Quantification of WT1 mRNA by Competitive NASBA in AML Patients

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Summary: The measurement of Wilms’ tumor gene (WT1) mRNA levels by reverse transcriptase-polymerase chain reaction (RT-PCR) is useful in detecting minimal residual disease (MRD) in leukemia patients. In the present study, we quantified the level of WT1 mRNA in the peripheral blood and bone marrow of patients with acute myelocytic leukemia (AML) at initial onset, remission and recurrence by the use of nucleic acid sequence based amplification (NASBA), and then ascertained the clinical usefulness of this method. At initial onset, the level of WT1 mRNA in the peripheral blood was above $10^3$ copies/µg and that in the bone marrow was above $10^4$ copies/µg. The level of WT1 mRNA was decreased in cases where therapy resulted in complete remission, but it was abnormally high in recurring cases. In AML (M3) patients, the relationship between the level of WT1 mRNA and the expression of the PML-retinoic acid α receptor (RARα) gene, assessed by fluorescence in situ hybridization (FISH), was investigated. When leukemia was in remission hematologically, the PML-RARα gene was negative and the level of WT1 mRNA decreased. These findings suggest that the quantification of WT1 gene expression by competitive NASBA is useful in assessing therapeutic effects and detecting MRD.

Key words WT1 mRNA, competitive NASBA, minimal residual disease

INTRODUCTION

Wilms’ tumor gene (WT1) is a cancer suppressor gene located on chromosome 11q13 that was isolated in 1990 as the etiologic gene of Wilms’ tumors, which are pediatric renal tumors [1]. This gene encodes a zinc finger transcription factor having a molecular weight of 52-54 kDa [2,3]. There have been several reports on the expression of the WT1 gene in hematopoietic organ tumors, and Call and colleagues documented its expression in a human leukemia line, K562, in 1990 [1]. Later, Inoue and colleagues quantified the level of WT1 mRNA in leukemia patients by reverse transcriptase-polymerase chain reaction (RT-PCR), reporting that WT1 mRNA expression was high in almost all leukemia patients and correlated to the disease state of leukemia. They concluded that the level of WT1 mRNA expression could be an indicator of minimal residual disease (MRD) [4,5]. In the present study, we quantified WT1 mRNA expression in the peripheral blood and bone marrow of patients with acute myelocytic leukemia (AML) by competitive nucleic acid sequence based amplification (NASBA) [6-8], and then investigated its clinical usefulness.

SUBJECTS AND METHODS

Subjects were 14 AML patients (11 men and 3 women; age range, 25-75 years; mean age ± SD, 56±13.84 years) admitted to the First Department of Internal Medicine at Kurume University Hospital between November 1998 and October 2000. Two patients had FAB: M1, five had M2, two had M3, one had M4, one had M4E, one had M5a, one had M5b, and one had M6 (Table 1). The level of WT1 mRNA expression was quantified by competitive NASBA. For comparison, a peripheral blood sample...
was collected from 60 healthy individuals. In the present study, total RNA samples extracted from the peripheral blood of the healthy individuals and the peripheral blood and bone marrow of the leukemia patients were used in analyses. In competitive NASBA, RNA (1 µg of extracted total RNA and competitor RNA, respectively), primer and NASBA reaction solution (40 mM Tris, 12 mM MgCl₂, 1 mM dNTPs and 2 mM NTPs) were mixed and kept warm at 65 °C for 5 min. The resulting solution was placed in an incubator at 41 °C for 5 min, and three enzymes (RT, RNase and T7RNA polymerase) were then added and mixed gradually to allow the solution to react at 41 °C for 5 more min. Next, a portion of the resulting solution was diluted 100-fold, and the level of WT1 mRNA was quantified based on the level of fluorescence using the standard calibration curve. As reported by Sugiyama and colleagues [4], the level of WT1 mRNA has usually been expressed in relation to the level of WT1 mRNA in human leukemia cell line K562, which is 1.00. Since the level of WT1 mRNA expression was stated as the number of copies per microgram of sample in the present study, a comparison was made between these two systems. The expression of WT1 mRNA in K562 quantified by competitive NASBA was 3.0×10⁶ copies/µg. Hence, the number of copies corresponded to the relative assessment values as follows:

Relative assessment: 1.00=3.0×10⁶ copies/µg
10⁻¹=3.0×10⁵
10⁻²=3.0×10⁴
10⁻³=3.0×10³
10⁻⁴=3.0×10²
10⁻⁵=3.0×10¹

Through the use of the above system, 1) the level of WT1 mRNA in the peripheral blood of the healthy individuals was quantified, 2) the level of WT1 mRNA in peripheral blood and bone marrow samples was quantified at different times, 3) the correlation of WT1 mRNA in the peripheral blood to that in the bone marrow was assessed, and 4) the relationship between WT1 mRNA and the PML-retinoic acid α receptor (RARα) gene in the bone marrow was evaluated [9]. The expression of chimeric mRNA (PML-RARα gene) in bone marrow samples was assessed by fluorescence in situ hybridization (FISH) [10].

Statistical analysis of the levels of WT1 mRNA in the peripheral blood and bone marrow samples collected at different times was performed by a paired t-test, and a simple regression model was used to determine the correlation of WT1 mRNA in the peripheral blood to that in the bone marrow.

RESULTS

The level of WT1 mRNA in the peripheral blood of all healthy individuals was less than 10² copies/µg. Table 1 and Fig. 1 shows measurement results at different disease states. Table 1 shows the disease type, age, sex, chromosome, gene marker, prognosis and the level of WT1 mRNA (copies/µg) of patients at

<table>
<thead>
<tr>
<th>CaseNo.</th>
<th>FAB</th>
<th>age</th>
<th>sex</th>
<th>Chromosome</th>
<th>gene marker</th>
<th>sample</th>
<th>initial onset (WT1)</th>
<th>remission (WT1)</th>
<th>recurrence/PR (WT1)</th>
<th>prognosis</th>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>50</td>
<td>M</td>
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<td>-</td>
<td>BM/PB</td>
<td>4.1×10⁹/1.5×10⁹</td>
<td>3.2×10⁸/2.4×10⁸</td>
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<td>2</td>
<td>M</td>
<td>48</td>
<td>M</td>
<td>46XY</td>
<td>-</td>
<td>BM/PB</td>
<td>3.1×10⁹/2.5×10⁹</td>
<td>3.7×10⁸/3.7×10⁹</td>
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<td>recovered 13 months after CR</td>
</tr>
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<td>65</td>
<td>F</td>
<td>46XY, t(15;17)(q22;q21) PML/RARα</td>
<td>BM/PB</td>
<td>BM/PB</td>
<td>9.0×10⁶</td>
<td>1.4×10⁶</td>
<td>-</td>
<td>CR+19M</td>
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<td>4</td>
<td>M3</td>
<td>42</td>
<td>M</td>
<td>46XY, t(15;17)(q22;q21) PML/RARα</td>
<td>BM/PB</td>
<td>BM/PB</td>
<td>3.4×10⁶</td>
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<td>-</td>
<td>CR+14M</td>
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<tr>
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<td>66</td>
<td>M</td>
<td>46XY</td>
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<td>BM/PB</td>
<td>1.6×10⁸/8.0×10⁷</td>
<td>2.4×10⁷/0.0</td>
<td>-</td>
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<td>6</td>
<td>M2</td>
<td>61</td>
<td>M</td>
<td>46XY</td>
<td>-</td>
<td>BM/PB</td>
<td>2.1×10⁹/1.1×10⁹</td>
<td>1.0×10⁹/1.3×10⁹</td>
<td>1.1×10⁸/6.1×10⁸</td>
<td>recovered 17 months after CR</td>
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<td>46XY</td>
<td>-</td>
<td>BM/PB</td>
<td>2.6×10⁹/1.4×10⁹</td>
<td>1.2×10⁹/3.4×10⁹</td>
<td>-</td>
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<td>F</td>
<td>46XX, del(9)(?), add(q22?)</td>
<td>BM/PB</td>
<td>BM/PB</td>
<td>1.4×10⁹/9.2×10⁸</td>
<td>1.8×10⁹</td>
<td>-</td>
<td>CR+17M</td>
</tr>
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<td>9</td>
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<td>M</td>
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<td>1.1×10⁹</td>
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<td>M4E</td>
<td>74</td>
<td>M</td>
<td>46XY, inv16(p13q22)</td>
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<td>BM/PB</td>
<td>1.7×10⁹</td>
<td>2.4×10⁹</td>
<td>not measure</td>
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<td>66</td>
<td>M</td>
<td>46XY</td>
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<td>-</td>
<td>1.3×10⁹/9.8</td>
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<td>M1</td>
<td>48</td>
<td>F</td>
<td>46XY</td>
<td>-</td>
<td>BM/PB</td>
<td>3.9×10⁸/5.2×10⁹</td>
<td>5.2×10⁷/5.5×10⁹</td>
<td>-</td>
<td>CR+1M</td>
</tr>
<tr>
<td>13</td>
<td>M2</td>
<td>52</td>
<td>M</td>
<td>46XY, t(8;21)(q22;q22)</td>
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<td>BM/PB</td>
<td>2.0×10⁹/7.9×10⁹</td>
<td>3.4×10⁹</td>
<td>-</td>
<td>CR+1M</td>
</tr>
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<td>14</td>
<td>MSa</td>
<td>75</td>
<td>M</td>
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<td>3.3×10⁹</td>
<td>6.6×10⁹</td>
<td>-</td>
<td>CR+7M</td>
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BM: bone marrow; PB: peripheral blood; M: month; CR: complete remission; PR: partial remission

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WT1 mRNA MEASUREMENT BY COMPETITIVE NASBA IN AML PATIENTS

Fig. 1. WT1 mRNA levels in the peripheral blood and bone marrow at different stages in AML patient.
- WT1 level in bone marrow
- WT1 level in peripheral blood

Fig. 2. WT1 mRNA levels in the peripheral blood and bone marrow of AML patients.

WT1 mRNA levels were measured by competitive NASBA in AML patients. Figure 1 shows the level of WT1 mRNA (log copies/μg) in the peripheral blood and bone marrow samples collected at different disease states. At initial onset, the mean WT1 mRNA level (log copies/μg) in the bone marrow was 5.7161±1.0262 and that in the peripheral blood was 6.0303±1.2063. At complete remission, the mean WT1 mRNA level (log copies/μg) in the bone marrow was 3.6878±0.6821 and that in the peripheral blood was 2.8668±0.7288. At recurrence, the mean WT1 mRNA level (log copies/μg) in the bone marrow was 5.8896±0.5387 and that in the peripheral blood was 5.1458±0.5895. The level of WT1 mRNA for the leukemia patients at initial onset was significantly higher than that for the healthy individuals. The level of WT1 mRNA at complete remission was lower than that at initial onset, and that at recurrence was higher than that at complete remission. Of those patients who had a low WT1 mRNA level at complete remission, the level of WT1 mRNA in the bone marrow for cases 2, 6 and 7 was somewhat higher than that for the other cases, and leukemia recurred in two of the three cases (cases 2 and 6). The paired t-test revealed the level of WT1 mRNA in the bone marrow differed significantly between initial onset and remission (p=0.000002) and between remission and recurrence (p=0.0170). However, there was no significant difference in the level of WT1 mRNA in the bone marrow between initial onset and recurrence. In addition, the level of
WT1 mRNA in the peripheral blood differed significantly between initial onset and remission (p=0.0007) and between remission and recurrence (p=0.0325), but there was no significant difference between initial onset and recurrence.

A simple regression model was used to compare the level of WT1 mRNA in the peripheral blood and that in the bone marrow of eight AML patients in whom the levels of WT1 mRNA in the peripheral blood and bone marrow were quantified at the same time. As shown in Fig. 2, there was a fine correlation with a coefficient of $r=0.9245$.

The expression of the PML-RARα gene was assessed by FISH and compared to that of WT1 mRNA in the AML (M3) patients (Fig. 3). The results showed that the PML-RARα gene was positive and the level of WT1 mRNA was high in these patients before therapy, and the PML-RARα gene was negative and the expression of the WT1 gene decreased when leukemia was in remission hematologically.

**DISCUSSION**

Competitive NASBA quantifies the expression of mRNA within a 50-10^8 range during 90 min of NASBA reaction and after about 2 hrs of detection reaction, and it is, therefore, a faster and more sensitive quantification method than RT-PCR. In the present study, we quantified the level of WT1 mRNA in AML patients by competitive NASBA.

The level of WT1 mRNA in the peripheral blood was less than 10^8 copies/μg for every healthy individual. Although the level of WT1 mRNA in the bone marrow of the healthy individuals was not quantified in the present study, it has been reported that the expression of WT1 mRNA in the bone marrow is about 10-100 times greater than that in the peripheral blood [5].

The level of WT1 mRNA in the peripheral blood and bone marrow of AML patients at initial onset was significantly higher than that in the peripheral blood of the healthy individuals. In addition, we quantified the level of WT1 mRNA in the peripheral blood and bone marrow of AML patients at different times, finding that the level of WT1 mRNA was significantly high at initial onset, significantly low at remission, and significantly high at recurrence, thus suggesting that monitoring the level of WT1 mRNA would be useful in assessing the clinical course of AML patients. However, when therapy resulted in complete remission, the level of WT1 mRNA decreased in every patient, but in some of these patients (cases 2, 6 and 7), the level of WT1 mRNA in the bone marrow was somewhat higher than that of other patients. The WT1 gene is a cancer suppressor gene, and the loss or mutation of this gene is involved in the onset of leukemia in some cases, thus it is necessary to keep in mind that low levels of WT1 mRNA do not necessarily represent remission [7-9]. Leukemia recurred in two of the above-mentioned three cases in which the level of WT1 mRNA in the bone marrow was relatively high (cases 2 and 6). Therefore, even when complete remission is achieved morphologically, leukemia can recur when the level of WT1 mRNA is high, suggesting that it will be necessary to monitor closely any shifts in the level of WT1 mRNA if its level is high at complete remission.

The detection of a chimeric gene is reportedly useful in diagnosing some translocation-type leukemia and detecting MRD with a high degree of sensitivity. In the present study, we quantified the expression of WT1 mRNA and detected the presence or absence of the PML-RARα gene by FISH in AML (M3) patients. The results showed that at initial onset, the PML-RARα gene was positive and the level of WT1 mRNA was high, whereas the PML-RARα gene was negative and the level of WT1...
mRNA low at complete remission. These findings suggest that the level of WT1 mRNA could be a marker of MRD. The FISH technique used in an analysis of chimeric genes can quickly identify chromosomal changes not only in dividing cells but also in interphase cells, thus making it possible to monitor MRD. However, there are problems with false-positive and false-negative cases and MRD cannot be quantified [15]. In the future, it will be necessary to quantify the expression of chimeric mRNA by RT-PCR and assess its correlation to the expression of WT1 mRNA so that therapeutic outcomes can be assessed and recurrence predicted more accurately.

Recurrence prediction and early diagnosis are extremely important factors in leukemia therapy. As far as leukemia diagnosis and follow-up observations are concerned, the detection of a chimeric gene is extremely useful in some translocation-type leukemia (AML (M2) AML1/ETO [16-18], (M3) PML-RARα [19,20], and CML BCR/ABL [21] account for about 10-20% of leukemia), and it can detect MRD with a high degree of sensitivity. However, this type of chimeric gene is not detectable in every type of leukemia, and the type of chimeric gene differs depending on the type of leukemia. Therefore, a sensitive tumor marker that is useful in the detection of MRD in all types of leukemia is needed, and WT1 mRNA could serve as such a tumor marker in various types of leukemia [4,5].

In the present study, we quantified the level of WT1 mRNA by competitive NASBA in AML patients, and confirmed that the present method was convenient, fast and sensitive. Although only 14 patients were analyzed in the present study, the level of WT1 mRNA in the peripheral blood and bone marrow of these patients at the initial onset was high irrespective of the presence or absence of chromosomal abnormality or the type of AML. Hence, we were able to confirm that by measuring the level of WT1 mRNA, MRD can be detected in AML patients and their clinical course can be monitored. Since the level of WT1 mRNA in peripheral blood samples was a useful marker, test samples can be collected easily. In the future, it will be important to study more cases and analyze the level of WT1 mRNA at initial onset, complete remission and recurrence more thoroughly so that recurrence can be predicted and diagnosed early with greater accuracy.

In recent years, the level of WT1 mRNA was found to be useful for classifying myelodysplastic syndrome (MDS) [22]. It will be necessary to investigate the usefulness of the level of WT1 mRNA measured by the present method in other hematological diseases.

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