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ORIGINAL ARTICLE

Is Wilms' tumor gene 1 a useful biomarker for detecting minimal residual disease in chronic myeloid leukemia (BCR-ABL1-p210-positive) patients?

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Abstract Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the presence of Philadelphia (Ph) chromosome. Wilms' tumor gene 1 (WT1) plays an important role in leukemogenesis and can be useful as a molecular marker to detect minimal residual disease (MRD). The goal of this study was to evaluate WT1 expression and compare it with BCR-ABL1 expression in peripheral blood (PB) of CML patients, in order to explore the utility of WT1 as an alternative marker for MRD detection. Eighteen newly diagnosed CML patients (BCR-ABL1-p210-positive), 16 chronic CML patients with a history of imatinib therapy, and 18 normal individuals (BCR-ABL1-p210-negative) as controls were enrolled in this study. WT1 and BCR-ABL1 expression was evaluated by quantitative real-time polymerase chain reaction (Q-RT-PCR). Analysis of RT-PCR data was performed using REST Software (2009, QIAGEN, Valencia, USA). Data were analyzed by SPSS software (v.16.0) using Spearman's rho and Kruskal-Wallis methods. WT1 expression in all PB samples of newly diagnosed CML patients was significantly higher than that of the normal individuals ($P=0.003$). WT1 expression was not different in patients on imatinib therapy compared to normal individuals ($P=0.6$), but it was significantly lower than that of the newly diagnosed CML patients ($P=0.001$). There was no significant correlation between expression of WT1 and BCR-ABL1 and hematological findings in newly diagnosed CML patients. We confirm the oncogenic role of WT1 by WT1 upregulation in CML. Due to lack of significant correlation between BCR-ABL1 and WT1 expression, using WT1 as an additional marker for CML monitoring could not be applicable.

Keywords Chronic myeloid leukemia · Wilms' tumor gene 1 · BCR-ABL1

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Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell (HSC) disorder and is characterized by the Philadelphia (Ph) chromosome as a result of reciprocal translocation between long arms of chromosomes 9 and 22 [t(9;22)(q34;q11)](Zheng et al. 2006; Babashah et al. 2012), which leads to generation of BCR-ABL1 fusion oncogene and translation into a constitutively active tyrosine kinase protein(Smith et al. 2003). BCR-ABL1 protein promotes cell proliferation and survival through activating different pathways, including JAK/STAT, Ras/Raf/MEK/ERK, and PI3/Akt (Fig. 1) (Jabbour and Kantarjian 2014).

Wilms' tumor gene 1 (WT1) was first recognized as a tumor suppressor (TS) gene involved in the pathogenesis of Wilms' tumor, a pediatric renal neoplasm (Call et al. 1990; Yang et al. 2007). WT1 gene is located on the short arm of chromosome 11 (11p13). Its product is a zinc finger transcription factor that regulates the expression of different genes, including PDGF-α (platelet-derived factor) chain, CSF-1 (colony-stimulating factor 1), IGF-II (insulin growth factor II), IGF-1R (insulin growth factor receptor), C-myc, RAR-α (retinoic acid receptor alpha), and Bcl-2 (B cell lymphoma 2)(Inoue et al. 1998). WT1 controls the proliferation and

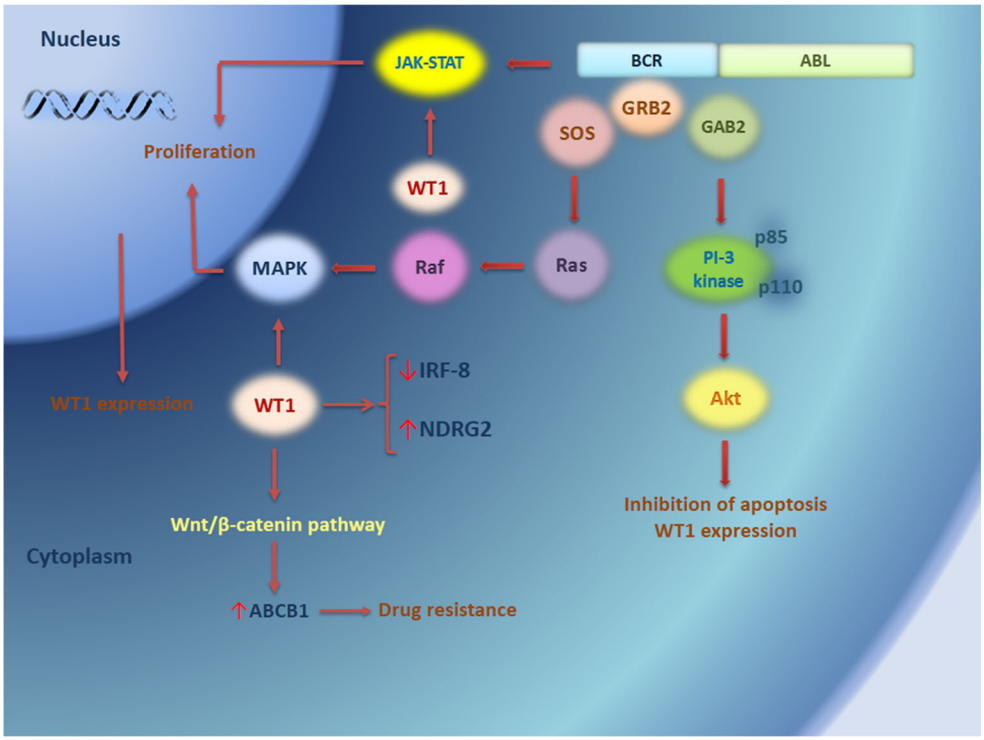

Fig. 1 The role of WT1 in regulation of involved signaling pathways in CML pathogenesis. BCR-ABL protein promotes proliferation and survival of a cell through activating different signaling pathways such as JAK/STAT, MAPKinase, and PI3/Akt. The expression of WT1 is induced by these pathways. On the other hand, WT1 activates MAPK and JAK/STAT pathway-related genes. WT1 downregulates and upregulates IRF-8 and NDRG2, respectively. WT1 upregulation increases ABCB1 expression through Wnt/β-catenin. ABCB1 is involved in drug resistance in CML. *WT1* Wilms' tumor 1, *IRF-8* interferon regulatory factor 8, *NDRG2* N-myc downstream-regulated gene 2, *MAPK* mitogen-activated protein kinase, *JAK/STAT* Janus kinase/Signal transducer and activator of transcription

differentiation of several cells such as HSC (Menssen et al. 1997). The pattern of WT1 expression is restricted to CD34+ progenitor cells and early hematopoietic precursors, which is downregulated following differentiation (Heesch et al. 2010; Maurer et al. 1997). WT1 is highly expressed in early myeloid progenitors, but it is downregulated during differentiation (Nomdedéu et al. 2013). According to WT1 over-expression in leukemic cells, it is recently considered as an oncogene (Sugiyama 2010; Hatta et al. 2005). High expression of WT1 has been reported in hematological malignancies, including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and acute lymphocytic leukemia (ALL) (Tamaki et al. 1999; Cilloni et al. 2009; Asgarian Omran et al. 2008). Therefore, it is a useful and good marker for detecting minimal residual disease (MRD) and can be applicable as a prognostic marker for treatment monitoring and predicting the relapse in leukemic and MDS patients (Sakamoto et al. 2009). There is a high expression of WT1 in the blastic phase rather than the chronic phase of CML (Inoue et al. 1994). According to recent studies related to the usefulness of WT1 as a marker for MRD in other leukemias, we decided to evaluate its expression and possible utility in CML patients in Iran. The goal of our study was to evaluate the expression pattern of WT1 in peripheral blood of CML patients at diagnosis (BCR-ABL1-p210-positive) and during imatinib therapy by sensitive real-time quantitative polymerase chain reaction (PCR) method compared to normal individuals (BCR-ABL1-p210-negative), and we have tried to study the relationship between BCR-ABL1 and WT1 gene, in order to demonstrate WT1 as a prognostic and additional marker for monitoring CML in parallel with BCR-ABL1 expression.

Materials and methods

Patients and controls Eighteen newly diagnosed CML patients (BCR-ABL1-p210-positive) at the Shafa Hospital of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, were enrolled in this study between January 2013 and January 2014 among patients with suspected myeloproliferative disorders referred for the first time. They were nine males and nine females, randomly. All the patients were in the first chronic phase of CML according to the WHO criteria (Swerdllow et al. 2008) and before the start of treatment. Sixteen chronic CML patients (nine males, seven females) with a history of imatinib therapy which were referred to our Laboratory for treatment monitoring were also chosen. Peripheral EDTA blood samples (5 mL) were obtained from all patients. Diagnosis was based on morphological examination of peripheral blood (PB) and bone marrow (BM) films. CML was confirmed by the presence of the BCR-ABL1-p210 fusion gene by PCR. Eighteen normal individuals without any history of hematological disease which were referred for health checkup were also selected as controls. Their BCR-ABL1-p210 fusion test was also negative.

This study was approved by the local ethics committee of the Ahvaz Jundishapur University of Medical Sciences

(ajums.REC.1392, 341), and written informed consent was obtained from all patients and normal individuals.

Cell lines The K562 cell line was used as a positive control of Ph+ CML and WT1 expression. The Jurkat cell line was used for BCR-ABL1 expression, and the U937 cell line was used as negative control for BCR-ABL1 fusion. The cell lines were purchased from the Pasteur Institute of Iran and cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10 % fetal calf serum (FCS), 50 U/mL penicillin, 50 g/mL streptomycin, 2 mM/L L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 25 mM/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at 37 °C in 5 % CO_2.

Isolation of mononuclear cells PB EDTA samples were layered onto Ficoll-Paque (Lymphodex, inno-Train, Germany) gradient. Isolated mononuclear cells were washed twice with phosphate buffer saline (PBS).

RNA extraction and cDNA synthesis Total RNA was extracted from 10^6 isolated cells from PB EDTA samples as well as from the mentioned cell lines using RNeasy Mini Kit (RIBO-prep, Russia) based on the manufactures' instructions. RNA was spectrophotometrically quantified at 260 nm. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA in 20 mL reaction mixture using a cDNA synthesis kit (Bioneer, Korea) according to the manufactures' instructions. The mixture was incubated at 20 °C for 30 s, 42 °C for 4 min, and 55 °C for 30 s, followed by 95 °C for 5 min performed at 8 cycles.

Quantitative real-time polymerase chain reaction HPRT gene was used as a reference gene because it was validated according to our experimental conditions. In addition, its expression was invariant and stable by experimental factors. The use of a reference gene as an internal control is essential for normalizing real-time data (messenger RNA (mRNA) concentration of target gene/reference genes), and it should be experimentally validated for specific cell types or tissues (Bustin et al. 2009). For BCR-ABL1 fusion, the ABL gene was used as the reference gene. The expression level of genes was determined by the Step One Plus Real-Time RT-PCR system (ABI, USA) using SYBR Green Master Mix (Takara, Japan) according to the manufacturers' instructions. The following specific primers were used: WT1 forward (F): 5′-AGGGTACGAGAGCGATAACCACAC-3′; WT1 reverse (R): 5′-CTCAGATGCCGACCGTACAAGA-3′; HPRT1 F: 5′-CCTGGCGTCGTGATTAGTG-3′; and HPRT1 R: 5′-TCAGTCCTGTCCATAATTAGTCC-3′. Twenty-two microliters reaction mixture of PCR was prepared containing 11 μL SYBR Green, 0.5 μL each primer, 2 μL cDNA, and 8 μL DEPC water. WT1 real-time polymerase chain reaction (RT-PCR) was performed in 40 cycles, and each cycle consisted of 95 °C for 20 s, 95 °C for 3 min, and 60 °C for 30 s. BCR-ABL1 RT-PCR was performed according to NanoGene kit (Alert, Italy) in 50 cycles, and each cycle consisted of 50 °C for 2 min, 95 °C for 2 min, and followed by 95 °C for 15 s and 60 °C for 1 min.

The data of RT-PCR were analyzed by the $2^{-\Delta\Delta CT}$ method and were presented as fold change in gene expression (Livak and Schmittgen 2001). The results were presented as a relative index of the WT1/HPRT1 ratio. BCR-ABL1 expression was presented as a copy number. All samples were tested in duplicate.

Statistical analysis Analysis of RT-PCR data was performed using REST Software (2009, QIAGEN, Valencia, USA). Data were analyzed by SPSS software for Windows v.16.0. Spearman's rho method was used to examine the correlation between quantitative data. The Kruskal-Wallis method was used to compare quantitative data between three groups of CML patients. P value <0.05 was considered statistically significant.

Results

WT1 expression in newly diagnosed CML patients

WT1 expression level was evaluated in PB EDTA samples of 18 newly diagnosed CML patients (9 females and 9 males) with a mean age of 48.12 ± 18.77 years. The WT1/HPRT ratio was calculated for each patient in comparison to normal individuals with REST software, separately. WT1 was upregulated and downregulated in 13 (72.2 %) and 2 (11.1 %) newly diagnosed CML patients in comparison to the control group, respectively ($P < 0.05$).There was no difference of WT1 expression in three (16.6 %) newly diagnosed CML patients compared to the control group ($P > 0.05$) (Fig. 2). Taken together, WT1 expression in all PB samples of newly diagnosed CML patients was significantly higher than that of the normal individuals ($P = 0.003$). WT1 expression was not related to BCR-ABL1 fusion copy number (Spearman's correlation coefficient = −0.205; $P = 0.414$).

The demographic and clinical characteristics of chronic-phase CML patients are listed in Table 1. There was no significant correlation between WT1 expression and hematological findings (P values >0.05) (Table 2). Moreover, there was no significant correlation of hematological findings and BCR-ABL1 fusion copy number between three groups of patients according to WT1 expression level (P values >0.05) (Table 1).

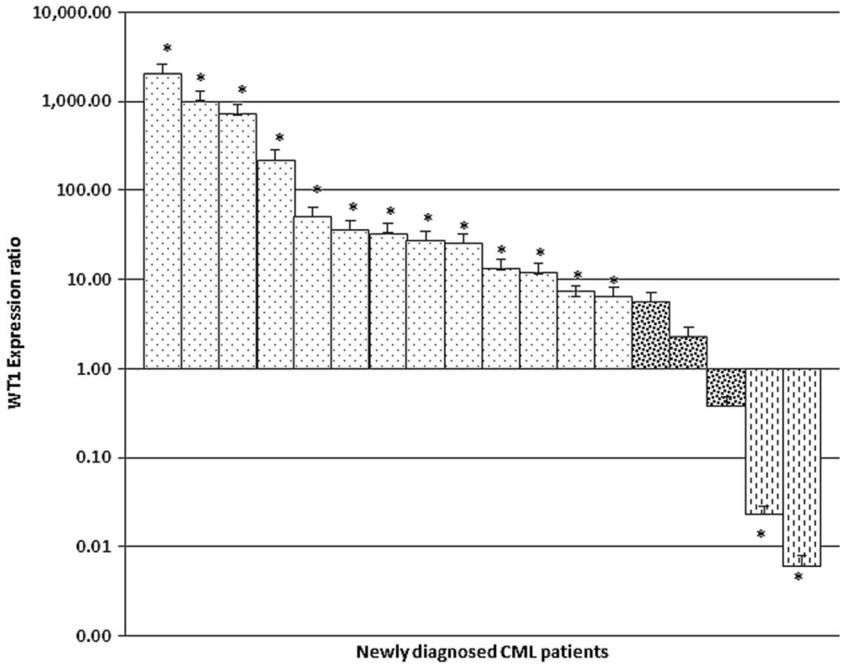

Fig. 2 Relative expression of WT1 in 18 newly diagnosed CML patients in comparison with normal individuals

WT1 expression in CML patients on imatinib therapy

WT1 expression level was evaluated in PB EDTA samples of 16 CML patients on imatinib therapy. WT1 expression was downregulated, upregulated, and not different in three (18.75 %), three (18.75 %), and ten (62.5 %) of these patients in comparison to normal individuals, respectively (Fig. 3). Taken together, WT1 expression was not different in CML patients on imatinib therapy in comparison to normal individuals ($P=0.6$). WT1 expression in these patients was significantly lower than that of the newly diagnosed CML patients ($P=0.001$), which was downregulated on imatinib therapy (Table 3.).There was no significant correlation between WT1 and BCR-ABL1 in newly diagnosed CML patients, so it was not evaluated in patients on imatinib therapy.

WT1 expression in cell lines

WT1 is highly expressed in K562 (b3a2) and Jurkat cell lines. Its expression in the Jurkat cell line is less than that in the K562 cell line. There was no expression of WT1 in the U937 cell line. No significant difference was observed for WT1/HPRT ratio between the K562 cell line and newly diagnosed CML patients ($P=0.06$).

Discussion and future directions

CML is a myeloproliferative disorder associated with the presence of Philadelphia chromosome and elevated tyrosine kinase activity of BCR-ABL1 fusion protein (Jabbour and Kantarjian 2014). The presence of oncogenes as molecular indexes of tumor or absence of tumor suppressors in combination with BCR-ABL1 is likely the reason for CML

Table 1 Demographic, laboratory, and clinical characteristics in newly diagnosed cases according to WT1 expression

Character	Total	Upregulated group ($N=13$, 72.2 %)	Equally expressed group ($N=3$, 16.6 %)	Downregulated group ($N=2$, 11.1 %)	*P* value
Sex (male/female)	9/9	–	–	–	–
Age (years)	47 (18–87)	–	–	–	–
WBC (10^3/mm^3)	77.05 (3.5–205.70)	82.4 (3.5–205.7)	78.65 (75.4–81.9)	17.65 (11.8–23.5)	0.423
RBC (10^6/mm^3)	3.98 (2.01–5.44)	3.78 (2.01–5.1)	4.04 (3.88–4.20)	5.02 (4.59–5.44)	0.087
Hb (g/dL)	9.58 (5.6–14.9)	9.55 (5.6–13.2)	9.4 (8.2–10.6)	13.8 (12.7–14.9)	0.635
Plt (10^3/mm^3)	334.5 (38–2711)	286 (38–2711)	516 (357–675)	251 (140–363)	0.111
BCR-ABL (copy number)	981.5 (13–7400)	963 (13–6333)	3866 (3301–7400)	273 (124–423)	0.113

WBC white blood cell, *RBC* red blood cell, *Hb* hemoglobin, *Plt* platelet (expressed in median and range)

Table 2 Correlation between WT1 expression and hematological findings and BCR-ABL fusion expression

		WBC	RBC	Plt	Hb	BCR-ABL
Expression	Correlation coefficient	0.203	−0.493	−0.068	−0.344	−0.205
	P value	0.451	0.05	0.803	0.192	0.414

WBC white blood cell, *RBC* red blood cell, *Hb* hemoglobin, *Plt* platelet

development. Therefore, recognition and evaluation of these markers could be helpful for better diagnosis, treatment, and control of disease. The research for understanding the involved molecules participating in CML development and CML monitoring continues. MRD monitoring is based on molecular markers which are present in leukemic but not in normal cells. Recent progress in molecular techniques has led to identification of leukemic genes known as tumor-associated antigens (TAA). They play a role in the development of leukemia and can be used as molecular markers for diagnosis and monitoring. WT1 is one of these genes (Asgarian Omran et al. 2008; Asgarian et al. 2005; Dong et al. 2014). Overexpression of WT1 has been reported in several hematological malignancies and acts as a good marker for detecting MRD in AML, ALL, MDS, and the blastic phase of CML; however, its role in leukemogenesis is yet unclear (Asgarian Omran et al. 2008; Siehl et al. 2004). The important role of WT1 in proliferation and survival of leukemic cells and antileukemic effect of its inhibition has been indicated. Glienke et al. identified downregulation of WT1 by siRNA which led to proliferation reduction and increased apoptosis of K562 and HL-60 cell lines (Glienke et al. 2007). In the study of Yamagami et al., downregulation of WT1 by WT1 antisense oligomers led to inhibition of cell growth in both K562 cell lines and fresh leukemic cells (Yamagami et al. 1996). Therefore, WT1 expression can be an appropriate molecular marker for malignant hematopoiesis (Mao et al. 2013) but its biologic function in hematopoietic cells and hematological malignancies is still controversial (Siehl et al. 2004).

Our study confirmed the previous studies, which have indicated that WT1 expression is increased as an oncogene in hematological malignancies such as CML (Tamaki et al. 1999; Cilloni et al. 2009; Asgarian Omran et al. 2008). There was no significant association between WT1 expression and BCR-ABL1 fusion expression in the present study, while there was an association between them in the bone marrow samples in Cao et al.'s study (Cao et al. 2007). This difference may be related to presence of accelerated and blastic-phase patients and WT1 evaluation in BM samples.

As all of our patients were BCR-ABL1-positive, the presence of the BCR-ABL1 fusion can induce CML in cooperation with WT1 overexpression. This may be the effect of tyrosine kinase activity of BCR-ABL1 on cellular signaling pathways (Jabbour and Kantarjian 2014). For example, BCR-ABL1 tyrosine kinase leads to overexpression of WT1 gene as well as increased mRNA and protein levels through PI3K-Akt pathway (Svensson et al. 2007a). BCR-ABL1 transfection leads to increased expression of WT1, and it was decreased after imatinib incubation (Cilloni et al. 2004). Also, incubation of K562 cells with imatinib caused inhibition of BCR-ABL1 tyrosine kinase activity and downregulation of WT1 expression (Cilloni and Saglio 2003). Therefore, inhibition of BCR-ABL1 tyrosine kinase activity can be responsible for WT1 reduction; otherwise, WT1 overexpression is related to increased BCR-ABL1 tyrosine kinase activity. According to overexpression of WT1 in BCR-ABL1-negative leukemias such as AML, BCR-ABL1-activated signaling pathway is not the only mechanism and reason for WT1 overexpression (Yang et al. 2007). Nishida et al. indicated that AML-ETO gene, which is recognized as a common chimeric gene in AML, performs its leukemogenesis activity in cooperation with WT1 expression and AML-ETO could not induce AML by itself (Nishida et al. 2006).

The oncogenic activity of WT1 is mediated through regulation of different genes and signaling pathways (Fig. 1). Li et al. (Li et al. 2014a) reported upregulation of molecules associated with MAPK and JAK/STAT signaling pathways in WT1-isoform-transfected U937 cell lines. Interferon

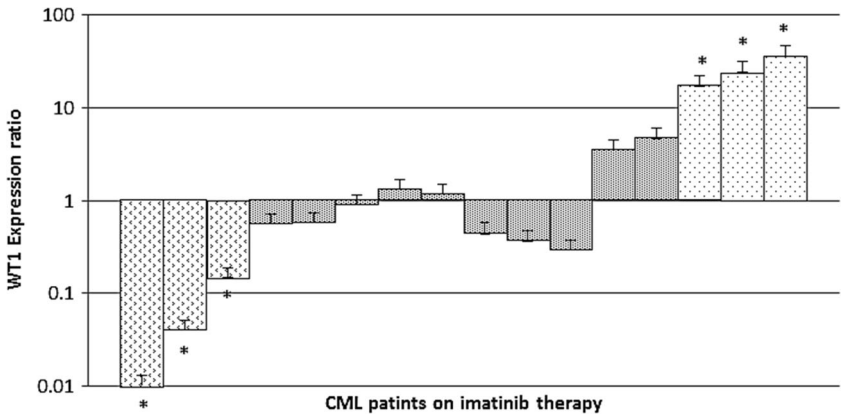

Fig. 3 Relative expression of WT1 in 16 CML patients on imatinib therapy in comparison with normal individuals

Table 3 Relative expression of WT1 in CML patients at diagnosis and at remission

Groups	Expression ratio	Result	*P* value
Newly diagnosed patients/control	12.6	Upregulated	0.003
Patients on imatinib therapy/control	0.74	Not different	0.6
Patients on imatinib therapy/newly diagnosed patients	0.05	Downregulated	0.001

regulatory factor 8 (IRF-8) and N-myc downstream-regulated gene 2 (NDRG2) are recognized as WT1 target genes. WT1 downregulates and upregulates IRF-8 and NDRG2 expression, respectively (Vidovic et al. 2010; Svensson et al. 2007b). IRF-8 leads to BCR-ABL1 antagonization and downregulation of antiapoptotic protein Bcl-2, increasing the expression of tumor suppressor p15 (Burchert et al. 2004; Schmidt et al. 2004). Therefore, deregulation of IRF-8 and NDRG2 expression by WT1 can be one of the oncogenic mechanisms in CML development. There was downregulation of β-catenin, Wnt/β-catenin pathway-related gene, and β-catenin target genes (including CCND1 and MYC in WT1-specific shRNA) in transfected K562 cells (Li et al. 2014b). In Corrêa et al.'s study (Corrêa et al. 2012), increased Wnt/β-catenin activity regulates ABCB1 gene expression, which is involved in drug resistance in CML (Corrêa et al. 2012). Therefore, WT1 gene has an oncogenic role by Wnt/β-catenin regulation in leukemogenesis.

Progression mechanism and molecular events involved in CML development are still unknown (Calabretta and Perrotti 2004). According to WT1 overexpression in most chronic CML patients in our study and higher expression of it in the blastic phase than the chronic phase in Inoue et al.'s and Varma et al.'s studies (Inoue et al. 1994; Varma et al. 2011), the value of WT1 as a possible and effective factor in progression of disease is indicated, and it can be used for predicting disease prognosis and progression. In the current study, there was also a significant reduction in WT1 in CML patients on imatinib therapy in comparison with newly diagnosed CML patients. No difference in WT1 expression was observed in patients on imatinib therapy compared to the control group. There are related studies about the effects of imatinib on WT1 expression in vitro (Table 4). For example, incubation of BM samples of pretreatment patients with imatinib for 18 h in vitro leads to decreased tyrosine kinase activity and eventual downregulation of WT1 in imatinib-responsive patients, while this was not observed in imatinib-resistant patients. Therefore, in vitro testing with imatinib to evaluate the inhibition level of WT1 expression before treatment can be the basis of a valuable test for predicting the response of patients to imatinib. It can also be used to recognize imatinib-resistant patients (Cilloni et al. 2004; Otahalova et al. 2008). Inhibition of BCR-ABL1 tyrosine kinase activity and downregulation of WT1 expression were also seen after imatinib incubation in K562 cell lines (Cilloni and Saglio 2003). In vivo study of Varma et al. (Varma et al. 2011) also demonstrated WT1 downregulation in 76 % of patients on imatinib therapy (decreased in 78.95 % of complete hematologic response (CHR) patients and increased in 66.67 % of non-CHR patients). The limitation of our study was inaccessibility of complete data of our patients and their paraclinical tests. So, we could not compare WT1 expression with other parameters.

Table 4 Relationship between WT1 expression and imatinib in current and previous studies

Methods	Cell types	Eventual results	References
Imatinib therapy	CML patients cells/in vivo	Significance reduction in WT1 in CML patients on imatinib therapy in comparison with newly diagnosed CML patients	Current study
18-h imatinib incubation	K562 cell lines/in vitro	Inhibition of BCR-ABL tyrosine kinase activity Downregulation of WT1 expression	(Cilloni and Saglio 2003)
18-h imatinib incubation	BCR-ABL-transfected COS cells/in vitro	Decreased expression of WT1	(Cilloni et al. 2004)
18-h imatinib incubation	BM samples/in vitro	Decreased tyrosine kinase activity, downregulation of WT1 in imatinib-responsive patients, while this was not observed in imatinib-resistant patients	(Otahalova et al. 2008)
Imatinib therapy	CML patients cells/in vivo	WT1 downregulation in 76 % of patients on imatinib therapy	(Varma et al. 2011)

WT1 Wilms' tumor, *CML* chronic myeloid leukemia

Conclusion

Our data confirmed the previous studies, which have indicated the oncogenic role of WT1 by its upregulation in hematologic malignancies, but this expected role was not seen in all cases. However, we could not demonstrate its relation to BCR-ABL1 expression at diagnosis and in patients on imatinib therapy. Overall, according to current and previous studies, imatinib can be effective in downregulating WT1 expression, but due to WT1 upregulation in just 72.2 % of CML patients at diagnosis and lack of significant correlation between BCR-ABL1 and WT1 expression, using WT1 as an additional marker for CML monitoring could not be applicable, although a longer follow-up of CML patients may validate the results. Therefore, future studies are required in which WT1 expression will be evaluated in combination with other molecular markers during treatment, after remission, or after the relapse phase of CML in order to optimize its possible role in CML, therapy efficiency, disease stage determination, WT1 peptide vaccination, and target therapy.

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the local ethics committee of the Ahvaz Jundishapur University of Medical Sciences (ajums.REC.1392, 341) and with the 1964 Helsinki declaration. Written informed consent was obtained from all patients and normal individuals.

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