RESEARCH ARTICLE

Wilms' Tumor Gene (WT1) Expression Correlates with Vascular Epithelial Growth Factor (VEGF) in Newly Acute Leukemia Patients Undergoing Chemotherapy

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Abstract

Background: Today, leukemia is one of the biggest problems worldwide. The Wilms' tumor gene (WT1) and the vascular endothelial growth factor (VEGF) gene are highly expressed in patients with various cancers. This study concerned the relationship between expression of WT1 and VEGF in patients with acute leukemia. Materials and Methods: We evaluated expression of WT1 mRNA and VEGF mRNA using real-time quantitative RT-PCR in the peripheral blood (PB) of 8 newly diagnosed AML and 4 newly diagnosed ALL patients, serially monitored for 2 months. A further 12 normal PB samples served as controls. Results: In the patient group, in comparison with the normal ranges, WT1 and VEGF gene expression was increased, the average values for the expression of these two genes being 0.2852±0.11 and 0.2029±0.018, respectively. While was no significant relevance between the two genes pre-treatment, a positive link between the two genes in 75% of patients with AML was noted during the procedure of chemotherapy, whereas in 75% of patients with ALL an antiparallel association was observed. Conclusions: Leukemia is associated with production of WT1, which may affect the expression of VEGF.

Keywords: WT1 - VEGF - acute leukemia - chemotherapy

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Introduction

The biggest human health problem in the world is cancer (Phipps et al., 2007). It is considered that cancer is the second cause of death, followed by deaths due to adverse cardiovascular events. Currently, one in every four deaths is caused by cancer. ALL and AML are most blood cancers in Iran (Zand A M et al., 2012). According to the results of Cancer Department State of Non Communicable Disease Management Center in 2010 reported that the incidence of blood cancer rates among women and men in Iran was 1330 and 2131, respectively. These figures for women and men in the Khuzestan province during the same year were 148 and 203, respectively (Korosh et al., 2012). This data indicated that the incidence of blood cancer rate in the Khuzestan province is significantly higher than that in other provinces. Some human characteristics are important in generation of blood cancer such as age, gender, ABO and Rh blood groups, weight and platelet counts are important in generation of blood cancer (Zand et al., 2012). Also the expressions of some genes are influenced by the change of developing cancer. Owing to treatment complications and high expenditure of diagnosis and treatment, the present study tried to apply a new approach in the treatment of cancer using the immunotherapy method.

Wilms' tumor 1 (WT1) gene locus is located in 11p13 (Gessler et al., 1990; Haber et al., 1990). The WT1 gene has 10 exons and coding zinc finger sequences, which provide the context for the connection to nucleic acid (Kreidberg et al., 1993). The effects of the down-regulation of this gene are very important on growth factors such as PDGF-α and TGF-β1 (Scharnhorst V et al., 2001). In other words, the WT1 gene affects growth and cell differentiation (Sugiyama, 2001; Oka et al., 2006) and exerts an oncogenic function in various types of leukemia. It is also over expressed in several solid tumors (Inoue et al., 1997; Oji et al., 1999; Miyoshi et al., 2002a; Oji et al., 2002; Keilholz et al., 2005), and therefore, has been considered as an attractive target for cancer immunotherapy. In addition, WT1 gene expression increases in various types of blood malignancies (Zamora-Avila et al., 2007; Wagner et al., 2008)

and different carcinomas such as lung, thyroid, breast (Zapata-Benavides et al., 2002; Caldon et al., 2008), testis (Hashiba et al., 2007), and ovary (Shimizu et al., 2000).

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Specific anti-WT1 immune responses have been described in which CD8+ cytotoxic T cells have been generated in vitro (Gao, 2000; 2003; Xue, 2005).

Formation of new blood vessels from pre-existing vasculature through sprouting or invagination is a complex process including many factors. The role of angiogenesis in the progression of cancer has been extensively observed, and it is now well established that increased levels of angiogenesis in some solid tumors correlates with poor prognosis (Sally et al., 2004). Vascular endothelial growth factor (VEGF) is another gene that belongs to the homodimer glycoprotein family and contains six members, namely, placental growth factor (PIGF) (Maglione et al., 1991), VEGF A, B (Olofsson et al., 1996), C (Lee et al., 1996), D (Achen et al., 2001), and E (Meyer et al., 1999). The VEGF gene promoter is regulated by the WT1gene (Kondo et al., 1994). Increased serum level of VEGF has been observed in several patients with cancer. Furthermore, patients with metastatic cancer have shown higher serum levels of VEGF than those with no metastatic tumor (Hayes DF, 2005).

The seminal in vitro study by Cash et al. showed that the DNA binding domain of WT1 within the tumor cells plays an essential role in the transcriptional regulation of VEGF, which is a principal factor in inducing tumor angiogenesis (Cash et al., 2007). Although the potentiality for angiogenesis associated with tumor-produced WT1 has been suggested, few reports are available on the effect of WT1 as an angiogenic inducer in the intra-tumoral microenvironment of human solid tumors. The VEGF gene expression has a significant relationship with the WT1 gene expression in sarcoma cell. The WT1 gene expression increases the expression of VEGF and enhances the activity of angiogenesis (McCarty G et al., 2011). In contrast, some studies have reported different results. The current study showed that "there is an inverse relation between WT1 and VEGF expression in Leukemia children" (Moazam et al., 2010). Because of the conflicting reports on the relationship between the expression of VEGF and WT1 genes, the present study investigated the changes in the expression of these two genes in patients with Ahvaz leukemia during the first chemotherapy administration. Notably, the results of this study, along with the relationship between WT1 and VEGF genes, could indicate the effectiveness of chemotherapy in these patients.

Materials and Methods

Patients and materials

PB samples from newly diagnosed leukemia were collected from hematology department of "Shafa" Hospital, Ahwaz Jondishapoor University affiliated during over October 2011 to November 2012. The samples then processed to extract total RNA described below.

PB samples were collected at diagnosis and during the first chemotherapy treatment. Samples from all 12 patients (48 samples) were collected at the department of Hematology in Shafa Hospital from October 2011 to November 2012. A total of 12 patients (samples) include of subjects of AML and ALL. This protocol was approved by the Ahvaz Jondishapoor University ethical committee.

Clinical criteria and treatment strategy

The ALL patients were treated by hematology department staff with drugs i.e. Endoxan, Danurobicin, Mesna, Cytosar, and VCR. Other group of the AML patients were prescribed with drugs i.e. Idarubicin, Cytosal and Danurobicin. FAB (French American British) classification was applied for morphological diagnosis. The patient's bone marrow was examined and the ratio of the blasts were determinate for judgment of the treatment effect. Complete remission (CR) was considered by standard criteria, based on morphological findings by the presence of less than 5% blasts in the bone marrow. the samples were divided into two groups; the pre-treatment group that the patients received no treatment at diagnosis, and during the first chemotherapy period group, who had already received no other types of treatment.

The samples from AL (Acute Leukemia) patients in the pre-treatment and during the first chemotherapy were analyzed and their percentage of blast was compared with WT1 and VEGF mRNA level. The change of treatment strategy and the result based on the WT1 and VEGF mRNA level were examined. A total of 12 samples as negative controls were considered that showed the over-expression in these two genes in patients as a comparison. The high risk patients who died during the chemotherapy treatment course or lymphoid deposition in their blood samples were not enough amount, so their OD of mRNA amount were not in the range of 1.8-2.0 removed from the sample set.

RNA extraction and cDNA synthesis

The PB from the patients was processed with RBC lysis buffer. Total RNA was extracted with RNX solution (Cinnagen, Iran) method, following the manufactures instruction. Qualify of RNA was checked by Biofotometer (eppendorf). The gel electrophoresis of RNA was performed through 1% agarose. cDNA synthesis: reverse transcription (RT) step was performed with transcripter .First standard cDNA synthesis kit (Thermo scientific) was applied according to the manufactures instruction. Oligo (dT) primer was mixed with template RNA and this mixture was incubated at 65˚C for 5 min. Transcriptase reaction buffer, transcriptase, deoxynucleotide mix, RNase inhibitor (all Thermo scientific) were added to previous mixture. Total mixture was incubated at 42˚C for 60 min, and 70˚C for 5 min. The residue of extracted RNA and cDNA were stocked at -70˚C.

Real time quantitative PCR of WT1 and VEGF

Total mixture was incubated at -70˚C for Quantitative real time -polymerase chain reaction (RQ-PCR). Reactions and fluorescence measurements were performed on the Applied Bio systems 7500. The Real time PCR system primer and SYBR Green master mix was used for quantitative assessment. The applied Primers were; WT1 (Forward: AGGGTACGAGAGCGATAACCACAC/ Reverse: CTCAGATGCCGACCGTACAAGA), VEGF (Forward: CACCATCGACAGAACAGTCC/ Reverse: GAATCCAATTCCAAGAGGGA), and

glyceraldehyde-3-phosphate dehydrogenase GAPDH (Forward: ACTGTGAGGAGGGGAGATTC/ Reverse: GCAAGAGCACAAGAGGAAGA) and SYBR Green master mix dye (from Takara Company). All assessments were carried out in duplicate along with appropriate negative controls. The PCR was performed on the Applied Bio systems 7500 Real time PCR instrument (ABI) as follows: Initial denaturation at 95˚C for 10 min; 40 cycles of annealing at 95˚C for 15 sec; extension at 68˚C for 60 sec. Also Rest 2008 v2.0.7 and excel software were used for analysis of gene expression . Based on the data presented in excel, data that were obtained from CP were introduced as the severity of gene expression. These data were considered to compare gene expression.

Statistical analysis

The analysis was performed using the SPSS 21. Comparing quantitative differences of WT1 (or VEGF) expression from patients and normal individuals, the Mann Whitney U test was used. The Exact Chi-square tests were appropriately used to compare the qualitative differences of the WT1 (or VEGF) expression in the two groups. Correlation of WT1 and VEGF expression between in PB of AL (Acute Leukemia) patients was determined by the Pearson Ranks Correlation Test. The changes rate of the WT1 (or VEGF) expression in the first chemotherapy period was determined by repeated measures test. The WT1expression was defined as the ratio of the density of the WT1 and VEGF to GAPDH PCR product amount. P-values of less than 0.05 were considered significant.

Results

Diagnosis of patients with AML was primarily based on FAB criteria (Table 1) supplemented with bone marrow biopsy. The results obtained by pathologic observations and aspiration of bone marrow.

WT1 expression at diagnosis time

To determine the baseline, expression level of WT1 in normal samples, PBMCs from 12 normal subjects were tested from which, 5 samples gave so weak expression. The remaining 7 samples were totally negative. The average rate of WT1 expression was 0.014 in normal cells. Space please number was considered as a baseline for WT1 expression. Based on this baseline value, all of acute patients' PB samples included in this study were totally positive with a mean 0.285 (Minimum: 0.054, Maximum: 0.399); significantly higher than that of normal subject (p=0.001), (r=0.321) (Figure 1).

The Spearman Rank Correlation test indicated that significant differences were observed for WT1 level in pre-treatment and post-treatment (p=0.001), (r=0.88, SD: 0.111). In addition there was a significant correlation between VEGF gene expression of pre-treatment and VEGF gene expression of the second (p=0), (r=0.853, SD: 0.107) and third samples (p=0.043, r=0.592, SD: 0.105). However, there was no significant correlation between VEGF gene expression of pre-treatment and the last sample of person (p=o.118), (r=0.476, SD: 0.110). According to the results that have shown in the table 2, WT1 gene level decreased gradually during the chemotherapy course. By contrast, the VEGF gene did not have a gradual decreasing.

VEGF expression at diagnosis time

The average of VEGF expression rate in normal cells was 0.157. The obtained quantity was considered as a baseline for VEGF expression. Based on this baseline value, the VEGF expression for AML and ALL was appointed at a little quantity. The average rate of VEGF over-expression was 0.231 in cancer cells of patients and these samples with VEGF expression levels were judged (p=0.002, r=0.34) above the cut-off value.

The VEGF expression differed significantly between patients and healthy individuals. The current study was conducted at diagnosis time (p=0.002, r=0.34, SD: 0.175) and in some patients, VEGF gene expression in during the chemotherapy process (p=0.001, r=0.187, SD: 0.158) was higher than its normal level. In additional; there was a significant difference between the VEGF gene expression in the last sample and its quantity in normal individuals

Table 1. Clinical Characteristics at Diagnosis of the 12 Patients Contributed in the Study. The Outcomes of BM Aspiration and Flow Cytometry that are Correspond with the Increased WT1 Gene Levels at Diagnosis, whereas VEGF Levels have Decreased as Compared with Normal

FAB classification	Age	Blast	WT1 (pre-treatment)	VEGF (pre-treatment)	Diagnostic test
L2	27	74	0.139	0.209	BM aspiration
L2	22	32	0.054	-0.101	BM aspiration
L2	23	88	0.199	0.043	BM aspiration
M5	25	70	0.242	0.31	BM aspiration
M2	28	62	0.378	0.417	BM aspiration
L2	22	40	0.255	0.134	BM aspiration
M3	32	78	0.373	0.435	BM aspiration
M3	40	32	0.348	0.225	BM aspiration
M3	50	70	0.367	0.139	Flow Cytometry
M4	36	84	0.394	0.006	BM aspiration
M4	44	60	0.399	0.449	BM aspiration
M3	47	62	0.275	0.169	BM aspiration

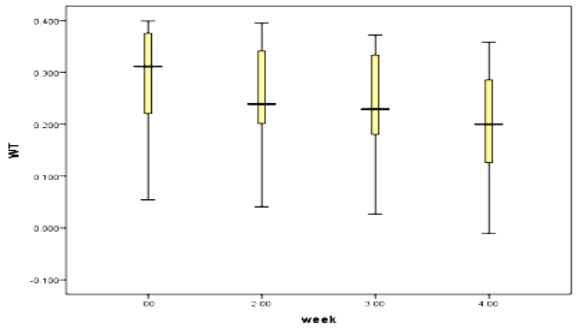

Figure 1. X Axis Shows the Time of Sampling and Y Axis Shows WT1 Expression Score (was Normalized with GAPDH Gene). This graph shows the increased average of WT1 gene expression of 12 patients at diagnosis and during the first chemotherapy period. According to the graph, its expression is decreased I during the first of chemotherapy period. While WT1 levels that were measured in the end of the first chemotherapy period were close to the normal range (no exactly within range)

(p=0.001, r=0.077, SD: 0.121).

The VEGF gene was originally recognized in different level for patients with acute leukemia. According to this survey, the VEGF gene expression in some ALL (Pt.'s number: 2, 3, 6) (median 0.088, range 0.101-0.209) or AML (Pt.'s number: 9, 10) (median 0.267, range 0.006-0.449) patients was lower than healthy volunteers (median: 0.005), (range: 0.182-0.193) (Table 3).

The average of VEGF expression was decreased in cancer cells of some patients (Pt's number: 1, 4, 5, 7, 10, 11) and this amount was even less than expression rate in normal cell (Table 3). However, VEGF was up-regulated for 1 AML and 3 ALL patients (Pt's number: 2, 3, 6, 9). Furthermore this level might be higher than gene expression levels in some patients. It is another notable point that the VEGF gene expression level was decreased in 75% of AML after chemotherapy course (Figure 2).

In contrast the VEGF level was decreased only in 25% of ALL patients in chemotherapy period. Therefore, the obtained results show that there is a constant situation with increasing process in the first course chemotherapy in many patients (75%) with ALL (Figure 3).

Correlation between WT1 and VEGF gene expression The Spearman Rank Correlation test indicated that there

Table 2. WT1 gene Expression Levels in Patients with Acute Leukemia: before any Treatment (WT0), During the Chemotherapy Course (WT1, WT2; Respectively 2th week and 4th week) and in the End of the First Complete Chemotherapy Course (WT3)

Number	WT0	WT1	WT2	WT3
1	0.139	0.114	0.104	0.09
2	0.054	0.041	0.026	Nearly zero
3	0.199	0.193	0.193	0.124
4	0.242	0.21	0.178	0.128
5	0.378	0.37	0.353	0.358
6	0.255	0.238	0.227	0.218
7	0.373	0.343	0.344	0.316
8	0.348	0.34	0.323	0.255
9	0.367	0.237	0.231	0.182
10	0.394	0.395	0.372	0.346
11	0.399	0.328	0.27	0.231
12	0.275	0.24	0.183	0.151

Table 3. VEGF Gene Expression Levels in Acute Leukemia Patients: Before any Treatment (VEGF0), During the Chemotherapy Course (VEGF1, VEGF2; Respectively 2th Week and 4th Week) and After the First Complete Chemotherapy Course (VEGF3)

Number	VEGF0	VEGF1	VEGF2	VEGF3
1	0.209	0.065	0.04	Nearly zero
2	Nearly zero	Nearly zero	Nearly zero	0.008
3	0.043	0.175	0.185	0.175
4	0.31	0.221	0.223	0.214
5	0.417	0.332	0.382	0.284
6	0.134	0.154	0.172	0.196
7	0.435	0.444	0.488	0.383
8	0.225	0.233	0.223	0.228
9	0.139	0.124	0.189	0.225
10	0.006	0.006	0.372	0.38
11	0.449	0.437	0.277	0.273
12	0.169	0.127	0.136	0.245

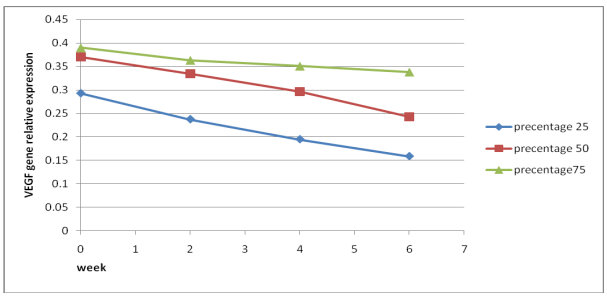

Figure 2. X Axis Shows the time of Sampling and Y Axis Shows VEGF Expression Score in 8 Patients with AML (Normalized with GAPDH Gene). VEGF gene relative expression is shown at diagnosis and during the first period of chemotherapy for 8 patients with AML. The VEGF gene expression level was totally decreased in 75% of AML after chemotherapy. According to this graph, in first week: 25% of patients have VEGF expression lower than 0.3 and higher than 0.4 and 75% of them have VEGF expression 0.4 >y >0.3. In twice week: 25% of patients have VEGF expression lower than 0.23 and higher than 0.37 and 75% of them have VEGF expression 0.37 >y >0.23. In third week: 25% of patients have VEGF expression lower than0.2 and higher than 0.35 and 75% of them have VEGF expression 0.35 >y >0.2. In fourth week: 25% of patients have VEGF expression lower than0.15 and higher than 0.34 and 75% of them have VEGF expression 0.34 >y >0.15, Also the navy red curve shows the median of VEGF gene expression in per week. Whereas the average of VEGF expression rate in normal cells was 0.157

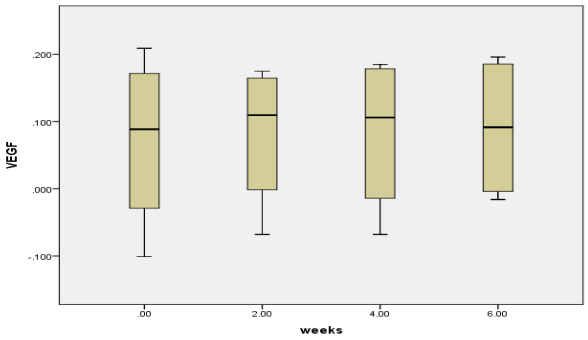

Figure 3. X Axis Shows the Time of Sampling and Y Axis Shows VEGF Expression Score (Normalized with GAPDH Gene): VEGF gene relative expression is shown at diagnosis and during the first period of chemotherapy for 4 patients with ALL. This chart showed VEGF level was decreased only in 25% of ALL patients in chemotherapy period and in 75% of patients with ALL, its expression was increased

was no significant correlation between WT1 and VEGF gene expression in healthy persons (p=0.994); however, the VEGF was expressed in several WT1 positive cancer blood cells. Typical VEGF expression within tumor cells is shown in table 3. The extracted results from the current study indicated that there was not a significant difference between the WT1 and VEGF levels for the first and second courses sampling (p=0.106, r=0.490) (p=0.124, r=0.469). By contrast, significant differences were observed in WT1 and VEGF express amount at the third and fourth courses sampling from patients (p=0.001, r=0.869).

Discussion

Recent studies have shown that tumor-produced WT1 influences tumor progression in various types of cancer.

Yamamoto et al. concluded that the WT1 protein might be an accelerator of the progression of severe ovarian adenocarcinoma (Yamamoto et al., 2007). Sera et al. found that over expressed WT1 was associated with tumor growth and resulted in worse prognosis of hepatocellular carcinoma (Sera et al., 2008). Hylander et al. showed that patients with WT1-positive tumors had a higher grade (p=0.006) and advanced stage (p=0.002) of epithelial ovarian cancer (Hylander B et al., 2006). Miyoshi et al. concluded that tumors >2 cm also demonstrated an orientation toward a rise (p=0.09) in WT1 (Miyoshi et al., 2002b). Furthermore, Dohi et al. (2010) revealed that the produced WT1 may regulate VEGF expression in endometrial cancer.

Based on the above-mentioned studies, it was noted in the present study that WT1 over expression was associated with illness onset (p<0.001). Clinical and experimental data revealed that WT1 over-expression could be considered as a simple prognostic factor and may indicate its important role in leukemogenesis. Many studies have demonstrated that the up regulation of WT1 expression positively correlated to tumor progression (Ozalp et al., 2003). Therefore, the aim of the present study was to investigate the effect of chemotherapy on WT1 expression. According to the obtained data, chemotherapy significantly decreased WT1 expression (p=0.002) and complete remission of the disease was observed in these patients, but not as much as in the normal range. In many treatment procedures, WT1 gene expression is considered as a pan leukemic marker, prognosis factor, and a useful index to identify minimal residual disease in patients with acute leukemia (Kusumoto et al., 1999). However, the high level of WT1 gene after first chemotherapy could be accounted as a possible relapse in future. The results obtained in the present study showed consistency with previous studies on other types of cancers (Daniela Cilloni et al., 2008). It has been reported that WT1, through upregulation of VEGF, plays a key role in allowing tumor cells to adapt to hypoxia and adopt an angiogenic phenotype (Gregory McCarty OA and Loeb, 2011. december). Hence, the present study examined three variables, including chemotherapy, WT1, and VEGF expression. VEGF, which has an important role in angiogenesis, is over expressed in some of the solid tumors. The importance of angiogenesis-targeting treatment is rather related to the efficiency of targeting blood vessel of endothelium tumor, which may be higher than the targeted tumor cells themselves (Rini, 2007).

The results obtained in the present study on WT1 and VEGF showed a strong correlation between the expression of these two genes in patients with leukemia, suggesting that WT1 may regulate tumor development and angiogenesis in acute blood cancer. WT1 was found to be co-expressed with VEGF in cancer blood cells at the time of diagnosis. However, there was a difference in the expression of these genes in patients with ALL and AML. In majority of the patients with AML (75%), decreasing VEGF expression resulted in reduced WT1 level during the treatment course, indicating the co-affectivity of chemotherapy on tumor-produced WT1 and angiogenesis. However, a decreasing trend in the expression of the two genes was observed in only 25% of the patients with ALL during the treatment. Thus, these results indicated that chemotherapy might be effective only as cancer immunotherapy, but not as an anti-angiogenesis treatment.

The obtained results showed that the expression of the WT1 and VEGF genes increased during the onset of the disease in both AML and ALL groups. However, different results were noted for the expression of these two genes during the chemotherapy course. In 75% of the patients with AML, the expression of WT1 and VEGF were accordingly reduced during the chemotherapy course. However, in 75% of the patients with ALL, along with the decreased WT1 expression during the course of chemotherapy, VEGF expression increased or remained unchanged. In other words, unlike patients with AML, there was no significant difference in the patients with ALL with respect to the effect of chemotherapy on VEGF expression.

In other similar studies, a positive correlation between WT1 and VEGF expression was demonstrated (Dohi et al., 2010; McCarty and Loeb, 2011), which is consistent with the results obtained in the present study. In most of the patients with AML, WT1 and VEGF expression decreased simultaneously. Silencing of WT1 in the high WT1-expressing cell lines resulted in a decrease in VEGF expression (McCarty and Loeb, 2011). Similarly, in another study by Hanson and Graham on patients with prostate cancer, WT1 unregulated VEGF expression in a positive situation (Graham et al., 2006; Hanson et al., 2007).

In view of the above-mentioned findings, it can be concluded that WT1 plays an essential role in the transcriptional regulation of VEGF gene in cancer cells (Dohi et al., 2010a; McCarty et al., 2011). Therefore, the WT1 peptide vaccine therapy and WT1 suppression could be an effective method to inhibit tumor metastasis. In the present study, in contrast to the patients with AML, WT1 unregulated VEGF before chemotherapy in patients with ALL; however, no positive correlation was observed in 75% of the patients after chemotherapy. In another study, Moazam et al. showed that WT1 down-regulated VEGF expression in children with ALL, which may be owing to the isoform type of WT1. It must be noted that WT1 occurs in various isoforms some of which cannot regulate VEGF expression (Moazam et al., 2010).

In conclusion, tumor-produced WT1 may regulate the VEGF gene expression and induce angiogenesis in patients with AML. Hence, angiogenic targeting therapy has begun to show promise as an effective treatment strategy for many types of solid tumors, and WT1 is also a main target for cancer immunotherapy in these patients. However, chemotherapy in patients with ALL only suppressed tumor-produced WT1, whereas angiogenesis inhibition was not observed.

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