Pathol. Oncol. Res. (2016) 22:217–221
DOI 10.1007/s12253-015-0002-0

ORIGINAL ARTICLE

WT1 Expression in Adult Acute Myeloid Leukemia: Assessing its Presence, Magnitude and Temporal Changes as Prognostic Factors

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Received: 25 July 2014 / Accepted: 28 October 2015 / Published online: 4 November 2015

Abstract Expression of the gene Wilms tumor 1 (WT1) has been suggested as a marker of minimal residual disease in acute myeloid leukemia (AML), but literature data are not without controversy. Our aim was to assess the presence, magnitude and temporal changes of WT1 expression as prognostic factors. 60 AML patients were followed until death or the end of the 6-year observation period. Blood samples were taken at diagnosis, post-induction, during remission and in case of a relapse. Using quantitative real-time PCR, we determined WT1 expression from each sample, normalized it against the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and classified samples as negative, moderately positive or highly positive. We divided the patients into groups based on detected WT1 expression values, illustrated overall and disease-free survival on Kaplan-Meier curves, and compared differences between each group by the logrank test. Disappearance of WT1-positivity during chemotherapy had a favorable effect on survival. Interestingly, no difference was seen between the survivals of WT1-positive subgroups that expressed moderate or high levels of WT1 mRNA. A 1-log decrease in WT1 expression without becoming negative did not affect prognosis, either. Our results suggest that defining a cut-off value for WT1-positivity, rather than just using logarithmic figures of changes in gene expression, might have prognostic use in post-induction AML patients. We encourage further, larger-scale studies.

Keywords WT1 gene · Overexpression · Quantitative real-time PCR · Acute myeloid leukemia

Introduction

The Wilms tumor 1 (WT1) locus in chromosomal region 11p13 encodes a DNA-binding protein with 4 zinc finger domains [1, 2]. The WT1 protein is a unique transcription factor that can activate or repress target promoters depending on the cellular cofactors it binds to [3], and regulates its targets on the posttranscriptional level as well [4]. The protein's role in development and tumorigenesis is very complex, mainly due to the large number of possible isoproteins [5, 6].

Overexpression in acute leukemias was reported as early as 1992, only 2 years after the gene's discovery [7]. The role of WT1 in the pathogenesis of acute myeloid leukemia (AML) is generally accepted, but its molecular details are largely unknown: one possible explanation is that WT1 blocks the expression of interferon regulatory factor 8 (IRF8), an important tumor suppressor in myeloid leukemogenesis [8].

Early reports on the usefulness of WT1 expression as a prognostic marker in AML were somewhat controversial [9, 10]. Currently, most authors suggest that although WT1-positivity at diagnosis may not have a reliable predictive value in either pediatric or adult AML [11, 12], it is a useful tool for monitoring minimal residual disease (MRD) in cases where a remission can be achieved [13–17]. WT1 expression may be the most universal MRD marker available in AML, as it is independent of age, subtype and cytogenetic risk group [13, 18].

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Here we present a study to evaluate the magnitude and temporal changes of WT1 expression as prognostic markers in adult AML.

Materials and Methods

Patients

Between October 2006 and January 2013, we followed a group of 60 patients: 41 with *de novo* AML, and 19 with secondary AML, most of which had developed from myelodysplastic syndrome (MDS). Patients' age range was 19 to 80 years (median age: 57.5 years). All diagnosed cases were confirmed by morphology and flow cytometry of bone marrow samples, while cytogenetics and mutation screening for the genes Fms-like tyrosine kinase 3 (FLT3) and nucleophosmin (NPM) were also performed. Written informed consent was obtained from all patients according to the Declaration of Helsinki.

Laboratory Methods

Peripheral blood samples were taken at diagnosis, after induction therapy, regularly in remission, and if a relapse was diagnosed. We continued to take samples until the end of the study, last contact with the patient, or death. Volume of analyzed samples was 4 ml, the number of evaluated cells in that volume ranged from 2.08×10^6 to 1.24×10^9.

Samples were collected in PAXgene Blood RNA Tubes, and stored at −20 °C. PAXgene Blood RNA Kits were used to extract RNA. We performed reverse transcription by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems), and detected cDNA levels using an Applied Biosystems 7500 Real Time PCR System with an assay containing primers and a fluorescent TaqMan probe for WT1 detecting a sequence at the boundary of exon 6 and 7 covering all known splice variants (also by Applied Biosystems, ID: Hs00240913_m1). While there may be differences between the biological roles of certain isoforms, assays using a consensus primer detecting all variants are currently considered standard in the literature [13–17]. A reference assay (ID: Hs99999905_m1) for a gene showing high and relatively constant expression is also required: it was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in our case. We validated the assays using plasmids (by BRC Riken) containing DNA clones of WT1 and GAPDH. We normalized WT1 expression against GAPDH, and used the resulting value (number of WT1 mRNA copies per 10^4 GAPDH mRNA copies) for further calculations.

Controls and Statistical Methods

To determine cut-off values for WT1-positivity, we studied a control population consisting of 35 healthy individuals of various age and sex, who provided blood samples at different times through the study. WT1 expression values (normalized against GAPDH) from the control samples ranged from 0.002 to 0.109. We performed a logarithmic transformation on the control data, followed by a chi-square goodness-of-fit test for normal distribution: normality could be accepted ($p = 0.9923$). We took the value at 97.5 percentile (0.108), and used the closest round value (0.1) as the threshold of moderate WT1-positivity in our study. The spectrum of relative gene expression seen in our samples spans several orders of magnitude (from 0.0077 to as high as 239.0), so 2 logs over the first threshold, we defined another cut-off value for high positivity (10.0). Kruskal-Wallis tests were used to analyze association of sample parameters and WT1-positivity.

We used the software MedCalc (version 12.4.0) for statistical analysis of all data. Overall survival (OS) was defined as the time in weeks between the date of diagnosis and death or the end of the observation period. Disease-free survival (DFS) was calculated as the number of weeks spent in complete remission until relapse, death or the end of observation. Figures in our paper were also drawn by MedCalc.

Results

WT1-Positivity at Diagnosis

53 of the 60 patients were found WT1-positive at diagnosis. WT1-positivity did not show association with either blast count or white blood cell count based on the results of Kruskal-Wallis tests. This was an expected finding, as the qRT-PCR method is sensitive enough to detect transcripts even from a very small WT1-expressing fraction of cells, so positivity (if found) should be considered a feature of the disease mostly unrelated to cell count. OS in the positive and negative group showed some difference (Fig. 1), but the trend was not statistically significant ($p = 0.0812$). DFS was only applicable in 3 of the 7 WT1-negative cases, so significance was even lower ($p = 0.1232$). Similar trends were observed when studying only *de novo* AML, with $p = 0.1174$ for OS, and $p = 0.134$ for DFS.

Changes in WT1-Positivity over Time

Temporal changes in the expression pattern of WT1 were studied in 49 patients who survived induction therapy (allowing further samples to be taken after the initial one at diagnosis).

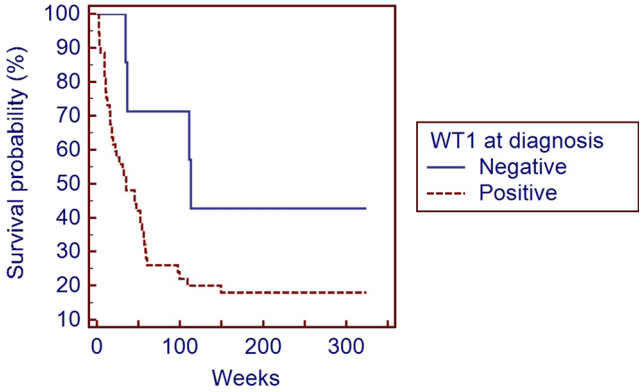

Fig. 1 Kaplan-Meier curves illustrating overall survival in AML patients shown to be WT1-positive (n = 53) and -negative (n = 7) at the time of diagnosis

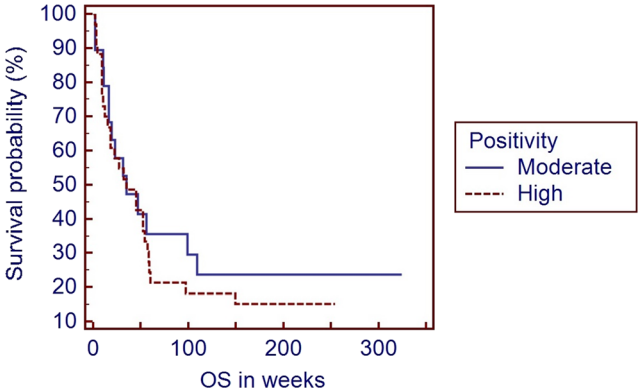

Fig. 3 Overall survival in the moderately (19 patients) and highly (34 patients) WT1-positive subgroups

Kaplan-Meier curves showed differences in OS and DFS between the 3 defined groups: the one found WT1-negative, the one which stayed WT1-positive throughout the study, and the one initially positive but turned negative at some point during the observation period (Fig. 2). The difference was found significant in the logrank test (OS: p = 0.0015, DFS: p = 0.0471).

In 11 out of the 25 cases in which a complete remission was achieved, we observed that a formerly positive WT1 expression turned negative. In a case, decreased WT1 expression built up again before a relapse occurred: we detected marginal positivity (0.149) at 10 weeks, and high positivity (53.9) 6 weeks before the relapse.

Degree of WT1-Positivity

To assess whether the magnitude of WT1-positivity affected prognosis, we divided all 53 WT1-positive patients into 2 groups: 19 cases that only produced WT1 expression values between 0.1 and 9.99 (moderately positive), and 34 cases that produced at least one sample over 10.0 (highly positive). Kaplan-Meier curves look remarkably similar (Fig. 3), and logrank tests showed no association between the degree of positivity and survival (OS: p = 0.5286, DFS: p = 0.4719). A prognostic difference between moderately and highly positive subgroups was also absent when we separately considered the group of patients who stayed WT1-positive throughout the study (OS: p = 0.4528, DFS: p = 0.401), and the group that became WT1-negative in response to therapy (OS: p = 0.3112, DFS: p = 0.3674). Interestingly, within the group of patients who stayed over the cut-off value for WT1-positivity, a decrease of gene expression by 1 logarithmic unit did not produce any difference in overall survival (p = 0.9228).

Discussion

Studies available in the literature [11–17] have used either bone marrow or peripheral blood samples for the detection of WT1 mRNA by qRT-PCR. According to some authors [16], using peripheral blood samples may achieve higher sensitivity, since the normal level of detectable WT1 expression as seen in leukemia-free samples (which may be interpreted as "background noise") is approximately 1 log lower in peripheral blood, due to the presence of WT1-expressing hemopoietic progenitor cells in the bone marrow. Further factors we considered when we decided to use blood samples were an

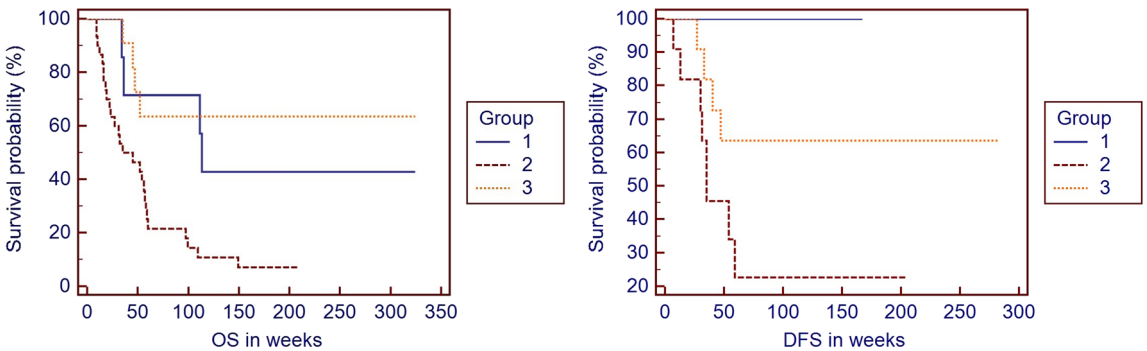

Fig. 2 Overall survival (OS) and disease-free survival (DFS) in 3 groups of patients. Group 1 only produced WT1-negative samples (n = 7, DFS applicable in 3 patients), while group 2 was positive until the end of observation (n = 31, DFS applicable in 11), and group 3 started as positive but turned negative at some point (n = 11 patients, DFS applicable in all cases)

easily obtainable pool of controls, and the need of subsequent samples from each patient in order to analyze temporal changes in the gene's expression pattern.

In November 2009, 3 years after our own study had been initiated, Cilloni et al. published a European Leukemia Net study [17], validating the efficiency of 9 different WT1 qRT-PCR assays in MRD detection. Even though the Applied Biosystems assay we used was not evaluated by the authors, we have no reason to doubt its sensitivity based on the differences we have demonstrated between WT1 expression in the majority of AML and healthy control samples.

The literature offers a variety of genes that performed well as controls for WT1 expression: GAPDH [11], Abelson murine leukemia oncogene 1 [13, 17] and β-actin [14]. It is not clear if any of these genes have an advantage in performance over the other two [19].

53 of our 60 patients (88.33 %) proved to be WT1-positive at the time of diagnosis. The found frequency of positivity is consistent with recent results produced by assays evaluated in the aforementioned Leukemia Net study [13, 17]. Our conclusions on the prognostic value of WT1-positivity at diagnosis are also similar to what has been reported [12]: a trend is apparent (Fig. 1), but not significant at a 95 % confidence interval, so prognosis may not depend on WT1-positivity at the time of diagnosis, but on whether the level of WT1 expression is affected by therapy (Fig. 2). Apart from monitoring minimal residual disease, early changes in WT1 expression during induction chemotherapy have also been suggested as important in the literature [20].

While previous studies often tracked changes in WT1 expression and linked them to a clinical response, most of them used simple logarithmic figures to represent a decrease in detected WT1 mRNA production. We tried a different approach by defining cut-off values to assess the level of WT1 positivity. In our study, once a sample was found positive, the magnitude of WT1 expression (moderate or high) did not affect prognosis in any of the observed groups, and a 1-log change in expression within the range considered WT1-positive did not seem to have any prognostic significance, either. On the other hand, WT1 expression dropping below the cut-off value of positivity was linked to a complete remission in each case and had a statistical impact on survival. In an observed case, WT1 expression seems to have increased over the cut-off value as a sign of imminent relapse.

Based on our results, we encourage further investigation; we believe that a larger-scale study should be performed to support the usefulness of a WT1 cut-off value for MRD detection in AML patients. Depending on the outcome of future experiments, WT1-positivity might even be established as a binary factor in MRD detection, its presence affecting prognosis more decisively than its degree.

WT1 is generally suggested as an oncogene in leukemogenesis [21]. This is based on many publications showing that its overexpression is present in the majority of cases, and has prognostic implications. However, approximately 5 to 7 % of AML cases show a mutation in the WT1 gene, resulting in an adverse prognosis [22, 23], which is a characteristic feature of tumor suppressors. According to some authors, the role of WT1 is so complex that it cannot be defined either as "oncogene" or "tumor suppressor" [24]. Perhaps after more evidence has accumulated on the gene's molecular function in AML, it will be easier to put expression and mutation data in context, establishing WT1 as a key component of a reliable survival scoring system.

Acknowledgments This work was supported by the Social Renewal Operational Programme of Hungary. The authors do not have a financial relationship with the sponsors of the research.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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