Quantitative monitoring of WT1 expression in peripheral blood before and after allogeneic stem cell transplantation for acute myeloid leukemia – a useful tool for early detection of minimal residual disease

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Overexpressed Wilms tumor gene 1 (WT1) has been found in a majority of patients with acute myeloid leukemia (AML). The aim of this study was to confirm the applicability of WT1 expression as a marker of minimal residual disease (MRD). The expression of WT1 gene was measured by real-time polymerase chain reaction in peripheral blood (PB) according to European Leukemia Net (ELN) recommendations. The WT1 expression was related to the expression of a reference gene Abelson (ABL) and the results were calculated as a number of WT1 copies related to 10^4 copies of ABL. The upper normal limit of WT1 expression was set at 50 copies of WT1 to 10^4 copies of ABL. Morphological, flow cytometry and chimerism examinations were evaluated according to standard protocols.

A total of 51 AML patients with overexpressed WT1 gene were analyzed. The median follow-up after transplantation was 14 (2-72) months. WT1 expression levels exceeding the upper normal limit were considered as a sign of impending hematological relapse, in accord with morphological, flow cytometry and chimerism data, as well as with the expression of the specific fusion genes. Moreover, in 7 patients the rise of WT1 expression preceded all other standard methods. Patients with high WT1 expression before allogeneic hematopoietic stem cell transplantation (allo-HSCT) had significantly worse outcome than patients with low WT1 level. Examination of WT1 expression in PB of patients with AML is a useful tool for MRD monitoring. Moreover, the WT1 gene expression before stem cell transplantation seems to be of prognostic significance.

Key words: WT1, MRD, allogeneic stem cell transplantation, acute myeloid leukemia
so called leukemia associated antigens and monitoring of these nonspecific markers could serve as an alternative in the absence of the specific markers. WT1 gene as one of them has been found overexpressed in 90% of acute myeloid leukemia cells [3]. It is located on the chromosome 11p13 and primarily it was isolated as a gene responsible for Wilms tumor [4]. The WT1 gene is preferentially expressed during embryogenesis and its expression is restricted to a small number of normal tissues such as testicle, kidney, ovary, uterus and other. Apart from normal functions the WT1 gene is considered as an oncosuppressor gene deleted in pediatric patients with WAGR syndrome, Denys Drash syndrome and concurrently it acts as a transcription factor with high expression found in mesothelioma, glioblastoma and leukemia [5, 6]. 24 protein isoforms have been described, which increase or decrease the function of many genes for growth factors or their receptors and these functions are probably associated with oncogenic properties such as inhibition of apoptosis via p53 and Bcl-2 and differentiation of leukemic cells [7]. According to many observations [8,9,10-15], the WT1 gene is considered as an appropriate MRD leukemic marker, and its expression in bone marrow or in peripheral blood correlates with disease status and reaches $10^4-10^8$ sensitivity of detection. Sensitivity and specificity of the WT1 quantification is of great importance, so huge effort was made in order to develop accurate and sensitive assay for WT1 quantification [16]. Beside the high expression of the WT1 gene at the time of diagnosis its expression is often elevated in advance of hematological relapse, which can be useful for early treatment approach. The aim of this study was to evaluate whether RQ-PCR monitoring of the WT1 gene expression in PB could serve as the MRD marker in acute leukaemia patients after allo-HSCT. Secondly, the prognostic significance of the WT1 expression before allo-HSCT seems to be important.

Patients and methods

Patients. Overall 51 patients were enrolled in the study between April 2005 and December 2010. All the treatment protocols, data collection and analysis methods were approved by the Institutional Review Board and the patients gave signed informed consent. Patients’ characteristics are shown in (table 1) and patients’ transplant characteristics are depicted in (table 2). The patients were divided into 2 groups according to the pre-transplant WT1 expression level with respect to the upper normal limit of WT1 expression (table 1). The overall survival (OS) was defined as the period from the day of transplantation until the day of death from any cause. Relapse incidence (RI) was defined as the time period from transplantation until relapse or disease progression. The definition of complete remission and relapse followed recommended criteria [17]. Prognostic subgroups of AML based upon presented cytogenetics and genetic lesions are defined according recent recommendation [18].

![Table 1. Patient characteristics](image)

<table>
<thead>
<tr>
<th>Total number of patients</th>
<th>51</th>
</tr>
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<tbody>
<tr>
<td>male</td>
<td>19</td>
</tr>
<tr>
<td>female</td>
<td>32</td>
</tr>
<tr>
<td>Age – median, years (range)</td>
<td>48 (21-63)</td>
</tr>
<tr>
<td>AML type (FAB)</td>
<td>M1 13x, M2 10x, M3 1x, M4 13x, M5 2x, M6 3x, NA 7x</td>
</tr>
<tr>
<td>Karyotype normal</td>
<td>23</td>
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<tr>
<td>abnormal</td>
<td>23</td>
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<td>5</td>
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<tr>
<td>Specific molecular marker</td>
<td>(2 x AML1/ETO, 5 x CBFβ/MYH11, 2 x MLL/AF9, 1 x PML/RARa)</td>
</tr>
<tr>
<td>WT1 status before HSCT</td>
<td>18 positive, 32 negative, 1 NA</td>
</tr>
</tbody>
</table>

* NA = not available

![Table 2. Transplant characteristics](image)

<table>
<thead>
<tr>
<th>Disease status at transplant</th>
<th>CR 1 (27%)</th>
</tr>
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<tbody>
<tr>
<td>CR 1 (after salvage therapy, primary induction failure)</td>
<td>16 (32%)</td>
</tr>
<tr>
<td>CR 2 (active disease)</td>
<td>15 (29%)</td>
</tr>
<tr>
<td>Donor</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>IS*</td>
<td>14 (27%)</td>
</tr>
<tr>
<td>Mismatched sibling</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>MUD</td>
<td>18 (35%)</td>
</tr>
<tr>
<td>MMUD (1-2 allelic mismatches)</td>
<td>18 (35%)</td>
</tr>
<tr>
<td>Conditioning</td>
<td>40 (78%)</td>
</tr>
<tr>
<td>myeloablative reduced intensity</td>
<td>11 (22%)</td>
</tr>
<tr>
<td>PBPC/BM+</td>
<td>41/10 (80/20%)</td>
</tr>
</tbody>
</table>

CR = complete remission, IS = identical sibling, MM= mismatch, MUD = matched unrelated donor, MMUD = mismatched unrelated donor, + PBPC = peripheral blood progenitor cells, BM = bone marrow

![Table 3. Follow-up](image)

| Follow-up after HSCT- median (range), months | 14 (2-72) |
| Disease status after HSCT | CR 32 (63%) |
| relapse | 17 (33%) |
| not evaluable | 2 (4%) |
| Status of patients at last follow-up | 33 (65%) |
| alive | 18 (35%) |
| death | 13 (25%) |
| Cause of death | NRM 5 (10%) |
| relapse/progression | 13 (25%) |
| NRM | 5 (10%) |

CR= complete remission, NRM = non relapse mortality

Samples preparation. Ten million white blood cells from peripheral blood were isolated by the red cell lysis method. The cell sediment was lysed and stabilized by guanidine isothiocyanate. Total RNA was extracted by the method of Chomczynski and Sacchi [19] modified according to Polak et al. [20].

cDNA Synthesis. The total of 0.5 – 1 µg isolated RNA was incubated with 50 pmol of random hexamers at 65°C for 10 minutes to release the possible secondary structure. Subsequently, 120 U of Mu-MLV reverse transcriptase (Promega,
The expression of WT1 was analysed before allo-HSCT. After allo-HSCT, the WT1 gene expression level was monitored every month in the first year and then four times per year, more often in case of relapse suspicion. Before introduction of the aforementioned protocol based on the ELN recommendations we had used the relative quantification of WT1 gene according to [20]. WT1 expression has been normalized to the ABL gene expression and results were depicted in relative units. Upper normal limit of 50 copies WT1/10000 copies ABL in ELN recommended protocol corresponds to relative WT1 expression = 0.02 according to the protocol described in [20]. Both methods have been found highly significantly correlated P<0.0001.

The expression of fusion genes CBFB/MYH11, RUNX1/RUNX1T1, PML/RARα and MLLT3/MLL was analysed according to previously described methods [20, 21].

Real-time PCR. The majority of the samples were analysed by the quantitative real-time PCR (RQ-PCR) of WT1 that was done by usage of the WT1 ProfileQuant KIT (Ipsogen S A., Marseille, France). The expression of the WT1 gene was related to the expression of the housekeeping gene ABL according to recommendations of ELN. Amplification and data analysis were carried out using the Rotor Gene 3000A thermocycler (Corbett Research, Sydney, Australia). The calibration curve, for quantitative assessment of WT1, was constituted by serial dilutions ranging from 10⁶ to 10⁴ molecules of a linearized plasmid obtained by cloning the target WT1 sequence. The results of WT1 expression are depicted as a number of WT1 copies related to 10⁴ copies of the reference gene ABL according to the manufacturer and ELN recommendations [16]. The sensitivity of this RQ-PCR method is defined as a possibility to detect 10 copies of WT1 gene. All samples were quantified in duplicates with defined measurement uncertainty and with appropriate negative control. In case of reference gene amplification, the samples with difference in particular Ct, larger than 0.6 cycle (that means measurement uncertainty) were re-amplified. Similarly, the maximal allowed differences, in particular for Ct values, i.e. measurement uncertainty, were defined for the particular levels of WT1 expression. The minimal expression of the reference gene ABL threshold was 3000 copies – the samples with lower expression of ABL gene were omitted. According to ELN recommendations the upper normal limit of WT1 expression is equal to 50 copies WT1/10⁴ copies of reference gene ABL in peripheral blood. The expression of WT1 was analysed before allo-HSCT. After allo-HSCT, the WT1 gene expression level was monitored every month in the first year and then four times per year, more often in case of relapse suspicion. Before introduction of the aforementioned protocol based on the ELN recommendations we had used the relative quantification of WT1 gene according to [20]. WT1 expression has been normalized to the ABL gene expression and results were depicted in relative units. Upper normal limit of 50 copies WT1/10000 copies ABL in ELN recommended protocol corresponds to relative WT1 expression = 0.02 according to the protocol described in [20]. Both methods have been found highly significantly correlated P<0.0001. The expression of fusion genes CBFB/MYH11, RUNX1/RUNX1T1, PML/RARα and MLLT3/MLL was analysed according to previously described methods [20, 21].

Morphological, flow cytometry and molecular chimerism examination. Flow cytometry analysis was performed on a FACS Aria III instrument equipped with FACS Diva v6.1.3 software and set up with CS&T calibration beads. Leukemia associated phenotypes (LAPs) were established in newly diagnosed AML and monitored using the following 4 tube/8 colour panel of monoclonal antibodies directly conjugated to Pacific Blue, Horizon V500, FITC, PE, PE-Cy5.5, PE-Cy7, APC and APC-H7 respectively:

1. HLA-DR/CD45/ CD2/CD13/CD34/CD117/CD7/CD14,
2. HLA-DR/CD45/CD36/CD56/ CD34/CD117/CD22/ CD19,
3. HLA-DR/CD45/CD15/CD33/CD34/CD117/CD11b/CD14, 4. HLA-DR/CD45/CD65/CD123/CD34/CD117/CD38/CD14. LAPs were identified in the CD45 low/SS-C1ow/CD34+ and/or CD117+ WBC compartment, following acquisition of at least 500,000 events. MRD% was corrected for the percentage of LAPs expression on leukemic blasts at diagnosis as described by Feller et al. [22].

Chimerism was analysed by quantitative determination of informative DNA polymorphisms that included VNTRs (variable number of tandem repeats) STRs (short tandem repeats) and sex-specific loci using standard protocols [23]. Complete chimerism was defined as >99% donor’s hematopoietic stem cells in recipient’s bone marrow or peripheral blood.

CR was defined as absence of any tumor and <5% BM blasts with polymorphonuclear cells >1.10^9/L, platelets >100.10^9/L and independence of transfusion. Partial remission was defined as 5-15% blasts in BM of adequate cellularity with evidence of trilineage regeneration. Patients who did not meet the criteria for CR or PR were categorized as resistant. Definition of response to therapy before and after transplantation was expressed according to the published criteria. [24]

Statistics. Estimation of median expression was performed by GraphPad Prism4 column statistics. Kaplan–Meier curves and two-sided long-rank test were used to estimate the distribution of overall survival and relapse incidence and to compare difference between survival curves. The tests were conducted at a level of significance of 0.05. Expression of specific chimeric fusion genes were correlated by using linear regression of GraphPad Prism4 software. Categorical patient characteristics were compared by using Fisher’s exact test and Pearson Chi-Square test, continuous characteristics were compared with the two-sample t test.

Results

After successful allo-HSCT, 32 patients remained in long-term remission and their WT1 expression stayed below the upper normal limit (fig. 3), i.e., low level; simultaneously the other markers i.e. morphologic, flow cytometry and chimerism examination were negative. Therefore we established complete agreement between low WT1 level, full donor chimerism (100%) and negativity by flow cytometry. On the other hand, many of followed patients (n = 17) relapsed with simultaneous rise of the WT1 expression (median of WT1 expression in remission was 3126 copies WT1/10^4 copies ABL) above upper normal limit (fig. 4). The WT1 gene expression rose not only together with morphologic, flow cytometry and chimerism findings, its expression even preceded expression of these markers with a median of 0.99 months (range: 0,69 – 1,87) in the follow-up samples of 7 patients (fig. 5 and table 4). A subset of patients (n = 10) displayed the presence of the specific fusion genes, i.e., CBFB/MYH11, RUNX1/RUNX1T1, PML/RARα and MLLT3/MLL. The close agreement between expression of WT1 and morphologic, flow cytometry and chimerism findings points to a highly significant correlation of the WT1 gene expression and of the fusion genes: (P<0.0001) in case of the CBFB/MYH11 fusion gene, the RUNX1/RUNX1T1 fusion gene (P=0.0114) and the MLLT3/MLL fusion gene (P<0.0001) (fig. 6). On the other hand, in one examined patients the expression of the PML/RARα fusion gene did not correlate...
with the WT1 expression. A total of 17 patients relapsed and 13 of relapsed patients died afterwards (tab. 3). Four relapsed patients were successfully treated by chemotherapy and DLI and they reached complete remission with simultaneous decline of the WT1 expression below the upper normal limit. Some relapsed patients (n=2) showed a relative slow increase of the WT1 expression with a longer WT1 expression doubling time. These patients might be the appropriate candidates for early immunomodulatory intervention (i.e., tapering of immunosuppressive therapy and/or DLI); the rest of the patients relapsed more rapidly with the short WT1 doubling time. For purposes of additional comparison we divided patients according to the WT1 expression level before transplantation. Both groups, i.e., WT1 positive patients before transplantation and

Table 4. Summary of 7 patients where the WT1 rised before positivity of morphology evaluation, chimerism and/or flow cytometry (FCM). The calculated predetection of WT1 expression is in „predetection by WT1“ column. Donor chimerism is depicted in percentage of the donor haemopoiesis. WT1 expression is measured in number of WT1 copies/10^4 copies ABL. 50 copies of WT1/10^4 ABL is the upper normal limit, HSCT is hematopoietic stem cell transplantation, micro means microchimerism.

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<th>Patient n.</th>
<th>months since HSCT</th>
<th>WT1 copies</th>
<th>morphology</th>
<th>FCM</th>
<th>chimerism percentage</th>
<th>Predetection by WT1 (months)</th>
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</table>

Figure 3. Monitoring of MRD of AML patients in permanent remission after HSCT by measuring relative expression of the WT1 gene in peripheral blood. The dotted line represents the upper normal limit.

Figure 4. MRD monitoring of AML patients who relapsed after HSCT by measuring relative expression of the WT1 gene in peripheral blood. The dotted line represents the upper normal limit.
WT1 negative patients before transplantation were compared in terms of followed parameters: age, sex, FAB subtype, disease status at allo-HSCT, donor type, graft type, conditioning regimen, incidence of acute and chronic GVHD and finally genetic risk. There have not been found statistically significant differences. We have found that the patients with a high pretransplant level (WT1 positive) of the WT1 gene (n=18) compared to those with low expression of the WT1 gene (WT1 negative) (n=32) differ significantly in terms of overall survival and relapse incidence. The overall survival was significantly longer in patients with low WT1 expression before allo-HSCT (OS in 3-years 26% vs. 73% (p=0,002) (fig. 1): Hazard ratio: 0,2383 95% CI of ratio (0.06128 to 0.4988). Analogously, relapse incidence was higher in patients with high pre-trans-
plation level of the WT1 expression (cumulative RI in 3
years 82 % vs. 25% (p=0.007) (fig. 2): Hazard Ratio 0.2350
95% CI of ratio 0.05284-0.4571. The follow up data and disease
status are depicted in (table 3).

Discussion

The determination and accurate assessment of MRD in
AML patients is very important, because relapse remains the
main cause of treatment failure [25, 26]. Data obtained from
MRD analysis allow to determine the disease status and
could predict impending relapse in advance. The chimeric
fusion genes: CBFB/MYH11, RUNX1/RUNX1T1, PML/
RARα and translocations involving MLL gene are widely
used for MRD evaluation [27-31]. Using the real-time PCR
quantification approach, the sensitivity of detection of one
leukemic cell among 10^4 to 10^6 normal cells can be reached. Unfortunately, 50-60% of adult acute leukaemia patients lack
these specific markers, and morphological, flow cytometry
and chimerism examination have lower sensitivity compared
to specific real-time PCR methods. Intensive effort has been
done to find an alternative nonspecific marker of MRD. The
WT1 gene has been found to be highly overexpressed in the
majority of AML; it may serve as nonspecific marker. There-
fore, it could be applied particularly to those AML patients
who lack specific markers. The expression of the WT1 gene
was described as a useful marker of MRD in AML patients
due to its high specificity, sensitivity and most importantly
to its broad applicability by many authors [8, 9, 10-15]. There
exist some controversies concerning the use of the WT1 gene
for MRD monitoring, especially after allo-HSCT [32]. The
above-cited study did not confirm the use of the WT1 gene
as a suitable MRD marker; on the other hand, this study was
based on the application of the qualitative PCR in contrast
to the real-time PCR, which is generally more specific and
sensitive and allows accurate quantification. The second
obstacle was the WT1 expression in the subset of normal
CD34 positive cells from peripheral blood of healthy donors
[33]. That is why a strict cut-off value must be determined
to distinguish normal expression from an abnormal one.
Our protocol is based on the highly specific and sensi-
tive RQ-PCR quantification of the WT1 gene expression in
peripheral blood samples, based on the recommended
ELN protocol. In this protocol the upper normal limit of
the WT1 expression had been determined and validated by
the multicenter study [16] in order to eliminate the above
obstacle with WT1 expression in a subset of normal CD34+
cells. Exceeding this limit, in fact the molecular relapse level,
predicts impending relapse. ELN multicenter study therefore
introduced a highly sensitive and reliable tool to distinguish
normal expression from the aberrant one. Another requisite
parameter in MRD follow-up is a proper determination of
the time schedule for measurement of WT1 expression [34].
All our patients were analysed at the time of diagnosis and/or
in relapse, to determine the information value of the WT1
expression. The majority of patients were followed at least
once per month following transplantation during the first
year. In case of risk patients with the persistent MRD before
transplantation is WT1 followed 2 times per month in early
postransplant period (up to 3 months). Secondly, the more
frequent monitoring of WT1 expression is recommended in
case of suspected relapse. We observed an absolute correla-
tion between increased WT1 expression and the positivity
with alternative methods, i.e., polychromatic flow cytometry
and molecular chimerism examination. In addition, as also
described in another study [24], the WT1 expression grew
earlier than the other markers. Approximately 40-50% AML
patients express chimeric fusion genes and their expression
significantly correlated with expression of the WT1 gene
[10, 8, 15]. In our setting, we found the expression of the WT1
gene in a highly significant correlation with the expression
of specific fusion genes (except for one patient bearing the
PML/RARα fusion gene), confirming the applicability of
our approach. The evaluation of the WT1 expression after
allo-HSCT seems to be a useful tool for ascertaining the
disease status in agreement with published data [2, 25, 26].
Similarly to other authors we found a correlation between the
pretransplantation WT1 level and prognosis [26]. The overall
survival of patients with high pretransplant level of the WT1
expression was significantly worse than that of patients with
low expression of the WT1 gene; also, patients with high ex-
pression of the WT1 before transplantation displayed higher
cumulative incidence of relapse. A certain limitation of our
study is the fact, that WT1 gene analysis was performed in
peripheral blood. According to published data [34] it is
more suitable to measure the WT1 gene expression in bone
marrow samples, because the impending relapse is detect-
able sooner than in peripheral blood samples. This problem
could be solved by more frequent sampling after HSCT as
recommended by [34], i.e., 2 times per month. Moreover,
examination from peripheral blood is less stressful for the
patients than bone marrow aspiration. Consequently, when
our molecular relapse border was exceeded, we performed
further analysis as soon as possible, in order to confirm or
exclude the impending relapse of disease. Our study en-
compasses a relative low number of patients to allow more
precise analysis of the impact of the other factors like type
of conditioning regimen used or impact of AML prognostic
markers like presence of FLT3/ITD or the other molecular or
cytogenetic prognostics markers. In our study we confirmed
the significance of accurate measurement of the WT1 gene
expression level in peripheral blood after allo-HSCT as
a specific and highly sensitive MRD marker. Early detection
of MRD allows early therapeutic intervention, especially in
patients with slow WT1 expression increase. Moreover, WT1
expression immediately before transplantation seems to be
of a prognostic importance analogically to the prognostic
significance of WT1 expression at diagnosis [35]. Therefore
we consider WT1 monitoring to be an essential approach to
detect and manage impending relapse in AML patients after
allo-HSCT. Particularly, the prognostic importance of the pre-transplant WT1 level is of important significance and it needs to be confirmed on more patients in further studies in order to validate our results.

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References


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