Over-expression of Wilm's Tumor Gene 1 (WT1) in Iranian Patients with Acute Myeloblastic Leukemia

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ABSTRACT

Background: The Wilm's tumor gene 1 (WT1) encodes a zinc finger transcription factor that is inactivated in a subset of Wilm's tumors. It plays a crucial role in growth, proliferation and development of some embryonic and adult organs. WT1 is expressed as a tumor associated antigen (TAA) in various types of solid and hematopoietic malignancies and can be employed as a useful marker for targeted immunotherapy and monitoring of minimal residual disease (MRD). **Objective:** To investigate the profile of WT1 gene expression in Iranian patients with acute myeloblastic leukemia. **Methods:** RT-PCR method was used to determine the WT1 gene expression in bone marrow (BM) and/or peripheral blood (PB) samples from 11 patients with AML and PB samples of 36 normal subjects. Isolated cells from all patients were immunophenotyped by flow cytometry. **Results:** The leukemic cells from 10 patients (91%) were found moderately or strongly positive for WT1 expression whereas only 3 out of 36 normal subjects expressed WT1 at very low levels. A highly significant correlation was observed for WT1 expression between paired BM and PB samples of the AML patients. **Conclusion:** Our results indicate that WT1 is expressed in the majority of Iranian AML patients and may be employed for screening and monitoring of minimal residual disease in these patients.

Keywords: Wilm's Tumor Gene 1, Acute Myeloblastic Leukemia, Tumor-associated Antigen, RT-PCR

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INTRODUCTION

Identification and characterization of tumor-associated antigens (TAAs) in human malignancies and development of new methods for identification of these antigens have been the focus of intense research in the past decade (1).

Wilm's tumor Gene 1 (WT1) is a zinc finger transcription factor, which was originally identified for its role as a tumor suppressor gene in the pathogenesis of Wilm's tumor (2, 3). It is now considered as a universal TAA in a wide range of hematopoietic and solid malignancies (4). The human WT1 gene is located on the short arm of chromosome 11p13. The WT1 gene is comprised of ten exons and has different variants because of alternative splicing (5).

WT1 is expressed during mammalian embryonic development in many tissues, such as urogenital system, gonads, spleen, liver, brain, and spinal cord (6), but during adulthood its expression is restricted to a limited number of tissues, mainly the genitourinary system (7).

The expression pattern of WT1 indicates that it plays a crucial role in the development, growth and differentiation of several organs (8). In normal bone marrow (BM) WT1 is expressed at low levels in normal $CD34^+$ progenitor cells implying its role in normal hematopoiesis (9).

Expression of WT1 has been reported in several malignancies such as ovarian cancer (10), esophageal cancer (11), desmoid tumors (12), colorectal adenocarcinoma (13), head and neck squamous cell carcinoma (14), and a variety of human leukemias such as chronic myelocytic leukemia (CML), acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML)(15,16). Although the biological significance of WT1 overexpression in patients with leukemia is not well determined, but it is suggested that WT1 could be involved in the pathogenesis of human leukemia by interference with cellular growth differentiation and also by acting as a transcription repressor element (17).

Annually one third of the newly diagnosed leukemia cases in the United States are classified into AML (18). AML is a malignant neoplasm of hematopoietic cells characterized by an abnormal proliferation of myeloid precursor cells, decreased rate of self-destruction and an arrest in differentiation (19). AML is a disease of older adults and its incidence increases with age (19). Gene profiling studies have identified overexpression of many TAAs in AML (20, 21).

Many groups have studied the expression of WT1 in AML patients for screening (16) and monitoring of minimal residual disease (MRD) (15). Wide overexpression of WT1 in human tumors especially in leukemias has prompted many investigators to employ this gene as a target for effective immunotherapy of cancer (22).

Despite extensive investigation of WT1 expression in leukemic patients from different ethnic populations, little is known about the profile of its expression in Iranian patients. In this report we studied the WT1 gene expression as a TAA in Iranian AML patients and also in normal healthy donors.

SUBJECTS AND METHODS

Subjects. A total of 18 samples including preservative free heparinized bone marrow (BM) and peripheral blood (PB) samples (7 paired BM and PB, 3 BM, and one PB) were obtained from 11 newly diagnosed AML patients. Diagnosis of AML was based on cytomorphological findings (FAB criteria) (23) and immunophenotypic characteristics of BM leukemic cells. Major clinical features of patients are shown in Table 1.

Table 1: Major clinical and hematological findings in AML patients

Patients	age	sex	FAB	WBC (10^9/L)	RBC (10^{12}/L)	HB(g/dl)	PLT (10^9/L)	Current status
ND1	7	M	**NA**	5.8	2.9	9.1	75	Alive
ND2	23	F	M2	122.3	3.2	9.4	43	Alive
ND11	12	F	M2	10.7	2.1	7.0	31	Dead
ND13	17	M	M5	85.4	3.1	8.8	22	Dead
ND20	21	M	M4	18.5	3.1	9.3	17	Alive
ND23	12	M	M3	1.0	3.6	9.8	34	Alive
ND27	8	F	M1	3.2	2.6	7.9	<10	Dead
ND30	12	M	M0	2.1	NA	8.6	104	Alive
ND64	10	M	M1	1.3	2.7	7.2	21	Dead
ND66	33	F	M3	NA	NA	NA	NA	Alive
ND72	13	F	M3	8.7	2.3	7.6	45	Alive

FAB: French-American-British staging system; WBC: white blood cell; RBC; red blood cell; HB; hemoglobin; PLT; platelet count

Based on FAB criteria and immunophenotyping results one patients was classified as M0 (9.1%), two cases as M_1 (18.2%), two cases as M_2 (18.2%), three cases as M_3 (27.3%), one case as M_4 (9.1%) and one case as M_5 (9.1%). The FAB data for one patient was not available.

The main criterion for inclusion in this study was that the patients were at presentation stage and not having taken any chemotherapeutic regimens at the time of sampling.

The patients were selected from those attending the Hematology and Oncology Clinics of Vali-Asr and Ali-Asghar hospitals, affiliated to Tehran University of Medical Sciences and Iran University of Medical Sciences, respectively.

Heparinized PB samples collected from 36 normal healthy donors (with mean age of 28 years and a range between 2 to 79 years) served as controls to determine baseline WT1 expression and cut-off values.

This study was approved by Tehran University of Medical Sciences and informed consents were obtained from patients or their parents.

Isolation of Mononuclear Cells. Normal and leukemic mononuclear cells were isolated from bone marrow and peripheral blood using Histopaque (Sigma, USA) den-

sity gradient centrifugation, as described (24). Mononuclear cells were washed twice in RPMI-1640 medium (Sigma, USA) and a total of 3×10^6 cells were transferred to eppendorf tubes for RNA extraction.

Immunophenotyping of Tumor Cells. After separation of mononuclear cells from BM of AML patients, cells were immunophenotyped with FITC or PE conjugated monoclonal antibodies (DAKO, Denmark) specific for myeloid lineage antigens (CD13, CD14, CD33), B-cell lineage antigens (CD10, CD19, CD20), T-cell lineage antigens (CD2, CD3, CD5) and lineage non-specific antigens (CD34, CD45, HLA-DR, Tdt) using flow cytometry (Partec, Germany). Expression of at least 20% of the leukemic blast cell population for each marker was considered as positivity for that marker after subtraction of background staining with isotype matched conjugated monoclonal antibodies of irrelevant specificity.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from leukemic cells and normal PBMCs using RNA-Bee reagent (TEL. Test Inc, USA), according to the manufacture's instructions. First strand cDNA was synthesized using 1 μg of total RNA in 20 μl reaction mixture consisting of 4 μl of 5x RT – PCR buffer, 2 μl 20 mM dNTPs (Roche, Germany), 1 μl 20 pmol/μl random hexamer (N6) (Roche, Germany), 2 μl deionized sterile H2O and 1 μl M-Mulv reverse transcriptase (200 u/μl) (Fermentase, Russia). Thereafter the mixture was incubated at 40 °C for 45 minutes, followed by 90°C for 5 minutes.

WT1 and β-actin PCR. PCR amplification was performed using WT1 specific primers 5´-ATAACCACACAACGCCCATC-3´ as sense and 5´-ACTGGAATGGT TTCACACCTG-3´ as antisense (Genebank accession No. BC 032861) and β-actin specific primers as described (25).

Briefly, 25 μl PCR reaction mixture was prepared using 2.5μl 10x PCR buffer, 1 μl (for WT1) and 3 μl (for β-actin) 25mM MgCl2, 1.5 μl 10 mM dNTPS (Roche, Germany), 5 pmol of each primer, 0.5 unit Taq-DNA polymerase (CinnaGen, Iran) and 2 μl (for WT1) and 1 μl (for β-actin) cDNA. PCR was performed in 38 cycles for WT1 and 26 cycles for β-actin.

Each cycle of WT1 amplification consisted of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and finally 72°C for 10 minutes. Each cycle of β-actin amplification consisted of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and finally 72°C for 10 minutes. The amplicon sizes of WT1 and β-actin PCR products were 332 bp and 321 bp, respectively.

PCR products were finally visualized by running on agarose gel (1.5%) electrophoresis containing ethidium bromide. Both WT1 and β-actin products of each sample were simultaneously run in a single gel to minimize between- run variations and enhance precision of the assay.

After electrophoresis, images were taken by gel documentation system (UVP-LMS-20E, USA). WT1 and β-actin band densities were determined by Labworks 4.0 software (UVP, USA) and the ratio of the two bands was calculated for each sample. To obtain values higher than one this ratio was multiplied by 100 for all samples.

Statistical Analysis. The analysis was performed using the SPSS 11.5 for windows. For comparing quantitative differences of WT1 expression in patients and normal subjects, the Mann Whitney U test was used. Exact Chi-square or Fisher's exact tests were appropriately used to compare the qualitative differences of WT1 expression in the two groups.

Correlation of WT1 expression between paired BM and PB of AML patients was determined by the Spearman Ranks Correlation test. WT1 expression is defined as the ratio of the density of WT1 to β-actin PCR product bands. P-values of less than 0.05 were considered significant.

RESULTS

Diagnosis of AML in our patients was primarily based on FAB criteria (Table 1) supplemented with immunophenotypic results obtained by flow cytometry. Representative flow cytometry results of one patient (ND27) are illustrated in figure 1.

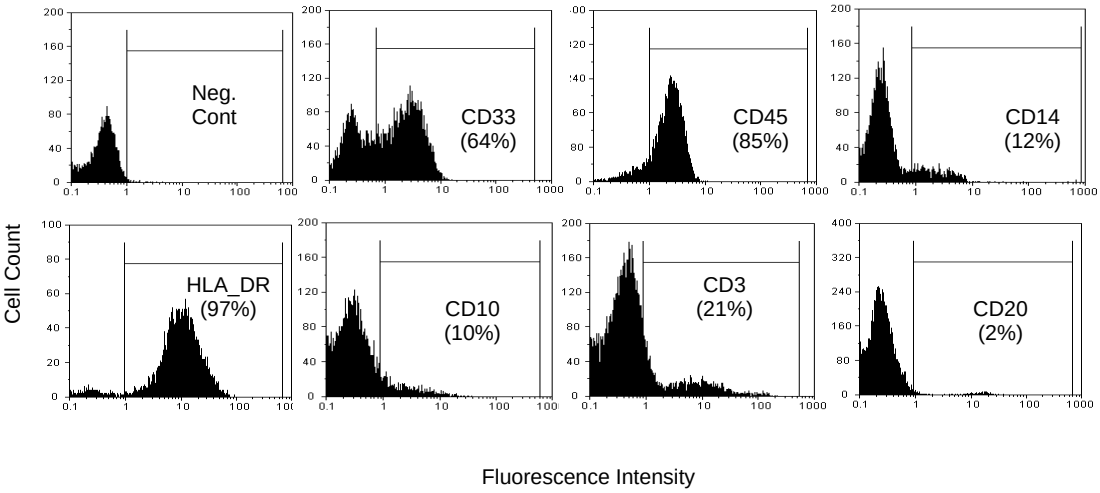

Figure 1. Immunophenotypic results obtained from an AML patient (ND27) by flow cytometry

The sensitivity limit of our RT-PCR method was determined using different proportions of a WT1 expressing cell line (K562) (15), used as a positive control, mixed with WT1 – negative PBMCs collected from a normal subject. The results demonstrate that this method can detect down to a single K562 cell among 2×10^5 negative normal PBMCs (Figure 2).

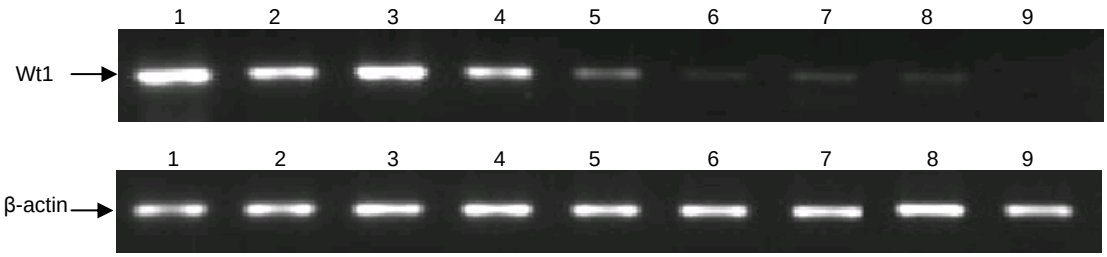

Figure 2. RT-PCR amplification of WT1 and β-actin in cDNA samples extracted from K562 cells titrated in normal PBMCs
K562 cells were mixed at 2×10^6, 0.5×10^6, 10^5, 2×10^4, 50×10^3, 10^3, 10^2, 10 and 0 cells (lanes 1-9 respectively) with a proportion of normal PBMCs to yield a total number of $2\times10^6$6 cells for all combinations. Upper row contains WT1 and lower row contains β-actin PCR products of the same samples.

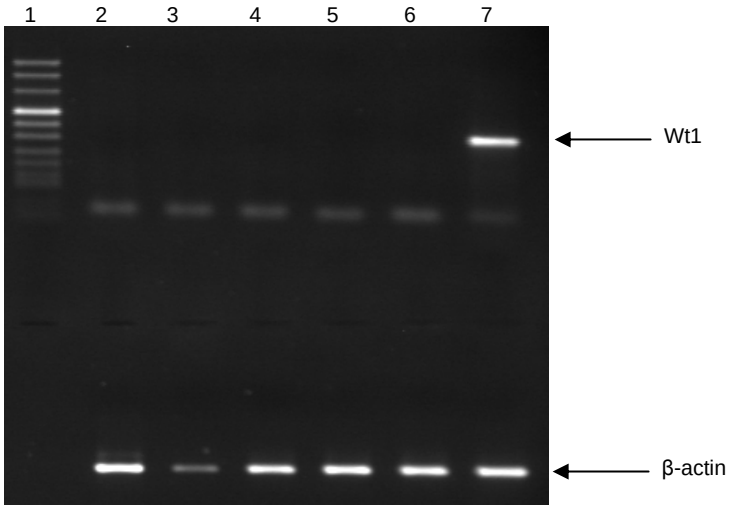

Figure 3. Expression profile of WT1 mRNA in PB of some normal subjects
Lane 1 represents size marker and Lane 7 represents K562 as positive control.

To determine the baseline expression level of WT1 in normal samples, PBMCs from 36 normal subjects were tested. Only 3 samples gave a weak band with ≈ 332 bp size corresponding to the WT1 amplicon size. The remaining 33 samples were totally negative (Figure 3). The ratio of WT1 to β-actin band densities was calculated for these three samples to obtain an arbitrary cut-off value for analysis of WT1 gene expression in patients. The calculated ratios were found to be 1.43, 5.78 and 2.47 (mean = 3.2). Based on this baseline value, BM and/or PB samples from 10 out of the 11 AML patients included in this study were positive with a mean WT1 / β-actin ratio of 56.6 (minimum: 7.7, maximum: 264) (Figures 4 and 5), significantly higher than that of the normal subjects ($p<0.0001$). A significant correlation was also observed for WT1 expression between paired BM and PB samples of the AML patients. However, no significant differences were observed for WT1 / β-actin ratios between the paired BM and PB samples (P=0.2).

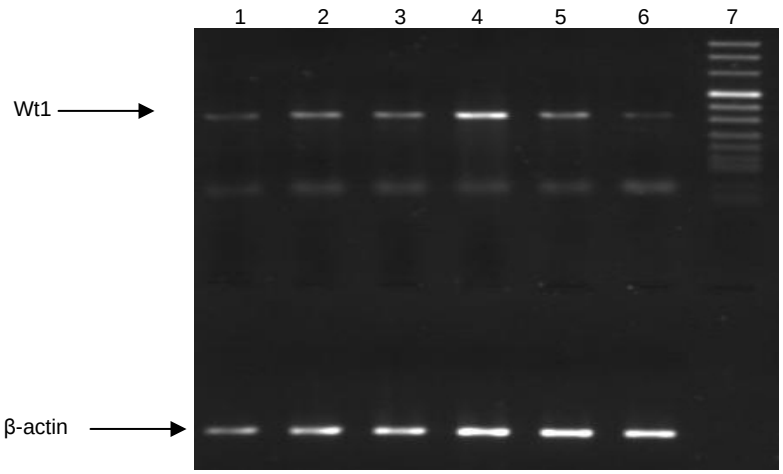

Figure 4. Expression profile of WT1 mRNA in BM and PB of some AML patients

Lanes 1-6 represent PCR products obtained from patients ND1 (BM), ND1 (PB), ND66 (BM), ND72 (BM), ND64 (BM) and ND64 (PB), respectively. Lane 7 denotes size marker.

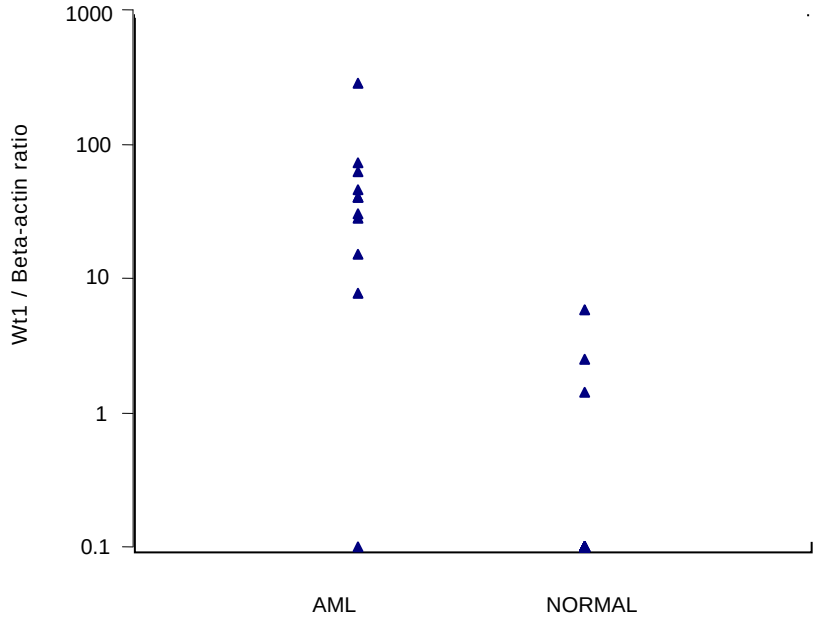

Figure 5. Relative expression of WT1 mRNA in AML patients and normal subjects

DISCUSSION

Recent advances in the field of molecular biology and tumor immunology have resulted in the identification of a large number of TAAs and their immunogenic epitopes from various types of malignant neoplasms (26). Many candidate TAAs are self proteins that are expressed by normal and malignant cells. These TAAs are classified into two groups, the TAAs expressed during fetal development but either undetectable or expressed at very low levels in adult tissues (such as MAGE1 and HER-2/neu) and those related to the stage of differentiation of malignant cells (such as tyrosinase) (27). Identification of TAAs may also be useful as targets for immunotherapy and molecular targeting therapy as well as development of diagnostic tools (1).

One of the TAAs that is expressed in various types of solid tumors and hematopoietic malignancies is WT1, currently known as a panleukemic marker. The main purpose of this study was to investigate the profile of WT1 expression in Iranian patients with acute myeloblastic leukemia. Most researchers have reported that WT1 is expressed in more than 90% of AML patients in different ethnic populations (2, 9). Our findings are very similar to previous reports.

Many studies have shown that WT1 is expressed at low levels in normal $CD34^+$ BM cells and also at very low levels in normal PB (8, 9, 28). Therefore, determination of the baseline expression levels of WT1 in normal PB cells is essential to identify overexpressed levels in leukemic cells. To accomplish this objective we determined the ratio of WT1 to β-actin band densities in PBMCs of 36 normal subjects with a broad range of age. Representation of WT1 expression as a ratio of the housekeeping gene β-actin was intended to normalize the data and avoid variations due to differences in cDNA concentration or application of different levels of PCR products to

electrophoresis gel. Further caution was taken by simultaneous electrophoresis of WT1 and β-actin PCR products of each subject on the same gel. This would minimize variations due to technical shortcomings. Taking these considerations into account, our results demonstrated that only 3 out of 36 normal samples expressed the WT1 gene at levels significantly lower than the minimum expression level of our AML patients (Figure 5). Of the 11 AML patients, 10 expressed WT1 at a level higher than that of the normal subjects. Despite the small sample size of our AML patients, statistical analysis indicated a highly significant difference between patients and normal subjects ($p<0.0001$).

Wide expression of WT1 in human neoplasia indicates that this gene may play a role in tumorogenesis and leukemogenesis. Many studies showed that WT1 plays an important role in growth and proliferation of immature leukemic cells [29,30] and during differentiation of K562 and HL60 leukemic cell lines, the expression of WT1 is downregulated (31, 32). WT1 expression in immature AML and ALL leukemic cells (according to FAB criteria) [15] and its expression in normal $CD34^+$ stem cells imply that WT1 may also play a crucial role in proliferation and regulation of normal hematopoietic progenitor cells and is downregulated during differentiation (30, 31, 32).

The WT1 gene was originally isolated from Wilm's tumor as a tumor suppressor gene, but its overexpression in hematopoietic and solid tumors and its role in growth and proliferation of these tumor cells indicate that WT1 may act as an oncogene (30). Differences in the interaction of WT1 with other regulatory and adaptor proteins might determine whether the WT1 gene acts as tumor suppressor gene or oncogene (30). Some studies have reported that WT1 expression increases during diagnosis and relapse phases of acute leukemias and decreases during remission phase (2, 9, 15). Thus, its expression can be a useful marker for prognosis and monitoring of MRD in these patients. Recently real-time quantitative RT-PCR for WT1 expression has widely been used to determine residual leukemic cells enabling prediction of the molecular relapse before the clinical relapse (33). It has also been used as a target for selective immunotherapy with promising results (4).

In conclusion our results show that WT1 is expressed at high levels in Iranian patients with AML and may be used for screening and monitoring of MRD in these patients.

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