

## Real-time quantitative PCR detection of WT1 and M-BCR-ABL expressions in chronic myeloid leukemia

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### Abstract

The Philadelphia chromosome and the resulting BCR-ABL fusion gene represent the hallmark event in chronic myeloid leukemia (CML) and their discoveries radically changed the management of these patients. Currently Wilms tumor 1 gene (WT1) is intensively investigated as high WT1 expression levels have been demonstrated in case of multiple solid tumors and malignant hematological syndromes (acute myeloid and lymphoid leukemia, myelodysplastic syndromes and chronic myeloid leukemia). The aim of our study was to investigate the WT1 expression in CML patients and its possible contribution to disease evolution. *Patients and Methods:* In the Laboratory of Molecular Biology, University of Medicine and Pharmacy of Tirgu Mures, Romania, we regularly determined the M-BCR-ABL and WT1 expression levels by RQ-PCR (real-time quantitative polymerase chain reaction) testing in case of 19 CML patients: six patients monitored from the diagnosis and 13 patients first tested during therapy. *Results:* Eight CML (four advanced stage and four CP) patients showed high WT1 expression level, and in case of 11 patients the WT1 expression levels were undetectable or lower than 0.02%. The only significant difference between the high and low WT1 expression groups was represented by the clinical stage. In the majority of pretreated patients (10 out of 13 patients), the WT1 expression levels were low or undetectable. *Conclusions:* High WT1 expression in CML patients is detected especially in the advanced stages of the disease. Efficient Imatinib therapy may contribute to low WT1 levels in CP patients.

**Keywords:** chronic myeloid leukemia, WT1, M-BCR-ABL, RQ-PCR.

### Introduction

Chronic myeloid leukemia (CML) is a malignant clonal disease of the pluripotent hematopoietic stem cell. Its course has two or three phases: the disease is diagnosed most frequently in chronic phase (CP), followed by – with or without an acceleration phase (AP) – a blast phase (BP), similar to acute leukemia and characterized by poor prognosis.

CML is a unique pathology among malignant diseases, because it was the first neoplasia with a demonstrated genetic background: the Philadelphia chromosome – t(9;22) (q34;q11), which is detectable by conventional cytogenetic testing in more than 90% of the cases, and the BCR-ABL fusion gene resulting from the translocation. There are several variants of the hybrid gene, depending on the breakpoint of the BCR gene: the most frequent (95%) the M-BCR-ABL (with two sub-variants: b2-a2 and b3-a2), while the m-BCR-ABL and  $\mu$ -BCR-ABL gene variants occur more rarely in CML. Regardless of the variant type, the BCR-ABL fusion gene encodes a tyrosine-kinase with increased activity that is responsible for the uncontrolled proliferation of cells.

Molecular level characterization of pathophysiological mechanisms contributing to development of CML lead to significant changes in the management of the disease:

according to the recommendations of the *European LeukemiaNet*, targeted tyrosine-kinase inhibitors (TKI): Imatinib, Nilotinib, Dasatinib are the gold standard therapy, and molecular testing (PCR) plays a pivotal role in diagnosis and monitoring of therapeutic efficacy [1, 2].

In recent years, several research groups focused on the Wilms tumor 1 (WT1) gene. The gene is located on chromosome 11, and encodes a zinc finger transcription factor that regulates the expression levels of a variety of growth factors, differentiation factors and transcription factors (IGF-II, PDGF- $\alpha$ , IGF-I-receptor, CSF-1, TGF- $\beta$ 1, cyclin E, PAX-2, syndecan-1, c-myc, bcl-2, c-myb), and thus it is deeply involved in cell proliferation and differentiation. The WT1 gene is expressed in different cell and tissue types of the healthy adult, like gonads, myometrium, podocytes, Bowman's capsule, mesothelial lining of body cavities and internal organs (heart, lungs, liver, bowels) and immature CD43+ hematopoietic progenitor cells.

The WT1 gene plays a variety of roles in the development of different tumors. Initially, it was regarded as a tumor suppressor because of the causality between the Wilms tumor (childhood nephroblastoma) and mutations of the WT1 gene. Subsequently, high WT1 expression

levels have been demonstrated in case of multiple solid tumors (lung, breast, thyroid, gastrointestinal, head-neck tumors) and malignant hematological syndromes (acute myeloid and lymphoid leukemia, myelodysplastic syndromes and chronic myeloid leukemia). Consequently, it has been suggested that in these cases the WT1 gene instead of being a tumor suppressor, it acts as an oncogene [3–5].

Several research groups have demonstrated that the WT1 protein could represent an attractive target for anti-tumor immune therapies, as it is able to elicit humoral (anti-WT1 IgM and IgG have been detected) and cellular immune responses [6, 7]. The therapeutic potential is supported by the survey of the *National Cancer Institute* (USA), which ranked potential tumor antigens based on current research results and WT1 made the top of the list (before well established markers like BCR-ABL) [8].

As WT1 gene and its contribution to hematological malignancies was investigated mostly in acute leukemia, less information is available regarding CML. The aim of our study was to investigate the WT1 expression in CML patients and its possible contribution to disease evolution.

## Patients and Methods

Nineteen CML patients' peripheral blood sample from the Center of Hematology and Transplant of Țirgu Mureș, Romania, were regularly tested at the Laboratory of Molecular Biology, University of Medicine and Pharmacy of Țirgu Mureș, for both BCR-ABL and WT1 gene between July 2008 and July 2013.

After mRNA extraction using QIAmp RNA Blood Mini Kit 50 (QIAGEN), we performed cDNA transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The M-BCR-ABL transcript level quantification by RQ-PCR (real-time quantitative polymerase chain reaction) analysis was conducted according to the recommendations of the *Europe Against Cancer Program* as described previously [9, 10]. For the determination of WT1 expression level, we performed RQ-PCR analysis with primers recommended by Cilloni *et al.* [11]. We carried out relative quantification for both markers using ABL gene as endogenous control. In addition, we tested a control group of 20 non-hematological patients' peripheral blood sample to determine the background WT1 expression level.

The statistical analysis was carried out with GraphPad Instat ver. 3.06 software using Fischer's test and Mann-Whitney *U*-test.

## Results

In case of the control group, we found very low or undetectable WT1 expression levels: mean value of 0.02% (0.001 to 0.041%).

The CML group comprises 19 patients: eight females and 11 males, with a mean age of 44.68 (27 to 65) years.

In case of six of these patients, we have been monitoring WT1 and M-BCR-ABL expression levels since the time of diagnosis, and for 13 patients we were able to perform the first RQ-PCR tests only after diagnosis, during therapy.

The majority of our patients (18) received medication therapy (Hydroxyurea, Imatinib, and four patients required second-generation TKI therapy), and one patient underwent hematopoietic stem cell transplantation (HSCT).

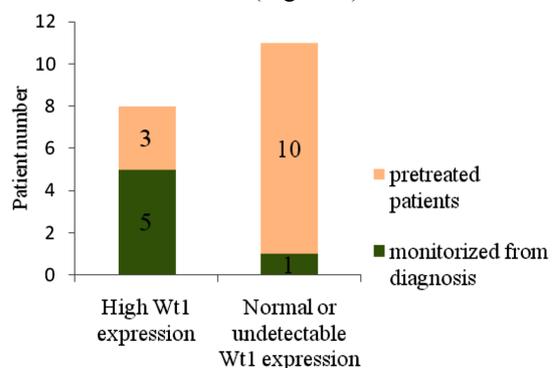
Based on the measured WT1 expression levels, we divided the patients into two groups: during follow-up, we measured higher values than the baseline mean values (>0.02%) in case of eight patients (four advanced stage and four CP patients), while in case of 11 patients the expression levels were undetectable or lower than 0.02%. When comparing the clinical and laboratory parameters, the only significant difference between the high and low WT1 expression groups was represented by the clinical stage (Table 1).

**Table 1 – The clinical parameters according to WT1 expression level**

	High WT1 level	Normal or undetectable WT1	<i>P</i>
Leukocytes $\times 10^9/L$	164.46 (18.04–391.1)	74.65 (4.4–305.0)	0.062
Hemoglobin [g/L]	11.22 (6.2–15.7)	11.52 (7.8–16.2)	0.0833
Platelets $\times 10^9/L$	344.0 (63.0–1399.0)	412.81 (29.0–1053.0)	0.968
<b>CML phase</b>	<b>No. of patients</b>		
CP	4	11	0.018
AP	1	0	
BP	3 <sup>+</sup>	0	

<sup>+</sup>Deceased; WT1: Wilms tumor 1 gene; CML: Chronic myeloid leukemia; CP: Chronic phase; AP: Acceleration phase; BP: Blast phase.

Classifying the patients according to the time of the first WT1 testing, we noted that in the majority of treated patients (10 patients out of 13) the WT1 expression levels were low or undetectable (Figure 1).

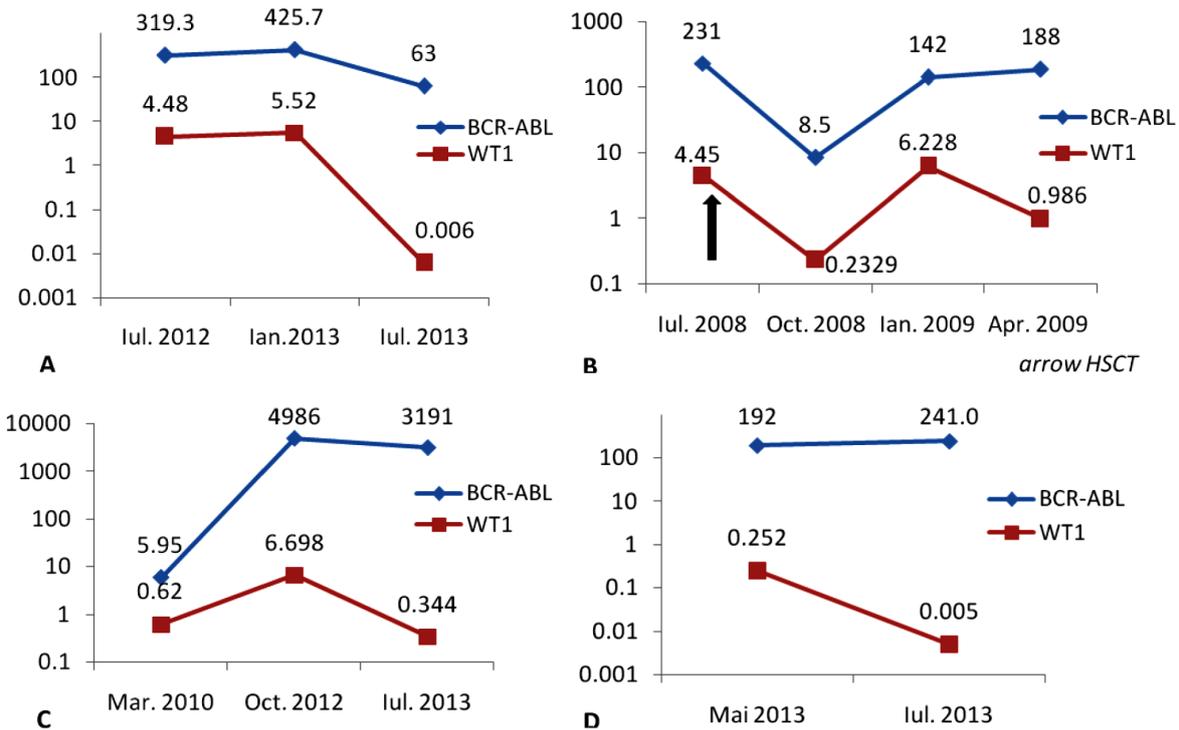


**Figure 1 – WT1 expression according to the first RQ-PCR analysis.**

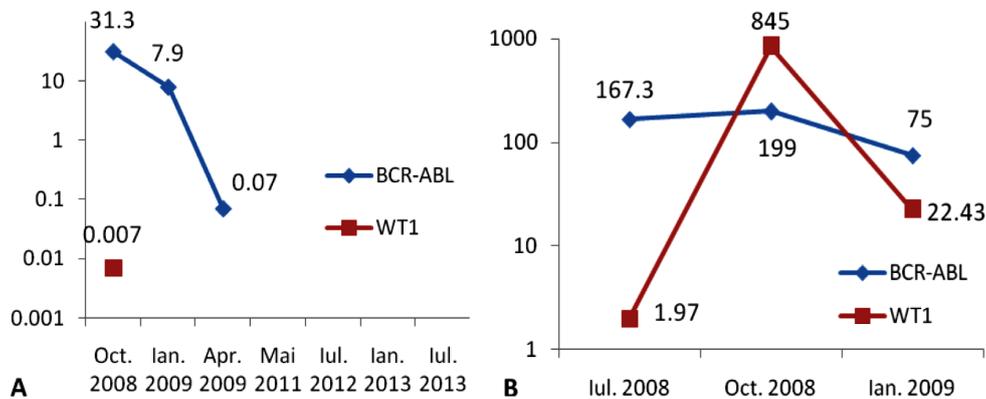
We analyzed separately the subgroup of patients followed-up from the time of diagnosis: in all six patients, the WT1 expression levels were 2–3 *log* lower than M-BCR-ABL levels, and this trend was maintained during follow-up as well. We present separately the dynamic changes of the two markers in case of four patients (Figure 2). Although baseline WT1 levels of patients A and B are similar, their progression is different: in case of patient A, the WT1 level decreased rapidly under Imatinib therapy, while in case of patient B with BP, the WT1 and M-BCR-ABL expression was still detectable after HSCT. Baseline WT1 levels of patients C and D are also similar, but in case of patient C the WT1 gene expression does not fall below 0.02%.

In the pretreated subgroup comprising 13 patients, we measured low or undetectable WT1 expression levels in the majority of patients (10), and in case of three patients

(one BP, two CP) expression levels were higher (Figure 3). WT1 levels were also lower than M-BCR-ABL levels.



**Figure 2 – BCR-ABL and WT1 kinetics in individual patients (diagnosis group):** (A) CP, high baseline WT1 levels that decreased to below the detectable threshold after initiation of Imatinib therapy; M-BCR-ABL gene expression modification was less evident; (B) BP, high WT1 levels at diagnosis, BCR-ABL and WT1 expression persisting after HSCT, then relapse in 2009 (80% myeloblasts in the periphery). The two curves are parallel; (C) AP, high WT1 expression levels that do not decrease below mean values under TKI therapy; dynamics of the two markers are parallel; (D) CP, after initiation of Imatinib (400 mg) therapy WT1 expression rapidly decreases.



**Figure 3 – BCR-ABL and WT1 kinetics in individual patients (pretreated group):** (A): CP, 4x100 mg Imatinib therapy, M-BCR-ABL expression gradually decreases, undetectable in the last four years; only one WT1 testing performed, and subsequently undetectable; (B): BP, under Imatinib 400 mg, then Dasatinib 2x70 mg therapy, the M-BCR-ABL levels of the second testing are comparable to baseline levels, while WT1 expression increases significantly. The following clinical visit confirms the BP (90% myeloblasts detected in the periphery).

**Discussion**

Among malignant hematological diseases, acute leukemia characteristically has high WT1 expression levels, and consequently most of the existing data is about this pathology. Although the prognostic value of high WT1 levels at diagnosis is controversial, several research groups suggested that the WT1 gene is suitable for follow-up of minimal residual disease, early detection of relapse, and high expression levels maintained or newly occurring after therapy represent a poor prognosis [12–16].

In case of CML, there is a well-established genetic marker (BCR-ABL), and its testing has been integrated in the management of the patients. Nevertheless, advanced stages of the disease (especially the PB) are similar to acute leukemia, so the role of the WT1 gene may be enhanced in these phases. After testing our patients, we found high WT1 expression levels in all AP and BP patients, and the data showed statistically significant correlation. Other tested parameters showed no difference.

Several research groups studied the relationship between WT1 and BCR-ABL expression levels, and the

significance of elevated WT1 values in advanced stages of CML. The published data is highly heterogeneous: according to some authors, WT1 levels change in parallel with BCR-ABL levels, while others found low correlation between the two markers [17–19].

Analyzing separately the cases followed from the time of diagnosis, we note that the majority of the patients have high WT1 expression levels, but they show different responses to therapy: in case of CP patients WT1 gene expression decreases, while in case of BP and AP patients WT1 levels are maintained higher than the mean values. The same occurs in acute myeloid leukemia: the prognostic value of high WT1 levels at diagnosis is minor, progression cannot be predicted, and high WT1 expression detected during/after therapy plays a much more important role [16, 20].

The only HSCT patient belongs to this group (Figure 2B): M-BCR-ABL and WT1 expression are still detected after HSCT, and the two markers change in parallel. Our result is similar to the two cases presented by Uzunel & Ringdén: after HSCT and before hematological relapse, expression levels of both markers increase and show similar kinetics [19].

In most of the pretreated patients (10 patients), we found low or undetectable WT1 expression. This correlates with the stage of the disease (CP, low number of immature cells), but efficient TKI therapy may also contribute to low WT1 levels. Cilloni *et al.* demonstrated that tyrosine-kinase inhibition of BCR-ABL decreases WT1 expression in Philadelphia-positive cell lines and bone-marrow samples of CML patients. This result is supported by the data published by Svensson *et al.*: Imatinib decreased WT1 expression in CML positive cell lines [21, 22]. All our above-mentioned patients (10 patients) receive tyrosine-kinase inhibitor therapy, and in case of the patients represented in Figure 2 and followed-up from the time of diagnosis (A and D), WT1 expression levels decreased spectacularly after initiation of Imatinib therapy.

Our only case where WT1 expression surpasses the BCR-ABL value at a certain time during follow-up (Figure 3B) is in the pretreated patients group. Analyzing the dynamics of the two markers, at the second testing the WT1 level increased significantly compared to the baseline, while BCR-ABL was maintained in the same logarithmic scale. The evolution was unfavorable, by the next clinical visit the patient progressed to BP and deceased. This is in agreement with the data of Na *et al.*, who suggested that in case of relapsing AP CML patients WT1 is significantly higher than in those responding well to therapy, while BCR-ABL values show no difference between these two groups [23].

This may be the most interesting question about the implication of WT1 gene in CML: is immune therapy against WT1 justified in this pathology, because it is well known that tyrosine-kinase therapy is highly efficient. As WT1 expression has also been documented in various solid and hematological neoplasias, there are several ongoing phase I/II studies about WT1 vaccine. The results are so far promising for both solid tumor and acute leukemia cases: clinical and/or immunological improvement has been documented in the majority of included patients, and the undesirable effects are negligible [24, 25]. As Narita *et al.* have reported, immune therapy targeting WT1 has

also been used in a CML patient: a 9-mer modified type WT1 peptide has been combined with the TKI therapy in the treatment of the Imatinib resistant CP patient, and the results were beneficial: BCR-ABL expression levels became undetectable [26]. Despite the initial encouraging results, clinical trials with large number of patients are needed, as there are a lot of questions about the application of immunotherapies: it is difficult to select the patients which will benefit the most from active immunotherapy and the interval of vaccine administration need to be clarified, as recent data suggest that too often vaccination leads to loss of high avidity WT1 specific CTLs [27, 28].

There is diverse and many times contradictory information available about the relationship between WT1 and CML. The first step towards the assessment and objective comparison of the results of different research groups would be the use of a standardized WT1 assay. The *European LeukemiaNet* offers the essential basis for this [29].

## ☐ Conclusions

In CML patients, high WT1 expression has been detected especially in the advanced stages of the disease. High WT1 detected at the time of diagnosis is not predictive of a poorer progression. In CP patients, the TKI therapy may contribute to low WT1 expression level. To elucidate the exact impact of high WT1 expression level on CML and the possible application of WT1 targeted immunotherapy, further research is needed.

## Conflict of interests

The authors declare that they have no conflict of interests.

## Acknowledgments

This study has been partially supported by the Internal Research Grant of University of Medicine and Pharmacy of Tîrgu Mureş, Romania, contract No. 21/11.12.2013.

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