Using Quantitative Assessment of the Wilms Tumor 1 Gene Expression in Residual Disease Monitoring in Patients with Acute Myeloid Leukemia

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ABSTRACT

Background. Despite considerable effort, a suitable specific molecular marker for monitoring minimal residual disease (MRD) has not been found for the majority of acute myeloid leukemia (AML) patients. The results of some studies suggest the Wilms tumor gene (WT1) as a possible molecular marker of MRD.

Methods and Results. We measured the expression of WT1 at diagnosis and during treatment of acute myeloid leukaemia (AML) patients. The expression of WT1 was measured by the quantitative real-time RT-PCR in peripheral leukocytes from 56 AML at diagnosis and 7 patients with AML transformed from myelodysplastic syndromes (MDS). The WT1 expression was significantly elevated (up to 3 orders of magnitude) in peripheral blood samples (PB) of AML patients at diagnosis compared to PB samples of healthy donors (P<0.0001). The level of WT1 expression depends particularly on FAB AML subtype, with the highest being found in AML patients with subtypes M4, M1, M3 and AML transformed from MDS. Conversely, AML patients with M2 and with the presence of AML1/ETO at presentation showed a significantly lower expression of the WT1 gene compared to the remaining AML patients at presentation (P = 0.005). Further, sequence samples of 12 AML patients under long-term surveillance were tested for the WT1 expression in parallel with the expression of specific MRD markers - fusion genes: AML1/ETO, PML/RAR and CBFB/MYH11. The levels of WT1 gene expression and the above specific fusion genes significantly correlated. Moreover, 14 patients without the specific MRD marker were tested for the WT1 expression. The results show that hematological relapses were associated with the rise of expression of the specific fusion genes and with the WT1 gene expression. As for the rise of WT1 expression above the level seen in leukocytes from peripheral blood and/or bone marrow of healthy donors, in four patients under long-term surveillance the “molecular relapse” predicted ongoing hematological relapses as early as 2 months in advance.

Conclusions. Our results, in accordance with some of the previously published ones, show that WT1 expression seems to be a suitable marker of minimal residual disease in AML patients.

Key words: leukemia, AML, WT1, MRD, PML/RARa, AML1/ETO, CBFB/MYH11, real-time PCR

found in less differentiated subtypes of AML M1-3 and lower in subtypes M4 and M5 (7), (8) and (9). Others (10), however, did not find a correlation between the WT1 gene expression and particular FAB subtypes, except for a significantly lower expression in AML M5. WT1 expression in patients with ALL is also increased, but one degree less than in acute myeloid leukemias (11, 12). Although a significantly increased expression of the WT1 gene is limited to leukemic blasts, its monitoring can be used for minimal residual disease surveillance detected best by quantitative RT-PCR (RQ-RT-PCR). In case of acute myeloid leukemias, it is possible to detect the WT1 gene using the above mentioned RQ-RT-PCR on a high level of sensitivity (detection of a single leukemic cell between $10^9 - 10^{10}$ normal peripheral blood cells). In another work (7), the authors systematically started to survey the level of MRD using a WT1 as a marker and concluded that it may be useful for the MRD monitoring. Even though some (13) confirmed the usefulness of the WT1 as a reliable marker of MRD, the majority of other studies, especially those using a highly sensitive, reliable and standardized method – PCR in real time (12, 8) and (11), confirmed the results published in (7). From the clinical point of view, it is important to note that the methods of RQ-PCR detection of the WT1 transcript make it possible to predict an oncoming relapse of a disease 3 months in advance (12, 11). A level of the WT1 gene expression in patients with AML is of a prognostic value in diagnosis: patients with a high level of the WT1 gene expression have a shorter overall survival and shorter disease-free survival (10), (7). A crucial fact is that there is a wider dynamic interval of the WT1 transcript level in peripheral blood cells compared with bone marrow cells, taking into account the fact that peripheral blood taking is less stressful for the patient (11, 14). Further, there is increased WT1 expression when chronic myeloid leukemia transforms to the blastic phase (11, 15).

**Purpose of the study**

Evaluation of some preliminary results on a group of patients with AML with clearly defined molecular markers: fusion genes AML1/ETO, CBFB/MYH11 and PML/RARalpha.

**METHODS AND PATIENTS**

We studied 57 patients with AML and 26 patients during treatment (median of surveillance = 11.51 months). Patients surveyed during treatment were divided based on the FAB subtype: 6 AML M1, 9 AML M2 (4 of them were carriers of a fusion gene AML1/ETO), 7 AML M3 with a fusion gene PML/RAR a 4 AML M4 (3 of them carrying a fusion gene CBFB/MYH11). The source material included leukocytes drawn from patients at the time of diagnosis and during the course of treatment. The elements were taken mostly from peripheral blood or, in retrospectively evaluated samples, from bone marrow. Leukocytes were prepared using standard methods of leukocyte isolation, selective osmotic lysis. A standard amount of leukocytes (10 million) were lysed in an ITG solution (16) kept at – 20 °C. Overall RNA was isolated in a lysate solution and was reversely transcribed into a cDNA using a method described previously (17). Regions of cDNA with tracked genes were amplified using quantitative polymerase chain reaction with specific starting oligonucleotides (primers) and a specific fluorescent-marked TaqMan probe for cDNA gene WT1 based on (11) using Hot Start polymerase FastStart (Roche, Mannheim, Germany). Quantification was performed on a thermal cycler RotorGene 2000 or 3000 (Corbett Research, Sydney, Australia) enabling the use of PCR in real time. For quantification purposes a dilutant series of a standard with an incorporated PCR gene WT1 with a known concentration was evaluated. This standard was prepared using cloning of a purified PCR product from a cDNA line K562 into a plasmid pCR®2.1-TOPO® using a method TOPO TA cloning (Invitrogen Corporation, Carlsbad, USA). After cloning the plasmid was isolated, cleaned, its concentration was determined and a dilutant series was prepared in a TE buffer, pH 8, with added Herring Sperm DNA with a resulting concentration of 100ng/l (Promega, Madison, USA). In parallel, a reference ABL gene was quantified, amplified using a RQ-RT-PCR with a specific TaqMan probe as described in (18). A standard for the ABL gene was prepared using the same method of cloning, isolation and purification. The results of quantification were expressed as relative values, comparing the WT1 values to ABL, which we obtained using a formula: $2^{(\text{Ct}_{\text{ABL}} - \text{Ct}_{\text{WT1}})}$, Ct standing for the amplification cycle where the fluorescence of the sample reached a threshold equal to all amplified samples during the run. Fusion genes PML/RARα, CBFB/MYH11 and AML1/ETO were quantified based on (24, 25) and (26).

Statistical evaluation of the results, i.e. medians and results of quantification differences and correlations were counted using GraphPad Prism with a Mann-Whitney linear regression.

**RESULTS**

**Patients at diagnosis**

The WT1 gene expression in the peripheral blood of patients with AML at diagnosis was significantly increased by 3 orders (median expression of WT1 = 1.211, number = 57) compared with expression in peripheral blood of 26 healthy donors (median expression of WT1 = 0.002, P<0.0001). After dividing the patients with acute myeloid leukemia based on the FAB classification, we found the highest expression in 14 patients with AML M1 (median expression of WT1 = 2.112) and in 14 patients with AML M4 (median expression of WT1 = 2.84) and lowest in 4 patients with AML M5 (median expression of WT1 = 0.577) and in 19 patients with AML M2 (median expression of WT1 = 0.276). Patients with AML M3 at the time of diagnosis had a relatively high WT1 expression (median expression of WT1 = 1.199). The highest expression of WT1 at the time of diagnosis was found in a patient with AML M4 (WT1 = 24) and the lowest expression of the WT1 gene was found in a patient with AML M5 (WT1 = 0.0004). After selecting a group of patients with AML M2 and with the presence of a fusion gene AML1/ETO, we found that this group showed a significantly lower expression of the WT1 gene at the time of diagnosis than in other patients with AML (P = 0.005). We also found a high expression of the WT1 gene in 8 patients with MDS transformed in AML (median WT1 gene expression = 2.595). In 6 patients with ALL at the time of diagnosis we found increased WT1 expression (median expression of the WT1 gene = 0.088).

**MRD surveillance**

In 102 retrospectively evaluated samples, mostly from bone marrow, we performed correlation between the WT1 gene expression during the course of the disease in 12 patients with AML and with a presence of a specific MRD marker (fusion gene). We found a highly significant correlation between the course of the WT1 gene expression and expression of other specific fusion gene markers ($r^2 = 0.63 – 0.99$). Considering that gene expression in bone marrow of 31 healthy donors (median expression of WT1 = 0.01) was significantly increased compared to the WT1 expression in peripheral blood in 26 healthy donors (median expression of WT1 = 0.002, P<0.0001), it is more convenient to monitor WT1 expression in leukocytes from peripheral blood.

In most patients successful therapy led to a decrease of WT1 expression by 3 orders reaching the values of normal donors and
complete remission; however, in some it later led to a relapse of the
disease. Concurrently, the relapse was accompanied with an
increased expression of the WT1 gene and in some patients this
preceded the hematological relapse. For example, in the case of one
patient with AML M4Eo, the WT1 expression started to rise about
2 months before the hematological relapse. This was followed by
therapeutical remission of the disease accompanied by a decrease of
the WT1 gene expression down to values one order higher
compared to the median WT1 expression in normal bone marrow
(see Graph. 1). Further on, the patient experienced another relapse
accompanied by a rapid increase of both markers of MRD.
After successfully overcoming the relapse and reaching complete
remission, the WT1 gene expression in parallel with increased expression of the specific marker for MRD – fusion gene PML/RARα followed by a relapse of the disease. Expression of both markers of MRD significantly correlated ($r^2 = 0.67$, $P = 0.011$). There was an analogous situation in another patient (see Graph. 3) where the remission was accompanied with a decrease in the WT1 gene expression in bone marrow samples by three orders together with a decrease of the CBFB/MYH11 gene expression by three orders. After approximately 10 months of remission the patient had another relapse accompanied by increased WT1 expression by one order (molecular relapse) preceding the hematological relapse by 32 days. The WT1 gene expression significantly correlated with CBFB/MYH11 gene expression ($r^2 = 0.63$, $P = 0.019$). Another patient (see Graph. 2) repeatedly experienced (3 times) major increase of the WT1 gene expression in parallel with increased expression of the specific marker for MRD – fusion gene PML/RARα followed by a relapse of the disease. Expression of both markers of MRD significantly correlated ($r^2 = 0.67$, $P = 0.011$). There was an analogous situation in another patient (see Graph. 3) where the remission was accompanied with a decrease in the WT1 gene expression in bone marrow samples by three orders together with a decrease of the CBFB/MYH11 gene expression by three orders. After approximately 10 months of remission the patient had another relapse accompanied by a rapid increase of both markers of MRD. After successfully overcoming the relapse and reaching complete remission (CR), the WT1 gene expression and the fusion gene CBFB/MYH11 expression both decreased to values equal to those found in normal bone marrow, and the patient reached a second complete remission. In this patient too, expression of both markers

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**Graph 1:** WT1 gene expression (---) and CBFB/MYH11 (-----) in CD in a patient with AML M4Eo

**Graph 2:** Graph of the WT1 gene expression (-----) and PML/RARα (-------) in bone marrow in a patient with APL
Hematological relapse: “h-rel”, 1 and 2 reinduction: “rein” (protocol IDA + ATRA), 3 reinductions (protocol AIDA) and 4 relapses – 2 therapies using Mylotarg: “MYL”.

(38)
of MRD showed high correlation ($r^2 = 0.90$, $P < 0.0001$). As mentioned above, in patients with AML M2 and a presence of the AML1/ETO gene we observed lower values of the WT1 gene expression at the time of diagnosis; therefore at the time of remission there is a relatively lower decrease of this marker compared to more pronounced decrease of the AML1/ETO gene expression. At the time of a relapse there is a higher increase in the AML1/ETO gene expression compared with the WT1 gene (see Graph 4). From this point of view, we can conclude that there is a higher dynamic interval of the AML1/ETO gene expression compared with the WT1 gene. An opposite tendency, i.e. higher dynamic interval of the WT1 gene expression compared with the PML/RARα gene, can be observed in patients with AML M3.

In the patients mentioned above, as well as some others, we were able to monitor the levels of the WT1 gene expression and levels of expression of both specific MRD markers – fusion genes. In most patients with AML (about 60 percent) these markers are not, however, present. Monitoring of the WT1 gene expression could replace them. In another 14 patients without the presence of a specific marker we monitored the MRD only by detection of the WT1 gene expression in peripheral blood compared with morphological-clinical features. Similar to a group of patients with AML with a specific marker of MRD, we found such individuals who, following successful therapy, reached complete remission (CR) accompanied with a decrease of the WT1 gene expression down to values measured in peripheral blood leukocytes of healthy donors.


where we observed a decrease of the WT1 gene expression on a value of 0.075, that is a decrease by 3 orders, however, not reaching the values of healthy individuals (note: the patient was refractory to induction therapy). This patient experienced a relapse of the disease preceded by an increase of the WT1 gene expression anticipating hematological relapse by 46 days (see Graph. 5).

We can summarize that in patients under surveillance we found a strong correlation between the WT1 gene expression and specific markers, fusion genes AML1/ETO, CBFB/MYH11 and PML/RARα and correlation of an increase of the WT1 gene expression before a hematological relapse. These results confirm the usefulness of monitoring the WT1 gene expression as a marker of minimal residual disease especially in those patients without a presence of specific markers.

**DISCUSSION**

Results of the WT1 gene expression in patients with AML show that the highest values were found in patients with a subtype M4 and lowest in patients with subtypes M2 and M5. In patients with AML M2 we also found the widest interval of values of the WT1 gene expression. In patients with AML M2 the median of the WT1 gene expression is relatively low compared with patients with subtypes AML M1 and M3, which is in contradiction with results published in (11) and (8). In these studies they found that the value of the WT1 gene expression in patients with AML M2 is relatively high. Patients with AML M2 and a presence of the AML1/ETO fusion gene show a significantly lower WT1 gene expression at the time of diagnosis compared with other patients with AML. This is in agreement with other published studies. From this point of view we can also explain the relatively lower median of the WT1 gene expression in all patients with AML M2 at diagnosis. Furthermore, 2 patients with AML M2 (without a presence of a fusion gene AML1/ETO) had a very low WT1 gene expression at the time of diagnosis, and the course of their disease was unfavorable, in contrast to results published in (7) and (8). One of these patients was most probably a patient with myeloproliferation with a presence of the t(5;12) translocation who transformed into an AML. Searching for correlation between the WT1 gene expression at the time of diagnosis and prognosis was not the goal of the study.

In accordance with published data (11, 9) and (8), we found

<table>
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<th>Patient number of retrospective samples</th>
<th>specific marker</th>
<th>level of significance</th>
<th>$r^2$</th>
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<tr>
<td>patient 1 8</td>
<td>AML1/ETO</td>
<td>P&lt;0.0001</td>
<td>0.98</td>
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<tr>
<td>patient 2 7</td>
<td>AML1/ETO</td>
<td>P&lt;0.0003</td>
<td>0.85</td>
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<tr>
<td>patient 3 6</td>
<td>AML1/ETO</td>
<td>P&lt;0.0001</td>
<td>0.99</td>
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<tr>
<td>patient 4 5</td>
<td>AML1/ETO</td>
<td>P=0.0002</td>
<td>0.99</td>
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<tr>
<td>patient 5 5</td>
<td>AML1/ETO</td>
<td>nekoreluje</td>
<td>0.46</td>
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<tr>
<td>patient 6 6</td>
<td>PML/RARα</td>
<td>P&lt;0.0001</td>
<td>0.998</td>
</tr>
<tr>
<td>patient 7 12</td>
<td>PML/RARα</td>
<td>P=0.011</td>
<td>0.67</td>
</tr>
<tr>
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<td>PML/RARα</td>
<td>P=0.0004</td>
<td>0.986</td>
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<tr>
<td>patient 9 13</td>
<td>CBFB/MYH11</td>
<td>P=0.019</td>
<td>0.63</td>
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<tr>
<td>patient 10 12</td>
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<td>0.901</td>
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<tr>
<td>patient 11 5</td>
<td>CBFB/MYH11</td>
<td>P=0.0001</td>
<td>0.99</td>
</tr>
<tr>
<td>patient 12 8</td>
<td>CBFB/MYH11</td>
<td>P&lt;0.0001</td>
<td>0.99</td>
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**Graph 5.** Graph of the WT1 gene expression (—–) in peripheral blood in a patient with AML M4.

a very high correlation between the high WT1 gene expression at the time of diagnosis in patients with AML M1, AML M3 and in patients with AML transformed from MDS. Although the number of patients with the AML M5 subtype was very small (4 patients), median WT1 gene expression showed a relatively low value and a wide interval of values, similar to the results published in another study (8).

Even though on a limited number of patients with ALL, we detected, in accordance with results published in (8) and (11), a significantly lower WT1 gene expression in 6 patients with ALL at the time of diagnosis compared with the WT1 gene expression in patients with AML at diagnosis. Monitoring the WT1 gene expression in peripheral blood and in bone marrow showed that the WT1 gene expression in bone marrow is significantly higher than in peripheral blood. The difference in the WT1 gene expression at the time of diagnosis and during remission is higher in peripheral blood samples than in bone marrow. This observation is in accordance with results published in other studies (14). Also, blood taking from peripheral blood is less stressful for the patient than bone marrow biopsy. It is again another reason why monitoring the WT1 gene expression from peripheral blood as a marker of the MRD is more useful.

Our results of the MRD monitoring using the WT1 gene expression showed highly significant correlation of the WT1 gene expression from bone marrow or peripheral blood with specific markers of the MRD – fusion genes AML1/ETO, CBFB/MYH11 and PML/RARα in all studied patients at the time of diagnosis and during the course of the disease. This is not in agreement with results published in study (13); however, it is in accordance with results published in (20) and (12). In these studies they also found a highly significant correlation of the WT1 gene expression with the above mentioned specific markers of MRD. In four patients in long-term surveillance we found that a molecular relapse (i.e. increase of the WT1 gene by one order over the median found in healthy donors) preceded the hematological relapse by almost 2 months five times.

In case of acute myeloid leukemias in children, it is important for prognosis to know the expression of the appropriate MRD marker 5 weeks after diagnosis (21). But in adults suffering acute leukemia it is not possible to prove correlation between the state of MRD after the first and second induction therapy and further prognosis of the disease (22). On the contrary, there was correlation found between prognosis factors, such as DFS (disease free survival) and OS (overall survival) and the WT1 gene expression after the induction and second consolidation therapy (23). The authors established a significantly better DFS and OS in patients with the WT1 gene expression values lower than the determined limiting expression equal to $10^3$ copies of the WT1/10^3 copies of the ABL gene. With regard to a common relapse in AML, it may be useful to find such a marker that would indicate a relapse early enough. Achieving the above mentioned limiting WT1 gene expression may provide a useful marker. However, the low number of patients in our study does not allow us to state a reliable conclusion, even though the results of MRD surveillance in patients with leukemia using quantitative monitoring of the WT1 gene expression show a good means of prediction value in case of a relapse.

List of used abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CIA</td>
<td>Czech Institute of Accreditation</td>
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<tr>
<td>EMDIS</td>
<td>European Marrow Donor Information System</td>
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<td>GvHR</td>
<td>Graft versus Host</td>
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<td>HLA</td>
<td>Human Leukocyte Antigens</td>
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<td>HR</td>
<td>high-resolution – (detection on the level of alleles)</td>
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<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
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<td>HSCT</td>
<td>Hematopoietic Stem Cells Transplantation</td>
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<td>HvGR</td>
<td>Host versus Graft Reaction</td>
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<td>IGMT</td>
<td>International Histocompatibility Working Group</td>
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<td>LR</td>
<td>low-resolution - (detection on the level of allelic groups)</td>
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<td>MUD</td>
<td>Marrow Unrelated Donor</td>
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<tr>
<td>PCR</td>
<td>SSP – Polymerase Chain Reaction- Sequence Specific Primers</td>
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<td>Sequencing-Based Typing</td>
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LITERATURE


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