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Monitoring of WT-1 Gene Expression in Peripheral Blood of Patients with Acute Leukemia by Semiquantitative RT-PCR; Possible Marker for Detection of Minimal Residual Leukemia

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The expression of the WT-1 gene which is found exclusively in human leukemic blasts frequently disappears from bone marrow of leukemia patients in complete remission (CR). Using semiquantitative RT-PCR, we investigated the expression of the WT-1 gene in peripheral bloods (PBs) of 33 patients with acute leukemia (AML 26; ALL 7) and monitored its expression after achievement of CR. None of the 6 normal controls expressed detectable levels of WT-1 transcripts ($<10^{-4}$, background level), whereas 31 (93.9%) of 33 patients expressed variable levels of WT-1 transcripts (range, 10^{-4} to 10^{1}) at diagnosis. The level of WT-1 expression was not different between AML and ALL. By monitoring WT-1 gene expression in PB of 31 patients during CR, 5 patients relapsed (two from the 18 patients with undetectable levels of WT-1 gene expression and three from the 13 with WT-1 gene expression in low levels). Three of the 5 relapsed patients showed preceeding reappearance or rise of WT-1 gene expression. From these results, we reconfirmed that the WT-1 gene is a pan-acute leukemic marker, which can be used to monitor minimal residual leukemia (MRL) after chemotherapy or in patients with CR.

Key Words: *Wilms' tumor gene (WT-1), acute leukemia, minimal residual*

Accurate assessment of minimal residual leukemia (MRL) in leukemic patients is essential to evaluate the efficacy of treatment regimens, to establish early diagnosis of impending relapse, and to individualize treatment protocols. Different techniques other than histologic examination, such as the classical cytogenetics, immunophenotyping, or the polymerase chain reaction (PCR), have been used to detect residual leukemic cells. However, PCR covers only 25~30% of all acute leukemia in spite of its prominent sensitivity, because leukemia-specific genetic markers are rare (van Dongen *et al.* 1992).

Wilms' tumor gene (WT-1) encodes a zinc finger transcription factor with a complex pattern of alternative splicing (Haber *et al.* 1991), is highly homologous with proteins of the early growth response (EGR) genes, and functions as a potent transcriptional repressor of insulin-like growth factor-II (IGF-II) (Drummond *et al.* 1992), platelet-derived growth factor-A (PDGF-A) chain (Gashler *et al.* 1992), colony-stimulating factor-1 (CSF-1) (Harrington *et al.* 1993) and IGF-receptor (IGF-R) genes (Werner

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et al. 1993). WT-1 also functions as a transcriptional activator, suggesting that it is a bifunctional regulator of transcription (Wang *et al.* 1993).

During development, the WT-1 gene is transcribed in urogenital tissue, kidneys, gonads, uterus, brain, and spleen (Call *et al.* 1990; Pritchard-Jones *et al.* 1990). The WT-1 gene is strongly regulated in a time- and tissue-specific manner. During ontogeny, it is expressed for a short period in the developing fetal urogenital tract (Pritchard-Jones *et al.* 1990). The WT-1 gene is highly expressed in Wilms' tumors. Recently, the expression of WT-1 has been demonstrated exclusively in blasts of human leukemia, and it has usually been lost in cells of patients with complete remission (CR) (Miwa *et al.* 1992).

In general, bone marrow is examined to investigate the residual leukemia or relapse. But, according to the report of Inogue *et al.* (1996), sensitivity for detection of WT-1 is more than 100 times higher in PB than in BM because of the presence of WT-1 background levels in BM, and the correlative relationship between the MRL in BM and PB.

In this study, we examined the expression of the WT-1 gene in peripheral bloods of patients in both newly-diagnosed acute leukemia and CR patients in order to detect MRL.

MATERIALS AND METHODS

Patients

Thirty-three patients with acute leukemia (AML 26, ALL 7) who reached CR after induction chemotherapy between Jan 1995 and Feb 1997 were enrolled in this study. Acute leukemia was classified according to the criteria devised by the French-American-British Committee (Bennett *et al.* 1985). The clinical features of patients examined are listed in Table 1. A combination chemotherapy of daunorubucin, 6-thioguanine, and cytarabine for AML and a combination of vincristine, prednisone, and daunorubicin for ALL were used for remission induction. Patients were considered to be in CR when their granulocyte count was greater than 1,500/μL, the platelet count was greater than 100,000/μL, the residual blast in the normocellular marrow was less than 5%, and if these conditions were maintained for at least 4 weeks (Yates *et al.* 1982). PB cells were obtained from leukemic patients at the onset of their disease and in CR. Normal PB and bone marrow (BM) cells were obtained from healthy volunteers and BMT donors.

Table 1. Characteristics of patients

	FAB	No.	Sex(M/F)	Age(yr)
AML	M1	7		
	M2	6		
	M3	5	10/16	42(27-69)
	M4	7		
	M5	1		
ALL	L1	6	5/2	30(17-49)
	L2	1		
Total		33	15/18	39(17-69)

Sample purification

To prepare mononuclear cells from leukemic patients, heparinized PB were mixed with isovolumes of phosphate-buffered saline (PBS) and centrifuged in Ficoll-Hypaque solution (Sigma, St. Louis, MO, USA). After density gradient sedimentation, the cells were washed twice in PBS. The purity of leukemic blasts in the samples was 30~95%.

Reverse transcription (RT) and PCR

We modified the method of Inogue *et al.* (Inogue *et al.* 1994). Total RNA was isolated with RNeasy total RNA purification system (QIAGEN, USA). RT was carried out according to the manufacturer's instructions. Briefly, one microgram of total RNA in 8.2 μL of diethylpyrocarbonate (DEPC)-treated water was mixed with 11.2 μL of RT buffer containing 20 U of AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany), 1 mmol/L of each deoxynucleotide triphosphate (dNTP), 1.6 μg of oligodT primer, 50 U of RNase inhibitor, 5 mmol/L of $MgCl_2$ and 2 μL of 10 × reaction buffer. The reaction mixture was incubated at 42°C for 60 minutes and heated at 99°C for 5 minutes. The 50 μL PCR reaction mixture contained cDNA derived from 100 ng of total RNA, 1.25 U Taq DNA polymerase, 0.2 mmol/L of each dNTP, 10 pmol of each primer, 1.5

mmol $MgCl_2$. PCR was performed for appropriate cycles with a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) under the following conditions: denaturation at 94°C for 1 minute, primer annealing at 64°C for WT-1 (or at 60°C for β-actin) for 1 minute and then chain elongation at°C for 2 minutes. When the PCR products of first-round PCR were not detected, the second-round of PCR was performed with the nested internal primers in a reaction solution containing 2.5 μL of the first-round PCR products. PCR products derived from 20 ng of total RNA (second round, 1 ng) were separated in 1% agarose gels containing 0.05 μg/mL of ethidium bromide, and examined. The sequences of the primers were: WT-1 (Call *et al.* 1990; Gessler *et al.* 1990): (outer sense primer for exon 7) 5'-GGCATCTGAGACCAGTGAGAA-3'; (outer antisense primer for exon 10) 5'-GAGAGTCAGACTTGAAAGCAGT-3'; (internal sense primer for exon 7) 5'-GCTGTCCCACTTACAGATGCA-3'; (internal antisense primer for exon 10) 5'-TCAAAGCGCCAGCTGGAGTTT-3'./ β-actin (Nakajima-Iijima *et al.* 1985): (sense primer) 5'-GTGGGGCGCCCCAGGCACCA-3'; (antisense primer) 5'-GTCCTTAATGTCACGCACGATTTC-3'. The expected size of PCR products were 481 bp for outer PCR product of WT-1; 333 bp for nested PCR product of WT-1; 540 bp for β-actin PCR product.

Statistical analysis

The difference of WT-1 gene expression levels according to type of acute leukemia was assessed by nonparametric Mann-Whitney test.

RESULTS

Determination of optimal conditions for semi-quantitative RT-PCR

The optimal condition for quantitative RT-PCR of the WT-1 gene expression was determined by using total RNA of K562 cells, which constitutively express the WT-1 gene. Complementary DNA prepared from 100 ng of the total RNA from K562 cells was amplified by various cycles of PCR using the first-round primer of the WT-1 gene. As exponential amplication was observed between 20 and 35 cycles of PCR, serial 1:10 dilutions of the K562 cDNA were amplified for 30 cycles. The amount of PCR product increased dependent on the loading amount of RNA (range from 1 to 1000 ng) (Fig. 1A). Thereafter, in the first-round PCR for WT-1, cDNA derived from 100ng of total RNA was needed as a substrate and amplified for 30 cycles. Ten

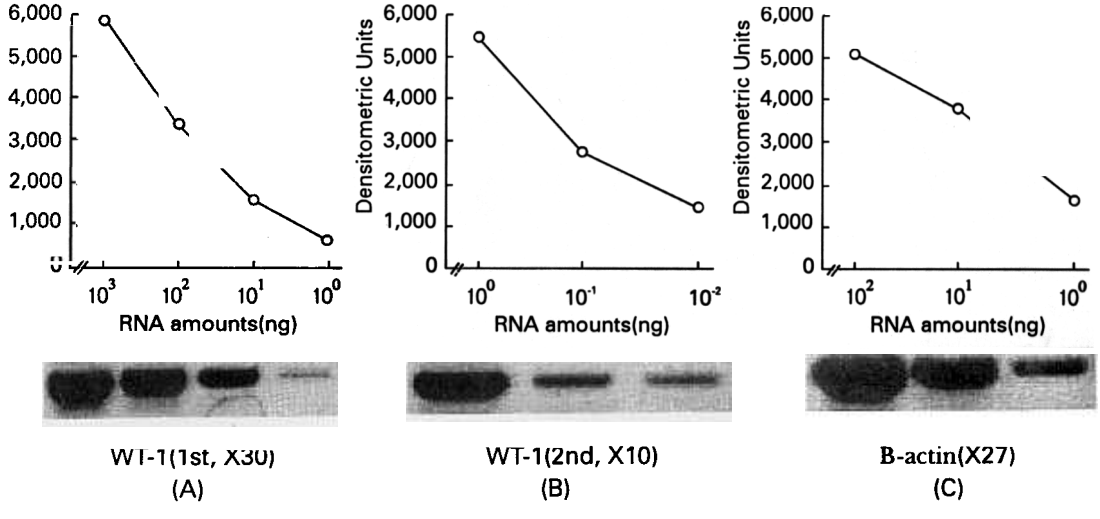

Fig. 1. Determination of optimal conditions for quantitative RT-PCR.

cycles of the second-round PCR which was started from 2.5 μL of the first-round PCR mixture was enough to show the dose-dependent amplification of PCR products (from 0.01 to 1 ng of RNA) (Fig. 1B). The condition for PCR amplification of β-actin gene was also determined as follows; the optimal cycles for exponential amplification of β-actin was 27. And, at cycle 27, dose-dependent exponential amplification from 1 to 100 ng of K562 RNA was present (Fig. 1C). To accommodate the difference in the loading amount of RNA for RT-PCR and of the degree of RNA degradation in each samples, the densitometric value of WT-1 gene expression was divided by that of β-actin gene expression. The level of WT-1 gene expression in K562 cells was defined as 1.00 ($=10^0$).

Expression of the WT-1 gene in healthy volunteers and patients with acute leukemia

None of the 6 normal PB expressed detectable level of WT-1 transcripts ($<10^{-4}$), whereas 9 normal BM samples expressed low levels of WT-1 transcripts. Thirty one (93.9%) of 33 patients with acute leukemia expresseded variable amounts of WT-1 transcripts in peripheral bloods at diagnosis (Fig. 2). However, there was no statistical difference of WT-1 expression between AML and ALL (P = 0.43) as well as between granulocytic (FAB M1, M2, M3 subtype) and monocytic AML (FAB M4, M5 subtype) (P = 0.21).

WT-1 expression in CR

Thirty-one patients with CR who had expressed

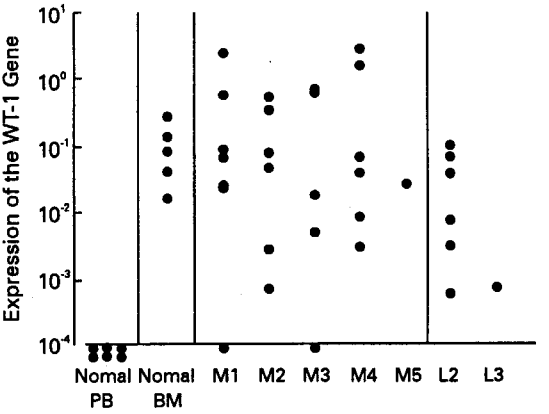

Fig. 2. Expression of the WT-1 gene in normal hematopoietic tissues and newly diagnosed acute leukemia.

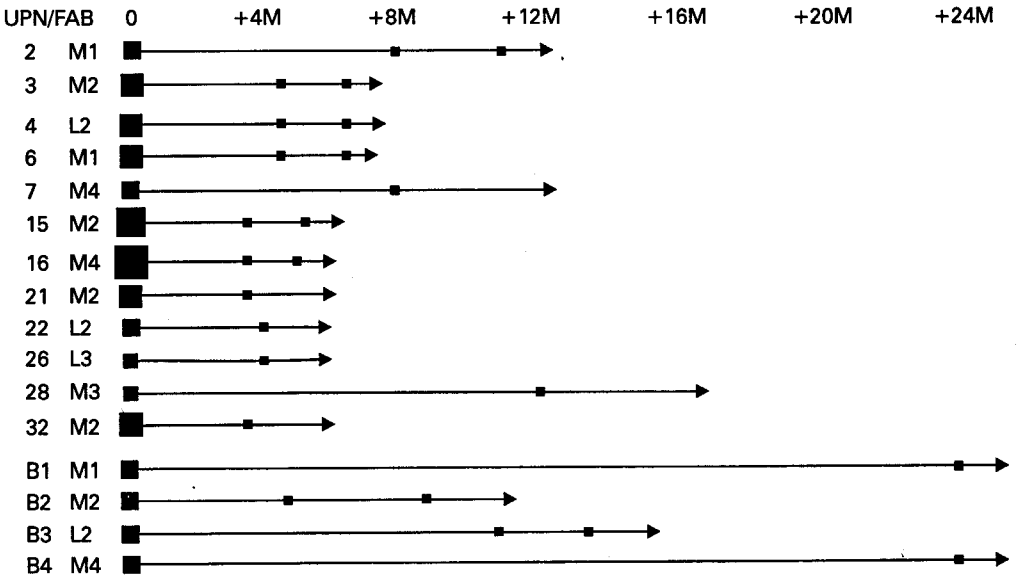

Fig. 3. MRL according to WT-1 expression in group 1.

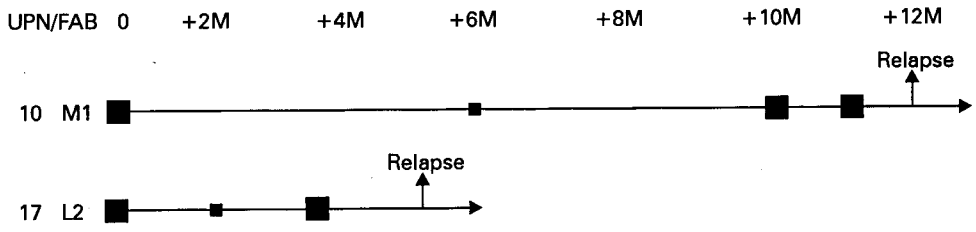

Fig. 4. MRL according to WT-1 expression in group 2.

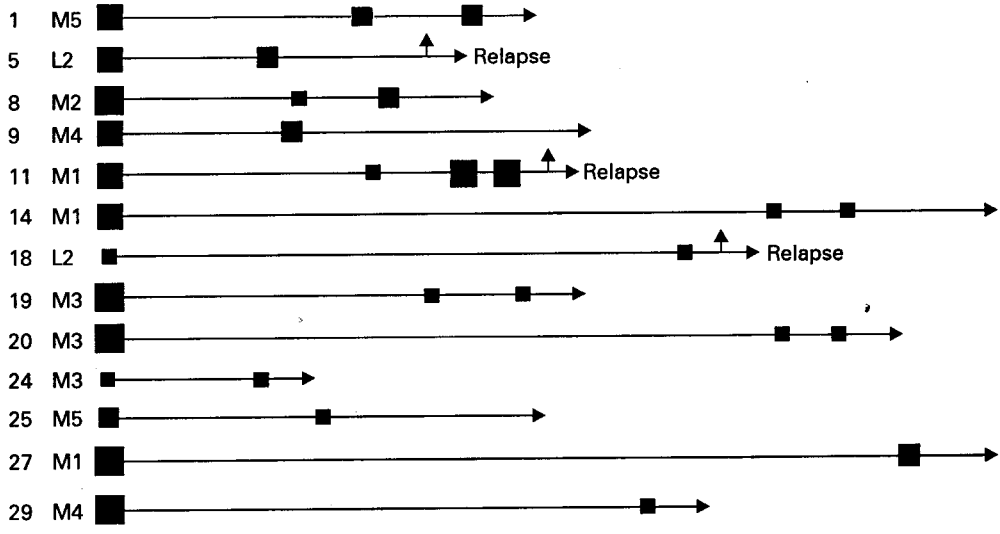

Fig. 5. MRL according to WT-1 expression in group 3.

WT-1 prior to induction therapy were studied. Although they remained in CR, according to the pattern of WT-1 expression, they were classified into three groups. In group 1 (n=16), WT-1 expression became undetectable (at background levels, $<10^{-4}$) after achievement of CR (Fig. 3). In particular, four who patients received allogeneic BMT were included in this group. In group 2 (n=2), WT-1 gene expression which had disappeared after CR rose again after interruption of consolidation chemotherapy, and they met clinical relapse 2 months later (Fig. 4). In group 3 (n=13), WT-1 gene expression persisted for extended periods (median 13.5; range, 5-24 months)(Fig. 5). Three of 13 patients with persistent WT-1 gene expression relapsed. Of interest, the rise of WT-1 gene expression preceded clinical relapse (UPN 11).

DISCUSSION

Expression of WT-1 has also been described in neoplasia such as Wilms' tumor (Varansi *et al.* 1994), ovarian cancer (Bruening *et al.* 1993), mesothelioma of the lung (Park *et al.* 1993), melanoma (Rodeck *et al.* 1994), and in blast cells of some human leukemias (Miwa *et al.* 1992; Miyagi *et al.* 1993; Brieger *et al.* 1994; Inogue *et al.* 1994), suggesting a broad range of expression in malignant tissues. Using Northern blot analysis, Miwa *et al* have demonstrated WT-1 gene expression in 7 (44%) of 16 patients with ALL, 15 (68%) of 22 with AML and 8 (80%) of 10 with chronic myelogenous leukemia (CML) in blast crisis (Miwa *et al.* 1992). Furthermore, RT-PCR demonstrated that WT-1 tran-

scripts appears in mononuclear cells of acute leukemia patients at a much higher frequency than was reported earlier (Brieger *et al.* 1994; Inogue *et al.* 1994). In this study, WT-1 transcripts were detectable in the majority (93.9%) of patients with acute leukemia. These results are consistent with the above results (Brieger *et al.* 1994; Inogue *et al.* 1994).

WT-1 is downregulated during differentiation of HL-60 cells (Sekiya *et al.* 1994) and K562 cells (Phelan *et al.* 1994), suggesting that repression of WT-1 may be necessary for differentiation of some hematopoietic lineages. For AML, the lowest levels of WT-1 gene expression were observed in the monocytic leukemias (M4 and M5). For ALL, the levels of WT-1 gene expression were more than 20 times higher in $CD19^+CD20^-$ pro-B-cell ALL than in $CD19^+CD20^+$ pre-B-intermediate B-cell ALL (Inogue *et al.* 1994). Meanwhile, we could not find any difference in WT-1 gene expression among FAB subtypes.

Detection of MRL after chemotherapy can be an important clinical parameter to design the next treatment as well as to predict the prognosis. The limit of morphologic detection is usually over 1% malignant cells, and that of molecular biologic technique, such as Southern blot analysis from 5% to 10% malignant cells. PCR, the recently developed technique, is able to detect one leukemic cell among 10^4 to 10^5 cells. However, it is applicable only to leukemias which bear tumor-specific genetic markers; for example, rearranged Ig and T-cell receptor genes, fused genes such as *bcr/abl* (Shtivelman *et al.* 1985), PML/RAR α (Giguere *et al.* 1987), AML1/ETO (Downing *et al.* 1993), and other fusion genes (Brown *et al.* 1990; Kamps *et al.* 1990). In contrast to specific fusion genes, the WT-1 gene can be applied to almost all leukemia patients. A recent report indicates that WT-1 expression levels per leukemic cell at the time of clinical relapse were approximately 5 times higher than those at the time of diagnosis (Tamaki *et al.* 1996).

Considering the result that WT-1 transcripts were undetectable in normal PB cells of volunteers, we suppose that the presence of WT-1 transcripts in PB of leukemia patients with CR may indicate MRL. Like the result of Inogue *et al.* (Inogue *et al.* 1996), the sensitivity for detection of WT-1 was more than 100 times higher in PB than in BM. The detection limit of our study (up to 10^{-4}) was lower than that of Inogue *et al.* (up to 10^{-5}). This discrepancy seems to be derived from the difference in the loading amount of cDNA.

From our results, serial monitoring of WT-1 expression may be beneficial to detect early relapse and to predict prognosis. Therefore, showing the rise of WT-1 gene expression without overt relapse will be a candidate of treatment for relapse of leukemia. Practically, specific treatment, such as reduction or withdrawal of cyclosporin A and donor leukocyte infusion, have been tried in the stage of molecular relapse after BMT.

Clinical relevance and the prognosis of patients in continuing CR whose MRL levels stayed low have been extensively studied with PCR study using other specific DNA markers. In acute promyelocytic leukemia (APL), negative RT-PCR assays for PML/RAR α are associated with prolonged disease-free survival, whereas a positive RT-PCR assay is highly correlated with subsequent relapse (Koller *et al.* 1995). In contrast to APL, MRL in CML and AML1/ETO-positive AML-M2 is less closely associated with clinical outcome, and *bcr/abl* or AML1/ETO transcripts were detected in a significant proportion of long-term remission patients (Kusec *et al.* 1994; Xu *et al.* 1994). In our study, the prognosis of the patients with detectable levels of WT-1 expression during CR is obscure because the persistence of WT-1 gene expression may not always predict later relapse. Actually, Inogue *et al.* reported that low levels of WT-1 expression (10^{-5} to 10^{-4}) in PB continued for up to 7 years after BMT in a patient in CR who was treated with BMT (Inogue *et al.* 1996). To determine the clinical significance of persistence of low levels of WT-1 gene expression in patients with CR, prospective study with many patients will be needed.

Although the optimal interval for monitoring MRL by RT-PCR is difficult to determine due to individual differences of regrowth and aggressiveness of leukemic cells, it is recommended to sample blood every two months because there was a latency of two months between the rise of WT-1 gene expression and the overt relapse.

Collectively, we reconfirmed that the WT-1 gene is a pan-acute leukemic marker which can be applied to monitor MRL after chemotherapy or even

in patients with CR.

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