Long-Term Follow-Up of Minimal Residual Disease in Leukemia Patients by Monitoring WT1 (Wilms Tumor Gene) Expression Levels

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Thirty-one patients (27 with acute myeloid leukemia [AML], 2 with acute lymphocytic leukemia [ALL], and 2 with acute mixed lineage leukemia [AMLL]) treated with conventional chemotherapy (CHT) and 23 patients (13 AML, 5 ALL, and 5 with chronic myeloid leukemia [CML]) treated with allogeneic bone marrow transplantation (BMT) were monitored for WT1 expression levels in BM and peripheral blood (PB) by reverse transcriptase-polymerase chain reaction over a long-term period (mean, 29 months for CHT and 24 months for BMT). Sixteen of the patients in the CHT group and 3 in the BMT group who had achieved complete remission suffered clinical relapse. In 10 of these patients, WT1 expression that had returned to normal BM levels (<10⁻²; the WT1 expression level of K562 cells was defined as 1.0) after complete remission (CR) either gradually or rapidly increased again to abnormal levels 1 to 18 months (mean, 7 months) before clinical relapse became apparent. In another patient, WT1 expression never returned to normal BM levels even after CR and the subsequent relapse was accompanied by a rapid increase in WT1 expression to levels higher than 10⁻² (10⁻³ levels in PB). On the other hand, the remaining 35 patients (15 CHT and 20 BMT) maintained their CR. In 20 of these patients (11 CHT and 18 BMT), WT1 expression either gradually or rapidly decreased to normal BM levels, whereas in the other 6 (4 CHT and 2 BMT), low or very low levels of WT1 mRNAs (10⁻³ to 10⁻² in BM and 10⁻⁵ to 10⁻³ in PB) remain detectable, but without any clinical signs of relapse.

A clear correlation was found to exist between the minimal residual disease (MRD) detected in the paired BM and PB samples for all types of leukemias (AML, ALL, and CML), with MRD in PB being approximately one-tenth of that in BM. WT1 quantitation of 168 paired BM and PB samples showed that PB samples were superior to BM samples for the detection of MRD. We conclude that monitoring of WT1 expression levels in BM and PB makes it possible to rapidly assess the effectiveness of individual treatment and diagnose clinical relapse in the early stage for all leukemia patients regardless of the presence or absence of tumor-specific DNA markers.

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A Chemotherapy (Relapse)

Fig 1. MRD detected by the quantitation of WT1 expression levels in the patients with clinical relapse (A) or in continuing CR (B) who were treated with CHT.
FOLLOW-UP OF MRD BY MONITORING WT1 EXPRESSION

B

Chemotherapy (CR)

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Fig 1 (cont’d). Top line, number of blast cells in BM on microscopic examination; center line, WT1 expression levels in BM; bottom line, WT1 expression levels in PB. Time 0 means the time when the patients were diagnosed as having leukemia.
tional chemotherapy (CHT) and 23 BMT) over a long-term period (7 to 124 months; mean, 29 months for CHT and 24 months for BMT). We conclude that the monitoring of WT1 expression levels facilitates the assessment of the effectiveness of individual treatment, early diagnosis of relapse, and prediction of prognosis.

MATERIALS AND METHODS

Patients. This study included patients with leukemia who were admitted between 1984 and 1995 to the institutions specified. Acute leukemia patients were treated with intensive chemotherapy, including daunorubicin, mitoxantrone, 6-mercaptopurine, etoposide, and cyclophosphamide preceded by conditioning therapy with high-dose cyclophosphamide (50 mg/kg; 4 doses) and busulfan, and other anticancer drugs. Allogeneic BM transplantation (BMT) with HLA-identical sibling BM cells was preceded by conditioning therapy with high-dose cyclophosphamide (60 mg/kg; 2 doses) and total body irradiation (2 Gy; 6 doses) or by high-dose cyclophosphamide (50 mg/kg; 4 doses) and busulfan (1 mg/kg; 16 doses).

Acute leukemia was classified according to the criteria devised by the French-American-British Committee.14,25 AMLL was defined as either leukemia differentiating along a specific lineage but coexpressing other lineage-associated markers or as biphenotypic but not well-differentiated leukemia (CD7+ stem cell leukemia).14,26

Thirty-one patients (27 AML, 2 ALL, and 2 AMLL) treated with CHT and the 23 patients (13 AML, 5 ALL, and 5 CML) treated with allogeneic BMT were monitored for the number of blast cells in BM and the WT1 expression levels in both BM and PB for 7 to 124 months (mean, 29 months for CHT and 24 months for BMT).

Sample preparation. To prepare mononuclear cells from leukemia patients, heparinized PB cells or BM aspirates were mixed with isovolumes of phosphate-buffered saline (PBS) and centrifuged on Ficoll-Isopaque solution (Pharmacia, Uppsala, Sweden). Mononuclear cells were collected by centrifugation and the pellet was washed twice with PBS to remove platelets. Cells (1 x 10^6 to 1 x 10^7) were then dissolved in 0.5 mL of solution D (4 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 mol/L 2-mercaptoethanol) and stored at -80°C until use.

Reverse transcription-polymerase chain reaction (RT-PCR). Quantitative RT-PCR was performed as described previously,14,27 with minor modifications. In brief, 2.0 μg of total RNA from leukemic samples was converted into cDNA in 30 μL of reaction buffer. The level of WT1 gene expression in K562 cells was defined as 1.0. Before the start of the experiments, large amounts of total RNA (>100 μg) from K562 cells were converted into cDNA, which was then used as the standard throughout our experiments. PCR was performed for 35 cycles with the outer primer to quantitate 10^-4 to 10^-1 levels of WT1 expression. To quantitate 10^-2 to 10^-6 levels, PCR was terminated at 27 to 30 cycles. For the quantitation of 10^-3 to 10^-6 levels, second-round PCR was performed for 10 to 14 cycles using nested internal primers; to quantitate β-actin levels, PCR was performed for 18 cycles. Serial 1:10 dilutions of standard K562 cDNAs were always amplified simultaneously with leukemic samples. All experiments were performed in duplicate. Standard errors for quantitation of WT1 in the same samples were ±15% at 10^-2 levels, ±25% at 10^-3 levels, and ±35% at 10^-4 levels. RT-PCR for bcr/abl,16-18 PML/RAR-α,19,22 and AML1/ETO12 transcripts was performed as described previously.

RESULTS

Assessment of the effectiveness of individual treatment, early diagnosis of relapse, and prediction of prognosis by monitoring of WT1 expression levels. The 31 patients (27 AML, 2 ALL, and 2 AMLL) treated with CHT and the 23 patients (13 AML, 5 ALL, and 5 CML) treated with allogeneic BM were monitored for the number of blast cells in BM and the WT1 expression levels in both BM and PB (Figs 1 and 2).

Sixteen (13 AML, 1 ALL, and 2 AMLL) of the patients in the CHT group had clinical relapse (Fig 1A). These patients were further classified into two groups according to the changes in WT1 expression levels in BM: RL1, WT1 expression levels either gradually or rapidly increased again to levels of 10^-2 or more after reduction to normal BM levels; RL2, WT1 expression levels never returned to normal BM levels even after complete remission (CR) and relapsed into leukemia after a short-term CR. In patients no. 51, 115, and 122, WT1 expression at normal BM levels after CR achievement increased to abnormal levels again after the discontinuation of chemotherapy due to its toxicity and this was followed by clinical relapse. WT1 expression in BM increased from normal to abnormal levels as early as 14 (patient no. 51), 18 (patient no. 115), and 8 months (patient no. 122) before clinical relapse. In patient no. 20, intermediate levels (10^-2 to 10^-3) of WT1 expression continued even after the second CR, which lasted for 3 months; this was
Fig 2 (cont'd).

RT-PCR

Microscopic Examination

\[
\begin{align*}
10^5 & \text{ w/w} \\
10^4 & \text{ w/w} \times \text{ w/w} \\
10^3 & \text{ w/w} \times \text{ w/w} \\
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10^1 & \text{ w/w} \times \text{ w/w} \\
10^0 & \text{ w/w} \times \text{ w/w} \\
\end{align*}
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Levels of cDNA gene expression compared with expression levels on admission
followed by clinical relapse and death of the patient. It should be noted that, in all patients with clinical relapse, abnormal levels of WT1 expression in PB continued throughout the clinical course, suggesting the persistence of MRD in PB. (Because at the beginning of this study we did not have the data described in Fig 5 showing that WT1 levels in PB correlative reflected those in BM, we did not quantitate WT1 levels in PB. Therefore, we did not have data for many patients on WT1 levels in PB at the early stage of the clinical course.)

Three of the 23 patients who were treated with BMT suffered clinical relapse and were also classified into two groups, RL1 and RL2, according to the same criteria as those for CHT (Fig 2A). WT1 expression for patients no. 242 and 235 changed from PCR negative to positive 2 to 3 months before clinical relapse.

The changes in the WT1 expression levels for the patients with relapse are shown in Fig 3A for BM and in Fig 4A for PB. In these patients, either rapid or gradual increase in WT1 expression levels occurred in both BM and PB before clinical relapse. These results show that either rapid or gradual increase in WT1 expression levels from normal to $10^{-2}$ levels in BM and to $10^{-3}$ levels in PB (RL1) or a further increase in WT1 expression over the persistent abnormal levels of WT1 (RL2) suggest clinical relapse in the near future regardless of the modality of treatment (CHT or BMT). The lead
FOLLOW-UP OF MRD BY MONITORING WT1 EXPRESSION

**A** MRD in PB (relapse)

![Graph showing MRD in PB (relapse)]

**B** MRD in PB (CR)

![Graph showing MRD in PB (CR)]

Fig 4. Time course of MRD in PB in the patients with clinical relapse (A) 12 CHT and 2 BMT; patients no. 20, 28, 102, 150, and 297 were not included) or in continuing CR (B) 15 CHT and 20 BMT). The horizontal axis represents the time from the diagnosis of the disease. The symbols are the same as in Fig 3.

On the other hand, the patients in continued CR after CHT were classified into two groups according to the changes in WT1 expression levels in BM (Fig 1B): CR1, WT1 expression decreased to and stayed at normal BM levels after CR; CR2, WT1 expression returned to normal BM levels once, but increased again and stayed at low levels (10^{-3} to 10^{-2} levels) or WT1 expression never returned to normal BM levels and stayed at low levels even after CR. The patients who are in group CR1 at present may deteriorate to CR2 due to a subsequent increase in WT1 expression over normal BM levels. On the other hand, CR2 patients treated with CHT may have to be reclassified as CR1 after more effective therapy such as BMT. In patient no. 164, AML1/ETO transcripts were measured simultaneously with WT1. Neither WT1 nor AML1/ETO transcripts were detected at 104 and 122 months, suggesting the absence of MRD. In patient no. 179, WT1 expression was abnormal at 35 months, but became undetectable 1 year later. In patient no. 25, WT1 expression was abnormal in PB at 26 months, but became undetectable 19 months later.

The patients in continuing CR after BMT were similarly classified into two groups, CR1 and CR2, according to the same criteria as those for CHT (Fig 2B). In patient no. 45, very low levels (<10^{-4}) of WT1 expression were still de-
tected in PB, but not in BM, 84 months after BMT. In patient no. 120, low levels (10^{-3} to 10^{-2}) of WT1 expression were detected in BM 25 months after BMT, but became undetectable 5 months later. The changes in WT1 expression levels and leukemia-specific bcr/abl transcripts occurred in parallel, confirming that WT1 expression levels reflect MRD. In patients no. 248 and 270, low levels (10^{-3} to 10^{-2}) of WT1 expression became detectable again 8 and 11 months, respectively, after BMT. In patient no. 64, low levels of WT1 expression could still be detected in both BM and PB 28 months after BMT, but became undetectable 7 months later.

Changes in WT1 expression levels in the patients in continuing CR are shown in Fig 3B for BM and Fig 4B for PB. For these patients, WT1 expression levels either rapidly or gradually decreased to normal (<10^{-5}) or low levels (10^{-1} to 10^{-2}) in BM and to undetectable (<10^{-5}) or very low (10^{-5} to 10^{-2}) to low (10^{-4} to 10^{-5}) levels in PB and then stayed at these levels.

**MRD is more sensitively detectable in PB than in BM.** Paired BM and PB samples obtained from leukemia patients were quantitated for WT1 expression (Fig 5). In 33 of 168 paired BM and PB samples (49 AML, 10 ALL, and 10 CML), WT1 expression was within normal BM levels in BM, but abnormal in PB, suggesting the existence of MRD. On the other hand, in 4 of 168 paired BM and PB samples, WT1 expression was abnormal in BM, but undetectable in PB (Fig 5A). Therefore, it can be concluded that PB samples are superior to BM samples for the detection of MRD. Similar results were obtained for the paired samples from the patients with AML (Fig 5B), ALL (Fig 5C), and CML (Fig 5D).

**Strong correlation between the MRD detected in paired BM and PB samples obtained from leukemia patients.** Correlation of WT1 expression levels was examined in paired BM and PB samples of patients expressing both greater than 10^{-3} levels in BM and greater than 10^{-8} levels in PB (Fig 5). Strong correlations (Spearman's correlation coefficient, r > .5) were observed for all types of leukemia (Fig 5B, C, and D). These results show that the degree of MRD in PB paralleled that in BM, but that MRD detected in PB was approximately 10 times lower than that in BM regardless of the type of leukemia (AML, ALL, or CML).

### DISCUSSION

A most important problem in the CHT and BMT for patients with leukemia is the uncertainty as to whether leukemic cells have been totally killed when CR is achieved. If we can monitor leukemia patients for MRD, we may be able to treat individual patients with different protocols based on the extent of MRD. Because the limit of MRD detection is only 1% to 5% for morphologic techniques and as little as 5% to 10% for Southern blot technique, these techniques are not practical for detection of MRD. The sensitivity for MRD detection by fluorescence in situ hybridization (FISH) is no more than 1% because it is limited by the presence of aneuploid (but not leukemic) cells and by certain inherent technical artifacts. Flow cytometry for leukemia-associated phenotypes, on the other hand, has a sensitivity of 1 leukemic cell in 10^4, but this method is applicable only to leukemia with limited phenotypes. PCR can detect leukemic cells at frequencies as low as 1 in 10^3 to 10^4 normal cells, but is applicable only to the leukemias that bear tumor-specific DNA markers, including rearranged Ig and T-cell receptor genes and fused genes such as bcr/abl, PML/RAR-α, AML1/ETO, and other fused genes. Furthermore, detection of MRD by PCR amplification of junctional regions of rearranged Ig and TCR genes can be applied only to B- or T-cell-lineage leukemia and requires cloning and sequencing of the junctional regions before PCR, thus making it too cumbersome for clinical application. Detection of MRD with the aid of PCR amplification of the tumor-specific DNA markers is useful for only 20% to 30% of leukemia patients. In strong contrast to the techniques described above, detection of MRD by means of the quantitation of WT1 gene expression is applicable to all leukemia patients, regardless of the type of leukemia (AML, ALL, or CML) or the presence or absence of tumor-specific DNA markers. Therefore, even in cases in which chromosomal and DNA analyses of fresh leukemic cells were unsuccessful or could not be performed, the WT1 assay can always be used for the detection of MRD. Thus, the WT1 assay has made it possible to rapidly assess the effectiveness of treatment and evaluate the degree of eradication of leukemic cells in individual leukemia patients. The WT1 assay will be beneficial to individualization of treatment protocols for all leukemia patients, although basing treatment on the WT1 assay has not yet been shown to be practically effective. Moreover, the monitoring of WT1 expression levels in BM has made it possible to diagnose relapse 1 to 18 months (mean, 7 months) before clinical relapse for all leukemia patients. We have recently found that WT1 expression levels per leukemic cell at the time of clinical relapse were approximately 5 times higher than those at the time of diagnosis (manuscript in preparation). This indicates that the sensitivity of MRD detection by quantitation of WT1 expression levels will increase as clinical relapse becomes imminent, thus making it possible
FOLLOW-UP OF MRD BY MONITORING WT1 EXPRESSION

A. All Patients

B. AML

C. ALL

D. CML

Fig 5. Strong correlation between the MRD detected in BM and PB. WT1 expression levels in the paired BM (vertical axis) and PB samples (horizontal axis) were plotted. The horizontal lines at the $10^{-2}$ level indicate the same items as in Fig 3. The vertical lines at the $10^{-4}$ level indicate the detection limit of WT1 mRNA in PB. (A) All leukemia patients examined, Spearman’s correlation coefficient, $r = .78, P < .001$; (B) AML, $r = .78, P < .001$; (C) ALL, $r = .85, P < .001$; (D) CML, $r = .75, P < .01$.

to detect MRD with enough sensitivity even in leukemia cases that expressed low levels ($10^{-2}$ to $10^{-3}$) of the WT1 gene at the time of diagnosis. The following conditions have been identified as indicating that clinical relapse is impending: (1) rapid or gradual increase in WT1 expression to levels of $10^{-2}$ or over after return to normal BM levels at CR ($10^{-3}$ levels in PB) and (2) the WT1 expression stays at levels near or over $10^{-2}$ in BM without return to normal BM levels even after CR. The absolute levels of WT1 that predicts with certainty that a patient will relapse are $10^{-2}$ levels in BM and $10^{-3}$ levels in PB. For the patients with these signs, treatment for relapse should be performed before obvious clinical relapse occurs. Especially in BMT, treatments such as reduction in dosage of cyclosporin A and donor leukocyte transfusion (DLT)$^{32-35}$ are often effective in the early stages of relapse, when the amount of residual leukemic cells is still small. Therefore, in case of an increase from normal to abnormal BM levels of WT1 expression after BMT, such treatment procedures should be implemented immediately. Thus, monitoring of MRD by the quantitation of WT1 expression levels after BMT is essential. Moreover, when a serial increase in WT1 expression levels is detected in patients treated with CHT, immediate BMT is recommended.

In the present study, the prognosis of the patients in continuing CR whose WT1 expression levels stayed low or very low ($10^{-3}$ to $10^{-2}$ in BM and $10^{-5}$ to $10^{-3}$ in PB) remain unresolved. However, it can be assumed that those whose WT1 expression gradually decreases and returns to normal BM levels can expect to enjoy prolonged disease-free survival, whereas the patients whose WT1 expression gradually or rapidly increases will suffer relapse. Actually, patients no. 51 and 115, who had been classified into group II in our previous report$^{14}$ (corresponding to RL1 in this report), relapsed several months after our publication. In patient no. 45 in continuing CR who was treated with BMT, very low
levels ($10^{-5}$ to $10^{-4}$) of WT1 expression in PB continued even 7 years after BMT. (Because this patient did not have any other tumor-specific markers, MRD could not be determined by other methods.) Clinical relevance of MRD has been thoroughly studied in acute promyelocytic leukemia (APL) (PML/RAR-a), $^{35,45}$ CML (bcr/abl), $^{45-47}$ and AML-M2 (AML1/ETO). $^{46,47}$ In APL, although all-trans retinoic acid induces CR in a high proportion of patients, it rarely eradicates molecular evidence of APL (PML/RAR-a transcripts). However, subsequent chemotherapy can result in changing the RT-PCR assay for PML/RAR-a to negative. Negative RT-PCR assays are associated with prolonged disease-free survival, whereas a positive RT-PCR assay is highly correlated with subsequent relapse. In contrast to APL, MRD in CML and AML1/ETO-positive AML-M2 is less closely associated with clinical outcome. As for CML, bcr/abl transcripts were detected in a significant proportion of long-term remission patients. In AML1/ETO-positive AML-M2, persistence of MRD in long-term survivors (5, 8, and 9 years) was reported, suggesting that complete elimination of AML1/ETO-positive leukemic cells is not necessary for the achievement of long-term remission. To determine the clinical significance of persistence of low WT1 expression levels, observation of more patients for several years will be needed.

We previously reported that WT1 expression was found in all the leukemia patients (45 AML, 22 ALL, 6 AML1, and 23 CML) we examined and that WT1 was identified as a new marker for the diagnosis of MRD. $^{14}$ Subsequently, Brieger et al. $^{48}$ reported that the WT1 gene was expressed in 79% (41/52) patients with AML and that WT1 expression was a possible marker for leukemia blast cells. Their results were basically consistent with those of our preceding report. However, there was an important difference in the incidence of WT1 expression in leukemic cells, which can be ascribed to the sensitivity and quantitativeness of the PCR used (they could not detect background levels of WT1 in normal BM cells).

PB samples were found to be superior to BM samples for the monitoring of MRD because (1) the residual leukemic cells flow from BM out into PB in a proportion corresponding to approximately one-tenth of the degree of MRD in BM and (2) the sensitivity for detection of MRD is more than 100 times higher in PB than in BM because of the presence of WT1 background levels in BM. Thus, instead of BM samples, aspiration of which is painful for patients and laborious for medical staff, PB samples are sufficient for monitoring MRD.

We have shown that approximately one-tenth of the residual leukemic cells in BM flows out into PB in AML, ALL, and CML. This is fundamentally consistent with the findings that bcr/abl titers in BM samples exceeded those in the corresponding PB samples in all sample pairs by at least 1 log (the mean difference was 1.55 log) in patients with bcr/abl-positive ALL. $^{49}$ However, to our knowledge, our study is the first to show clearly the correlative relationship between the MRD in BM and PB in a relatively large sample. Based on these findings, leukemia onset could be diagnosed in PB samples earlier than in BM samples by monitoring WT1 expression levels, making it possible to diagnose leukemia onset very early in persons at high risk for leukemia, ie., those who are accidentally exposed to radioactive rays or who have been treated with chemotherapy or radiotherapy for solid tumors. Furthermore, quantitation of WT1 expression levels in PB appears to be feasible in medical checkups for leukemia even for healthy individuals.

In conclusion, serial monitoring of WT1 expression levels should be considered indispensable for evaluation of the effectiveness of different therapies and individualization of treatment protocols for leukemia patients.

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Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels

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