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WT1 expression in peripheral leukocytes of patients with chronic myeloid leukemia serves for the prediction of Imatinib resistance

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The determination of patient's resistance to a particular drug contributes to more efficient therapeutical approach. The aim of this study was to evaluate if the responsiveness of Chronic Myeloid Leukemia (CML) patients to Imatinib therapy was predictable from WT1 gene expression in peripheral blood leukocytes. To examine the resistance we implemented an in vitro cultivation of the primary cells of 48 CML patients with Imatinib. The effect of Imatinib was characterized not only by the expression of WT1 but also by BCR-ABL, and proliferative factor Ki-67.

Our results showed that leukocytes of CML patients, clinically responsive to Imatinib treatment, significantly decreased WT1 expression after in vitro incubation with Imatinib. It was accompanied by an inhibition of expression of Ki-67 but not BCR-ABL. In leukocytes of CML patients clinically resistant to Imatinib, the expression of WT1, Ki-67, and BCR-ABL remained unaffected. The presented results showed that in vitro testing using peripheral blood cells enabled clinicians to predict responsiveness of CML patients to Imatinib.

Key words: CML; Imatinib; WT1; BCR-ABL; drug resistance

Chronic myeloid leukemia (CML) is characterized by a presence of the Philadelphia (Ph) chromosome and the BCR-ABL chimerical gene, molecular markers of the disease. The fusion protein BCR-ABL has a deregulated Tyrosine Kinase (TK) activity, which plays a critical role in the disease's development and progression [1]. Inhibition of BCR-ABL TK activity represents a modern and very powerful approach towards treatment of CML, as well as some other BCR-ABL – positive and BCR-ABL - negative myeloproliferative diseases [2, 3]. Imatinib (Gleevec, Glivec, formerly STI-571, Novartis; Switzerland) is a competitive inhibitor, selective for several tyrosine kinases, like ABL, PDGFR, and c-KIT [4, 5]. Recently, this drug has been the most frequent first choice for therapy of CML patients. It induces a very rapid hematological response in the majority of BCR-ABL- positive CML patients in the chronic phase, in half of the patients in the accelerated phase, and in a smaller percentage of the patients in the blast crisis. Almost 90% of the patients receiving Imatinib reach complete cytogenetic response within 60 months [6].

The main disadvantage of the Imatinib treatment is an increasing number of resistant patients. The resistance is caused by several molecular mechanisms, including mutations in the BCR-ABL gene (especially point mutations in the ATP or Imatinib binding site, the activation loop, and the catalytic domain of BCR-ABL). Other mechanisms contributing to Imatinib resistance include amplification or over-expression of the BCR-ABL gene, deregulation of multidrug resistance gene 1 protein, or organic cation transporter 1, and aberrations of other genes especially SRC kinases [7–11].

The ability to predict a patient's responsiveness to Imatinib, by an easy and reliable in vitro test, would enable the resistant patients to benefit from an alternative treatment with another TK inhibitor. Cilloni and Saglio [12] introduced a test based on the in vitro cultivation of patients' bone marrow cells with Imatinib followed by a subsequent estimation of WT1 gene expression. WT1 gene was discovered in Wilm's tumor as a tumor suppressor gene [13]. Its expression in normal cells is very low, with the exception of CD34+ hematopoietic progenitors; and it increases in various cancers including leukemia cells [14, 15].

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The aim of present study was to evaluate if a modified protocol using patient's peripheral blood cells was suitable for the determination of Imatinib resistance or responsiveness. Further, we investigated whether short-term cultivation of primary cells with Imatinib affected the mRNA expression of cell proliferation marker Ki-67 or pathogenic fusion of gene BCR-ABL. Imatinib inhibited gene expression of WT1 and Ki-67, but not BCR-ABL in the peripheral blood leukocytes of responsive patients. For the first time we showed that, the reliability of WT1 analysis for the prediction of Imatinib response was limited to patients not previously exposed to Imatinib treatment.

Patients and methods

Patient samples. Primary cells from peripheral blood of 48 CML patients, who obtained hydroxyurea and interferon α; and 6 healthy donors, were drawn after they signed an informed consent agreement. The obtained sample set contained 17 patients who had already received Imatinib previous to sample collection and 31 patients without prior Imatinib therapy. Each group of patients was divided into responders and non-responders. The responsive patients achieved at minimum complete hematological remission and the non-responsive patients exhibited sub-optimal or no response to Imatinib. Both groups of responders and non-responders contained patients tested at the time of diagnosis, before any Imatinib therapy, and patients who had already received Imatinib therapy. Detailed characteristics of patients included in the study are shown in Table 1. All patients were regularly monitored for at least 12 months after the initiation of Imatinib therapy. Hematological status was assessed monthly, BCR-ABL levels in PB were monitored every 3 months and cytogenetic tests of BM to determine Ph status and additional chromosomal changes were performed every 6 months.

Table 1. Detailed characteristics of patients used in the study

Groups of patients	Number of patients	Response after Imatinib therapy	Months of positive Imatinib response *median (min - max)*	Number of patients
R1	26	MCR	10 (5-16)	10
		CCR	9 (5-16)	16
R2	6	MCR	12 (7-16)	2
		CCR	12 (8-19)	4
NR1	5	PR		3
		SR		2
NR2	11	PR		6
		SR		5

The whole sample set used in this study was divided into subgroups along the response of particular patient to Imatinib therapy and the time when samples were drawn - before or during the therapy. R1, R2: responders tested before and during the Imatinib therapy, respectively; NR1, NR2: non-responders tested before and during the Imatinib therapy, respectively; MCR: major cytogenetic response; CCR: complete cytogenetic response; PR: primary resistance; SR: secondary resistance.

Short term incubation with Imatinib. BCR-ABL positive human leukemia cell line K-562 was used as an Imatinib sensitive control. This cell line was obtained from our laboratory repository. The cell line as well as primary patients cells, were maintained in RPMI 1640 medium (Invitrogene, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (Invitrogene) and antibiotics at 37°C in a humidified atmosphere with 5 % CO2. To determine an optimal concentration of Imatinib, the K-562 cell line was treated with increasing doses of Imatinib in a range between 0.1 – 25 μM for 24 and 48 hours. Primary cells were treated with 1 and 10 μM Imatinib for 24 hours. Cells of healthy donors (BCR-ABL negative) were used as negative controls.

Real-time RT-PCR. To quantify the expression of WT1, Ki-67, and BCR-ABL, we performed semi-quantitative real-time RT-PCR analysis. At the end of the Imatinib treatment, total RNA was isolated by the acid guanidium thiocyane-phenol-chloroform method [16] and 1 μg of RNA was used in reverse transcription employing Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA), as recommended by the manufacturer. Gene expression was determined by real time RT-PCR with a RotorGene 3000 (Corbett Research, Sydney, Australia). The reaction was carried out with a FastStart TaqMan Probe Master (Roche, Basel, Switzerland), a 0.2 μM TaqMan probe and 0.5 μM primers. Sequences of primers and probes used for amplifications of WT1, B-2-microglobulin (B2M), and BCR-ABL were described earlier [12,17]. Primers and probe for amplification of Ki-67 were designed in our laboratory using the on-line tool www.universalprobelibrary.com. Gene expression was normalized to the expression of B2M. For each experiment, the mRNA expression in untreated controls (cells treated with DMSO only) was set at 100 %; and the decrease of expression caused by Imatinib was monitored.

Statistical analysis. Statistical analysis was performed with GraphPad Prism software (GraphPad Software Inc., San Diego, USA) using nonparametric Mann–Whitney tests. The level of significance was set at a p-value < 0.05. P-values ≥ 0.05 were considered as statistically non-significant.

Results

To evaluate the effect of Imatinib on primary cells derived from the peripheral blood of patients with chronic myeloid leukemia, we monitored changes in the expression of WT1, BCR-ABL, and Ki-67 genes caused by Imatinib.

First, we determined time and dose effects of Imatinib on the K-562 cell line, which is BCR-ABL positive, and thus sensitive to Imatinib. Incubation of K-562 cells with Imatinib led to a dose dependent decrease of WT1 and Ki-67 but not BCR-ABL expression (Figure 1). For further studies of primary cells,

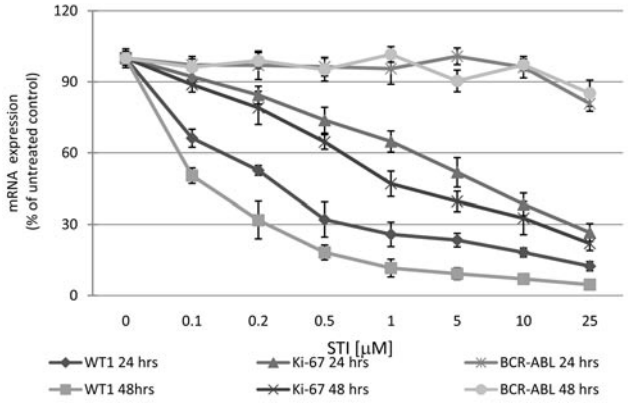

Figure 1. Gene expression of WT1, Ki-67, and BCR-ABL in the K-562 cell line treated with different concentrations of Imatinib for 24 and 48 hours measured by real-time RT-PCR. B-2 microglobulin was used for data normalization. The level of gene expression in untreated cells was set at 100%.

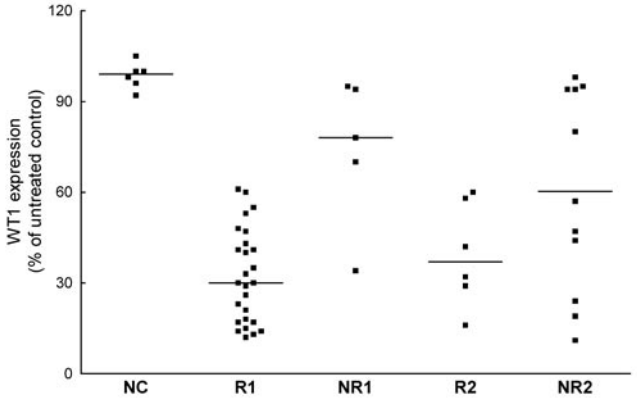

Figure 2. Real-time RT-PCR analysis of WT1 mRNA expression in primary cells incubated with 1 μM Imatinib for 24 hours. The level of gene expression in untreated cells was set at 100% and median of each group is indicated. NC: BCR-ABL negative controls, leukocytes from healthy donors; R1 and NR1: responders and non-responders tested before the initiation of Imatinib therapy, respectively; R2 and NR2: responders and non-responders tested during the Imatinib therapy, respectively.

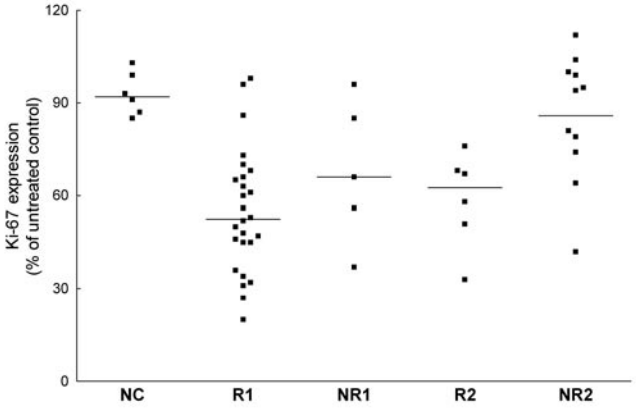

Figure 3. Real-time RT-PCR analysis of Ki-67 mRNA expression in primary cells incubated with 1 μM Imatinib for 24 hours. The level of gene expression in untreated cells was set at 100% and median of each group is indicated. NC: BCR-ABL negative controls, leukocytes from healthy donors; R1 and NR1: responders and non-responders tested before the initiation of Imatinib therapy, respectively; R2 and NR2: responders and non-responders tested during the Imatinib therapy, respectively.

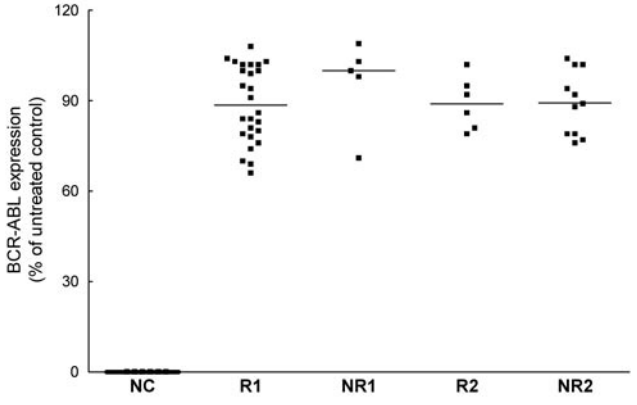

Figure 4. Real-time RT-PCR analysis of BCR-ABL mRNA expression in primary cells incubated with 1 μM Imatinib for 24 hours. The level of gene expression in untreated cells was set at 100% and median of each group is indicated. NC: BCR-ABL negative controls, leukocytes from healthy donors; R1 and NR1: responders and non-responders tested before the initiation of Imatinib therapy, respectively; R2 and NR2: responders and non-responders tested during the Imatinib therapy, respectively.

we chose an Imatinib concentration of 1 μM, corresponding to the clinically used dose of 400 mg of Imatinib per day. Treatment of the K-562 cell line with this concentration of Imatinib for 24 hours led to approximately 75 % and 35 % decrease of WT1 and Ki-67 expression, respectively.

Second, we evaluated the effect of Imatinib on WT1 expression in primary cells. We observed a significant decrease of WT1 mRNA expression in Imatinib responding patients (Figure 2). In the group of patients examined before Imatinib therapy, the level of WT1 mRNA decreased to 30% and 78% in responsive and non-responsive patients, respectively. This difference between responders and non-responders was rather significant at p-value=0,004. In the group of patients examined during Imatinib therapy, the level of WT1 mRNA decreased to 37% and 57% in responsive and non-responsive patients, respectively. This difference between responders and non-responders was not significant with a p-value=0,3. Thus, the decrease of WT1 expression observed in vitro correlated with the responsiveness to therapy in vivo, only in patients examined before the beginning of Imatinib therapy.

Furthermore, Ki-67 mRNA exhibited Imatinib-mediated down-regulation in primary cells of responsive (to 53%) and non-responsive (to 66%) patients examined before Imatinib treatment as well as responsive patients examined during Imatinib therapy (to 63%) (Figure 3). Non-responsive patients examined during Imatinib therapy did not display a significant decrease of Ki-67 mRNA expression (to 94%). The mRNA levels of Ki-67, between responders and non-responders from the group examined before therapy, were equal at p-value =0,2. When comparing levels of Ki-67 mRNA in responders and non-responders examined during Imatinib therapy, the difference was significant at p-value =0,05. However, the distribution of samples was too large to support a relationship between Ki-67 mRNA expression changes and responsiveness of patients to Imatinib therapy.

Finally, Imatinib did not affect the mRNA expression of BCR-ABL in any of primary cells (Figure 4). The effect on BCR-ABL expression was to 89% and 100% (p-value=0,3) in the group of responsive and non-responsive patients examined before Imatinib therapy, respectively. In the group of patients who were examined during Imatinib therapy, the decrease of BCR-ABL was equally to 89% (p-value=0,9) in both groups of responders and non-responders. Primary cells from healthy donors which didn´t possess any BCR-ABL mRNA expression (Figure 4), did not display any significant effect on WT1 or Ki-67 expression after incubation with Imatinib (Figures 2 and 3). The gene levels in these cells dropped to 99% and 92% of WT1 and Ki-67 expression in un-treated cells, respectively.

In summary, our results showed good correlation between down-regulation of WT1 mRNA in peripheral blood and responsiveness to Imatinib in patients examined before the beginning of Imatinib therapy. Nevertheless, there was no significant relationship between down-regulation of Ki-67 mRNA or BCR-ABL mRNA and patients' response to Imatinib.

Discussion

The present study evaluated the suitability of the WT1 gene for the prediction of Imatinib resistance after short-term cultivation of primary cells derived from peripheral blood of patients with chronic myeloid leukemia. We proved that the analysis based on WT1 mRNA expressional changes in peripheral blood cells was useful in the prediction of patient´s resistance to Imatinib therapy.

Up to 28% of patients may have to stop Imatinib because of intolerance or disease resistance, mostly due to point mutations of BCR-ABL [18]. WT1 gene was employed in this study based on its previously reported usefulness for prediction of therapy resistance in bone marrow samples [12]. Bone marrow extraction being a distinct inconvenience for patients, we wondered whether peripheral blood was a reliable tissue for substitution. The decrease of WT1 mRNA after Imatinib treatment in vitro significantly correlated with the responsiveness of patients to Imatinib therapy. This relationship was significant only in samples derived from patients who had not begun Imatinib therapy. Samples drawn from patients undergoing the Imatinib therapy did not exhibit the correlation, although possessed reduced level of WT1 mRNA also. Our results regarding WT1 expression performed with peripheral blood cells of patients before the initiation of Imatinib therapy, agree with those obtained by the group of Cilloni and Saglio [19] who used bone marrow cells. Using peripheral blood instead of bone marrow slightly reduced the assay sensitivity but had lower impact on patients during sample taking. This reduced sensitivity does not affect the test's reliability and usefulness. Application of the analysis of samples from CML patients already treated with Imatinib, caused the loss of its predictive value.

During final analysis, we also found that the presence of additional chromosomal aberrations affected the reliability of using WT1 as a resistance predictive marker. Part of clinically non-responding patients (one examined before Imatinib therapy, six examined during Imatinib therapy) showed a decrease of WT1 mRNA characteristic for responders. These patients possessed complex caryotype changes, monosomy of chromosome 7, additional Ph chromosome, or breast cancer. Therefore, presence of another chromosomal aberration appeared to influence the output result.

Ki-67 and BCR-ABL genes are involved in cell proliferation (Ki-67) or pathogenesis of CML (BCR-ABL) [1, 20] and thus we hypothesized that their expression would be changed by Imatinib incubation. The expression of Ki-67 mRNA decreased after Imatinib treatment in both groups of responsive patients and in samples from non-responsive patients examined before therapy. However we didn´t observe a significant difference between responders and non-responders. Non-responders examined after Imatinib therapy were the only subset of samples which did not exhibit decrease in Ki-67 mRNA. It may be due to other events that occurred during Imatinib therapy in these samples and thus made them less sensitive to Imatinib applied later in vitro, contrary to samples that were Imatinib free before the in vitro analysis. Furthermore, Imatinib did not affect the expression of BCR-ABL in any of tested groups. This discrepancy was caused by the primarily effect of Imatinib on BCR-ABL protein activity but not the transcription of BCR-ABL fusion gene [21].

In summary, the in vitro test based on quantitative measurement of WT1 expression in peripheral blood cells was a useful tool for prediction of Imatinib responsiveness in CML patients tested at diagnosis. This approach may be also applicable to other drugs considered for CML therapy resulting in increased benefit from suitable treatment for individual patients.

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