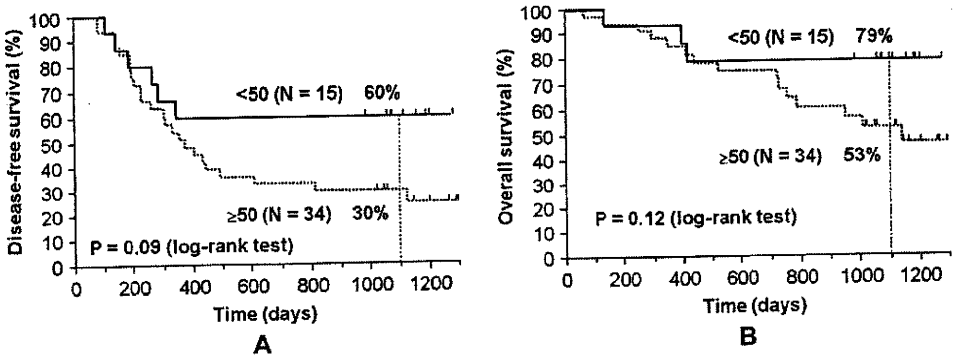

Figure 2. (A) Disease-free survival and (B) overall survival according to the detection of WT1 mRNA after CR.

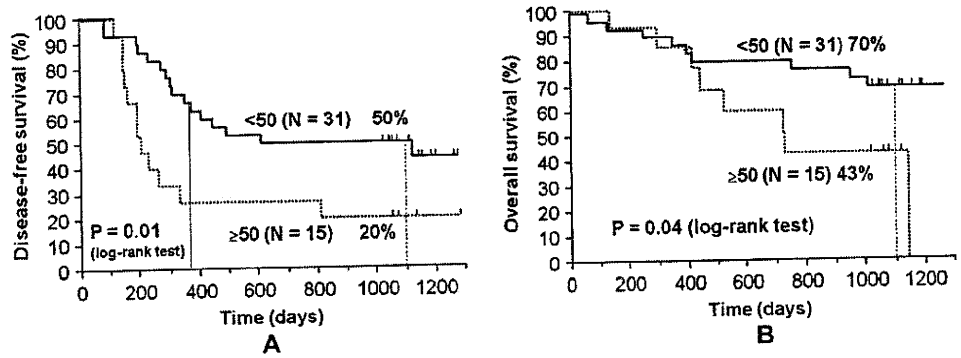

Figure 3. (A) Disease-free survival according to the detection of WT1 mRNA after consolidation. The solid line indicates the rate of relapse within 1 year after the date of CR: WT1 positive patients, 73%; WT1 negative patients, 33% ($p=0.01$, χ^2 test). (B) Overall survival according to the detection of WT1 mRNA after consolidation.

after achieving remission and then increased again when the disease relapsed. However, some reports [16,17] indicate that WT1 mRNA is not a reliable marker for MRD. This discrepancy may have arisen mainly because normal hematopoietic progenitor cells also express WT1 mRNA. Weisser *et al.* [9] evaluated WT1 mRNA in bone marrow and reported that high levels of WT1 mRNA were associated with short OS and event-free survival (EFS) within the intervals of 61–120 and 120–180 days after the start of chemotherapy, but not at diagnosis and from days 16 to 60. They further insisted that future studies to investigate the applicability of peripheral blood as the test material were necessary. Therefore, we evaluated the WT1 mRNA level in peripheral blood, because peripheral blood does not contain normal progenitor cells. Until now, only Cilloni *et al.* [18] and Nowakowska-Kopera *et al.* [19] have analyzed WT1 mRNA levels in peripheral blood instead of bone marrow for MRD.

Our results showed that WT1 mRNA levels at diagnosis did not correlate with the relapse rate or with DFS and OS. Cilloni *et al.* [18] also reported that no significant differences were observed in WT1 mRNA levels at diagnosis between patients with CR and those without CR, and patients who persisted in CR and those who relapsed. Inoue *et al.* [7] reported that high expression of WT1 was an adverse prognostic factor for AML. Conversely, it was also reported that WT1 mRNA levels were remarkably high in patients with favorable cytogenetics [10]. Hence, WT1 mRNA level at diagnosis is not an adverse prognostic factor.

Our results indicated that patients with positive WT1 mRNA at achievement of CR tended to relapse, in comparison with WT1 mRNA negative patients. Cilloni *et al.* [18] also reported that all patients in CR whose WT1 mRNA levels were above normal relapsed. Moreover, Lapillonne *et al.* [10] demonstrated that an elevated WT1 mRNA level (2 SD greater than that of normal control bone marrow) after induction is an independent prognostic risk factor for relapse and death. Therefore, in order to prevent relapse, more intensified consolidation therapy such as high-dose Ara-C or SCT is recommended for WT1 mRNA positive patients upon achievement of CR.

Until now, there have been only two reports on WT1 mRNA levels after consolidation therapy [12,19]. In this study, we found that the detection of WT1 mRNA after consolidation therapy is a poor prognostic factor for DFS or OS. Interestingly, WT1 positive patients suffered relapse after consolidation therapy earlier than negative patients, and the majority of the former suffered relapse within 1 year. The median CR duration of relapsed patients among the WT1 mRNA positive patients was 6.3 months. It is well known that the most important prognostic factor for relapsed AML is the duration of first CR, and unlike late relapsed patients, early relapsed patients fail to achieve a CR [20]. On the other hand, the results of SCT for patients in first CR did not differ from those of patients in second CR, and the results of SCT for patients in first or second CR differed considerably from those of patients without CR. On the basis of these data, WT1 positive patients should be recommended SCT after consolidation therapy in the first CR. In other words, we could define suitable patients and appropriate timing for SCT according to evaluation of the WT1 mRNA level in the peripheral blood after consolidation therapy.

In conclusion, we have shown that the detection of WT1 mRNA in the peripheral blood at achieving CR is closely correlated with the relapse rate, and the detection of WT1 mRNA after consolidation therapy indicates an early relapse and short survival. Although our results need to be confirmed with a large-scale prospective study, we consider that the detection of WT1 mRNA in the peripheral blood is essential for the prevention of relapse by therapeutic interventions such as intensified consolidation chemotherapy or SCT in CR. In a large-scale prospective study, the detection of WT1 mRNA in peripheral blood using the WT1 mRNA Assay Kit (Otsuka) can provide uniform results across hospitals, and this method is less stressful for patients.

Acknowledgements

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix. Names of leaders of the institutions who contributed to this study but are not included among the authors of this article (based on information at time of study)

No.	Name of institution and department	Investigator		Contract period (trial period*)
		Title	Name	
1	Department of Cell Therapy and Transplantation Medicine, Faculty of Medicine, The University of Tokyo Hospital	Lecturer	Shigeru Chiba	2001/06/21–2003/06/30 (2002/05/02–2003/05/19)
2	Department of Hematology and Cell Therapy, Aichi Cancer Center	Director	Yasuo Morishima	2001/08/01–2004/03/31 (2002/02/13–2003/07/30)
3	Internal Medicine, Nissay Hospital	Deputy Director	Masashi Nakagawa	2002/01/31–2003/09/30 (2002/04/22–2003/06/19)
4	Division of Hematology, Yaizu City Hospital	Director	Tadashi Tobita	2002/02/01–2003/09/30 (2002/02/21–2003/04/22)
5	Internal Medicine III, Hamamatsu University School of Medicine	Associate Professor	Kazunori Onishi	2001/08/20–2003/09/30 (2001/10/23–2003/05/21)
6	Department of Hematology and Oncology, Osaka University	Associate Professor	Hiroyasu Ogawa	2001/09/13–2003/12/31 (2001/12/12–2003/08/14)
7	Internal Medicine, Ogaki Municipal Hospital	Head	Hirofumi Kosugi	2002/02/20–2003/09/30 (2002/03/15–2003/08/12)
8	Internal Medicine, Nagoya National Hospital	Head	Motohiro Hamaguchi	2002/04/01–2003/03/31 (2002/04/25–2003/01/14)
9	Internal Medicine I, Tokyo Medical College	Professor	Kazuma Oyashiki	2001/11/15–2003/09/30 (2002/01/15–2003/08/19)

*Date of collection of samples from first patient–date of collection of samples from last patient.

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Prognostic potential of detection of WT1 mRNA level in peripheral blood in adult acute myeloid leukemia

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Abstract

We retrospectively analyzed the potential of Wilms' tumor gene 1 (WT1) mRNA levels in peripheral blood for predicting the prognosis of 50 patients with AML. After achieving complete remission (CR), 34 patients (69.4%) were determined to be positive and 15 (30.6%) were negative for WT1. The relapse rate of the positive and negative patients was 73.5% and 40.0% ($p = 0.02$), respectively. After consolidation therapy, only 15 patients (32.6%) were positive and 31 (67.4%) were negative for WT1. Although the relapse rate of the positive and negative patients was 80.0% and 54.8% ($p = 0.10$), respectively, the rate of relapse within 1 year was 73.3% in positive patients and only 33.3% in negative patients ($p = 0.01$), respectively. The disease-free survival (DFS) rate at 3 years was 20.0% for positive patients and 50.0% for negative patients ($p = 0.01$). The overall survival (OS) rate at 3 years was 42.8% in positive patients and 69.8% in negative patients ($p = 0.04$), respectively. WT1 mRNA levels in the peripheral blood can predict relapse after CR, and its levels after consolidation therapy are closely correlated with DFS, OS, and early relapse.

Keywords: *AML, WT1, prognosis, real-time RT-PCR, minimal residual disease*

Introduction

Approximately 70–80% of newly diagnosed patients with adult acute myeloid leukemia (AML) achieve a complete remission (CR) when treated with anthracyclines such as daunorubicin (DNR) or idarubicin (IDR), and cytosine arabinoside (Ara-C); however, relapse is common, and only about one-third of these patients remain free of disease for more than 5 years [1–5]. If left untreated, almost all patients in CR will suffer relapse and die [6]. The leukemic clone may regrow on account of persisting leukemic cells and cause relapse after CR. Therefore, minimal residual disease (MRD) is the most reliable marker for prognosis. If a subset of patients with a high risk of relapse are identified, these patients can be treated with intensified chemotherapy or stem cell transplant (SCT). However, MRD cannot be detected by standard methods, and therefore more sensitive methods such as polymerase chain reaction (PCR) or multi-dimensional flow cytometry are needed. Since half of AML cases do not contain any leukemia-specific genetic alteration, samples from these patients cannot be subjected to PCR. Recent reports suggest that adult AML is characterized by high expression of Wilms' tumor gene 1 (WT1), and that the monitoring of WT1 expression might be an appropriate method to detect MRD [7].

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The prognostic importance and the strong correlation of WT1 mRNA levels with the incidence of relapse and survival have been reported recently [8–12]. In most of these studies, WT1 mRNA level in the bone marrow, which contains normal CD34-positive cells expressing WT1 mRNA, was evaluated. Therefore, the background level of normal CD34-positive cells in remission limits the accurate assessment of WT1 mRNA levels in bone marrow cells. Therefore, we evaluated the WT1 mRNA levels in peripheral blood, which rarely contains normal CD34-positive cells and expresses low or undetectable levels of WT1.

In this study, we focused on the evaluation of WT1 expression in peripheral blood and its clinical significance for determining the relapse and survival of adult patients with AML by using a WT1 mRNA Assay Kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), which produces uniform results across hospitals.

Patients and methods

Patients

From 1 June 2001 to 30 October 2003, the clinical utility of the WT1 mRNA Assay Kit was determined for the early detection of relapse in 191 patients with AML [13]. In this study, we selected subjects who achieved a CR, and also had their WT1 expression analyzed at presentation, after induction (before consolidation), and after the final consolidation therapy. Patients of M3 type or who had undergone SCT before relapse were excluded. Table I shows the characteristics of these patients.

All patients received induction, consolidation, and maintenance therapies according to each institutional standard. Induction therapy consisted of cytarabine or behenoyl cytarabine and anthracyclines (idarubicin or daunorubicin); however, two patients received a separate induction therapy consisting of mitoxantrone or aclarubicin. All patients received three or four courses of consolidation therapy. Consolidation therapy consisted of the Japan Adult Leukemia Study Group (JALSG) post-remission regimen for AML (27/50) or high-dose cytarabine therapy (23/50) [14]. The definition of relapse was established when more than 5% blasts were observed in the marrow, or when blasts were detected in peripheral blood. The study was approved by the ethics committees of the participating institutions. Informed consent was obtained from the patients or their families prior to initiation of the study.

Table I. Patient characteristics.

Selected patients	50
Male/female	28/22
Age, median (range)	56 (22–86 years)
FAB	
M0	0
M1	9
M2	21
M4	12
M5	6
M6	2
M7	0
Karyotype	
Favorable	14
Intermediate	28
Adverse	7
Unknown	1

FAB, French–American–British classification.

Quantification of WT1 mRNA levels

Total RNA was extracted from peripheral blood using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). WT1 mRNA levels were determined using the WT1 mRNA Assay Kit (Otsuka). An outline of the estimation method described in the kit is as follows. One microgram of total RNA was converted into cDNA containing random hexamer using reverse transcriptase, RNase inhibitor, and dNTPs. Quantitative PCR was performed by the TaqMan method according to the previously described procedure [15]. The TaqMan probe was labeled with 6-carboxy-fluorescein phosphoramide (FAM) as the reporter dye at the 5′-end and with carboxy-tetramethyl-rhodamine (TAMRA) as the quencher dye at the 3′-end. For PCR analysis of WT1, 8 μL of cDNA mixture (corresponding to 400 ng of RNA) was used, and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression analysis, 1 μL of mixture (corresponding to 50 ng of RNA) was used. Known copy numbers of WT1 cDNA standard (5×10^3, 5×10^5, and 5×10^7 copies/mL) and GAPDH cDNA standard (5×10^5, 5×10^7, and 5×10^9 copies/mL) were simultaneously amplified by PCR, and standard curves for measuring WT1 and GAPDH mRNA levels in the samples were obtained. The WT1 mRNA level was adjusted according to that of GAPDH.

Procedures for assessing WT1 mRNA expression levels

The WT1 mRNA level was normalized with GAPDH mRNA level as follows. The ratio of WT1 transcripts to GAPDH transcripts was calculated, and the value of (WT1/GAPDH) $\times$ (2.7×10^7) (mean copy number/μg RNA of GAPDH in 94 normal human volunteers) was defined as copies/μg of WT1 RNA expression level in the samples. Next, 1 μg of RNA was dissolved in 20 μL of the reaction

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solution and used for reverse transcriptase reactions, as described in the kit. Therefore, the WT1 mRNA expression level was 50 copies/μg RNA at the lower limit of detection (2500 copies/mL). This value was regarded as the 'cut-off' value for assessment of the presence or absence of WT1 mRNA expression. In other words, patients were negative for WT1 if the mRNA levels were less than 50 copies/μg RNA, and were positive if the mRNA levels were equal to or higher than 50 copies/μg RNA.

Statistical analysis

Overall survival (OS) was defined from the date of diagnosis to the date of death. Disease-free survival (DFS) for patients who had achieved CR was defined from the date of CR to the date of the first event (either relapse or death). The Kaplan–Meier method was used to estimate OS and DFS. The log-rank test was used for the comparison of OS and DFS. The χ^2 test was used for ordinal data, such as the detection of WT1 and relapse. For the comparison of time to relapse, the Wilcoxon/Kruskal–Wallis test was used. Statistical analyses were conducted using JMP software (SAS Institute, Inc., Cary, NC).

Results

WT1 mRNA levels according to FAB subtype

WT1 mRNA was detected in 107 of 114 patients (93.9%). The percentage of detection of WT1 mRNA was low in M5 (6/9) and M7 (0/1) types; however, the percentage in other types was high, and ranged from 85.7 to 100%. In 92 of the 94 healthy controls (97.9%), the expression of WT1 mRNA was undetectable.

WT1 mRNA levels during the course of chemotherapy

The median follow-up period was 39 months (range 34–46 months). The median WT1 mRNA level at diagnosis was 48 327 copies/μg RNA (range 137–329 185). After achieving CR, the WT1 mRNA level ranged from <50 copies/μg to 30 732 copies/μg RNA. Thirty-four (69%) patients were positive and 15 (31%) were negative for WT1. After consolidation therapy, the WT1 mRNA level ranged from <50 copies/μg RNA to 49 174 copies/μg RNA. Only 15 patients (33%) were WT1 positive and 31 (67%) were negative.

Prognosis and expression level of WT1 mRNA at diagnosis

The WT1 mRNA levels did not correlate with the relapse rate (Table II) or with DFS [Figure 1(A)] and OS [Figure 1(B)].

Prognostic relevance of detection of WT1 mRNA after CR

Six of the 15 negative patients relapsed after a median 7.6 months (range 3.7–11.7 months). In contrast, 25 of the 34 positive patients relapsed after a median 10.0 months (range 2.6–37.4 months). No significant differences were observed with regard to time to relapse between the negative and positive patients ($p=0.21$). The relapse rate of positive patients and that of negative patients was 74% and 40% ($p=0.02$; sensitivity = 81%; specificity = 50%), respectively (Table II). The DFS rate at 3 years was 30% in positive patients and 60% in negative patients ($p=0.09$) [Figure 2(A)]. The OS rate at 3 years was 53% in positive patients and 79% in negative patients ($p=0.12$) [Figure 2(B)].

Prognostic relevance of WT1 mRNA detection after consolidation

Seventeen of the 31 negative patients relapsed after a median 10.2 months (range 2.6–37.4 months). In contrast, 12 of the 15 positive patients relapsed after a median 6.3 months (range 3.7–27.0 months). There were significant differences in

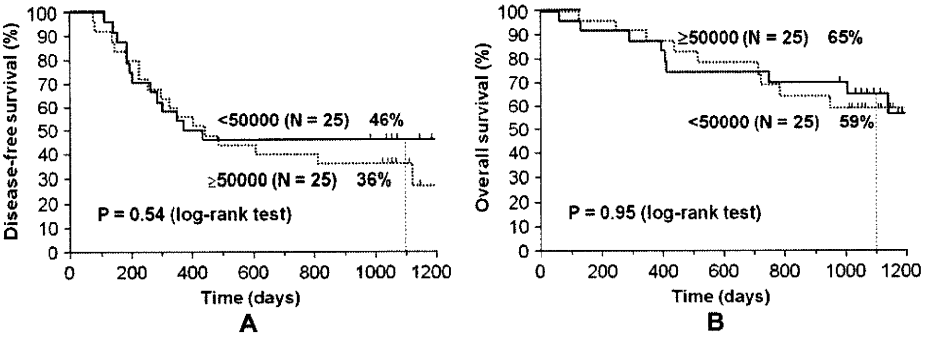

Figure 1. (A) Disease-free survival and (B) overall survival according to the expression level of WT1 mRNA at diagnosis.

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time to relapse between the negative and positive patients ($p = 0.04$).

The relapse rate of positive patients and that of negative patients was 80% and 55% ($p = 0.10$), respectively (Table II).

The DFS rate at 3 years was 20% in positive patients and 50% in negative patients ($p = 0.01$) [Figure 3(A)]. The rate of relapse within 1 year was 73% in positive patients and only 33% in negative patients ($p = 0.01$). The OS rate at 3 years was 43% in positive patients and 70% in negative patients ($p = 0.04$) [Figure 3(B)].

Table II. Relapse and expression level of WT1 mRNA.

Status	WT1 level	Relapse rate	*p*-Value
At diagnosis	<50 000 copies/μg RNA	14/25 (56%)	0.38
	≥50 000 copies/μg RNA	17/25 (68%)	
After CR	<50 copies/μg RNA	6/15 (40%)	0.02
	≥50 copies/μg RNA	25/34 (74%)	
After consolidation	<50 copies/μg RNA	17/31 (55%)	0.10
	≥50 copies/μg RNA	12/15 (80%)	

CR, complete remission.

Discussion

MRD in AML is one of the most important prognostic factors, and the evaluation of MRD by reverse transcriptase (RT)-PCR amplification of chromosome translocations is the most sensitive and reliable method. However, more than 50% of patients with AML lack a known chromosomal abnormality or genetic lesions suitable for MRD determination. Thus, multi-dimensional flow cytometry has been used for MRD measurement. However, this method is not popular because of technical complexity, non-uniform values, and changes in expression of surface markers.

In the light of this situation, WT1 mRNA would be an efficient marker for MRD. In this study, we evaluated WT1 mRNA levels in peripheral blood using the WT1 mRNA Assay Kit (Otsuka). Previously, we reported that 107 of 114 untreated patients with AML were positive for WT1 mRNA in the peripheral blood; levels of WT1 mRNA were reduced to fewer than 50 copies/μg RNA ('negative')

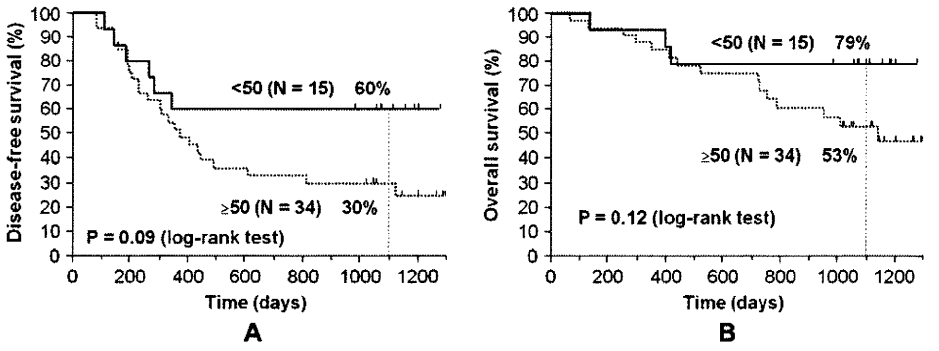

Figure 2. (A) Disease-free survival and (B) overall survival according to the detection of WT1 mRNA after CR.

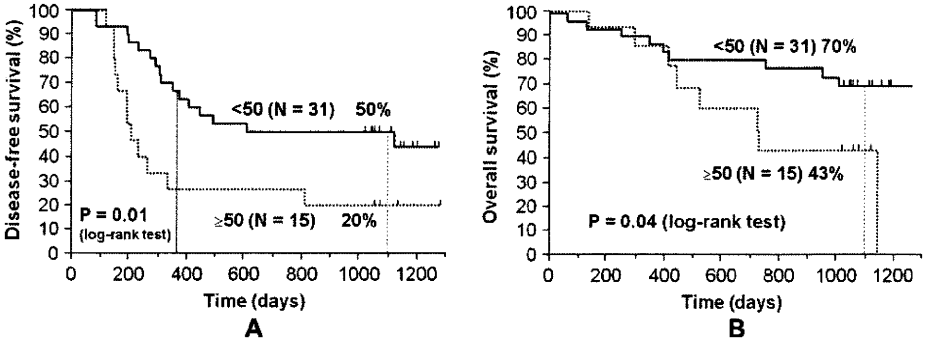

Figure 3. (A) Disease-free survival according to the detection of WT1 mRNA after consolidation. The solid line indicates the rate of relapse within 1 year after the date of CR: WT1 positive patients, 73%; WT1 negative patients, 33% ($p = 0.01$, χ^2 test). (B) Overall survival according to the detection of WT1 mRNA after consolidation.

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after achieving remission and then increased again when the disease relapsed. However, some reports [16,17] indicate that WT1 mRNA is not a reliable marker for MRD. This discrepancy may have arisen mainly because normal hematopoietic progenitor cells also express WT1 mRNA. Weisser *et al.* [9] evaluated WT1 mRNA in bone marrow and reported that high levels of WT1 mRNA were associated with short OS and event-free survival (EFS) within the intervals of 61–120 and 120–180 days after the start of chemotherapy, but not at diagnosis and from days 16 to 60. They further insisted that future studies to investigate the applicability of peripheral blood as the test material were necessary. Therefore, we evaluated the WT1 mRNA level in peripheral blood, because peripheral blood does not contain normal progenitor cells. Until now, only Cilloni *et al.* [18] and Nowakowska-Kopera *et al.* [19] have analyzed WT1 mRNA levels in peripheral blood instead of bone marrow for MRD.

Our results showed that WT1 mRNA levels at diagnosis did not correlate with the relapse rate or with DFS and OS. Cilloni *et al.* [18] also reported that no significant differences were observed in WT1 mRNA levels at diagnosis between patients with CR and those without CR, and patients who persisted in CR and those who relapsed. Inoue *et al.* [7] reported that high expression of WT1 was an adverse prognostic factor for AML. Conversely, it was also reported that WT1 mRNA levels were remarkably high in patients with favorable cytogenetics [10]. Hence, WT1 mRNA level at diagnosis is not an adverse prognostic factor.

Our results indicated that patients with positive WT1 mRNA at achievement of CR tended to relapse, in comparison with WT1 mRNA negative patients. Cilloni *et al.* [18] also reported that all patients in CR whose WT1 mRNA levels were above normal relapsed. Moreover, Lapillonne *et al.* [10] demonstrated that an elevated WT1 mRNA level (2 SD greater than that of normal control bone marrow) after induction is an independent prognostic risk factor for relapse and death. Therefore, in order to prevent relapse, more intensified consolidation therapy such as high-dose Ara-C or SCT is recommended for WT1 mRNA positive patients upon achievement of CR.

Until now, there have been only two reports on WT1 mRNA levels after consolidation therapy [12,19]. In this study, we found that the detection of WT1 mRNA after consolidation therapy is a poor prognostic factor for DFS or OS. Interestingly, WT1 positive patients suffered relapse after consolidation therapy earlier than negative patients, and the majority of the former suffered relapse within 1 year. The median CR duration of relapsed patients among the WT1 mRNA positive patients was 6.3 months. It is well known that the most important prognostic factor for relapsed AML is the duration of first CR, and unlike late relapsed patients, early relapsed patients fail to achieve a CR [20]. On the other hand, the results of SCT for patients in first CR did not differ from those of patients in second CR, and the results of SCT for patients in first or second CR differed considerably from those of patients without CR. On the basis of these data, WT1 positive patients should be recommended SCT after consolidation therapy in the first CR. In other words, we could define suitable patients and appropriate timing for SCT according to evaluation of the WT1 mRNA level in the peripheral blood after consolidation therapy.

In conclusion, we have shown that the detection of WT1 mRNA in the peripheral blood at achieving CR is closely correlated with the relapse rate, and the detection of WT1 mRNA after consolidation therapy indicates an early relapse and short survival. Although our results need to be confirmed with a large-scale prospective study, we consider that the detection of WT1 mRNA in the peripheral blood is essential for the prevention of relapse by therapeutic interventions such as intensified consolidation chemotherapy or SCT in CR. In a large-scale prospective study, the detection of WT1 mRNA in peripheral blood using the WT1 mRNA Assay Kit (Otsuka) can provide uniform results across hospitals, and this method is less stressful for patients.

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Appendix. Names of leaders of the institutions who contributed to this study but are not included among the authors of this article (based on information at time of study)

No.	Name of institution and department	Investigator		Contract period (trial period*)
		Title	Name	
1	Department of Cell Therapy and Transplantation Medicine, Faculty of Medicine, The University of Tokyo Hospital	Lecturer	Shigeru Chiba	2001/06/21–2003/06/30 (2002/05/02–2003/05/19)
2	Department of Hematology and Cell Therapy, Aichi Cancer Center	Director	Yasuo Morishima	2001/08/01–2004/03/31 (2002/02/13–2003/07/30)
3	Internal Medicine, Nissay Hospital	Deputy Director	Masashi Nakagawa	2002/01/31–2003/09/30 (2002/04/22–2003/06/19)
4	Division of Hematology, Yaizu City Hospital	Director	Tadashi Tobita	2002/02/01–2003/09/30 (2002/02/21–2003/04/22)
5	Internal Medicine III, Hamamatsu University School of Medicine	Associate Professor	Kazunori Onishi	2001/08/20–2003/09/30 (2001/10/23–2003/05/21)
6	Department of Hematology and Oncology, Osaka University	Associate Professor	Hiroyasu Ogawa	2001/09/13–2003/12/31 (2001/12/12–2003/08/14)
7	Internal Medicine, Ogaki Municipal Hospital	Head	Hirofumi Kosugi	2002/02/20–2003/09/30 (2002/03/15–2003/08/12)
8	Internal Medicine, Nagoya National Hospital	Head	Motohiro Hamaguchi	2002/04/01–2003/03/31 (2002/04/25–2003/01/14)
9	Internal Medicine I, Tokyo Medical College	Professor	Kazuma Oyashiki	2001/11/15–2003/09/30 (2002/01/15–2003/08/19)

*Date of collection of samples from first patient–date of collection of samples from last patient.

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Notch2 signaling is required for proper mast cell distribution and mucosal immunity in the intestine

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