Differential WNT Gene Expression in Various Subtypes of Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Dysregulation of WNT signaling has been reported in many malignancies. **Objective:** This study was conducted to investigate the expression pattern of 14 members of the WNT gene family in different immunophenotypic subtypes of ALL. **Methods:** Semi-quantitative RT-PCR was performed on samples from 71 ALL patients and 36 age-matched healthy individuals. The ALL patients were categorized into B-ALL (76%), T-ALL (22.6%) and mixed lineage (1.4%) and the B-ALL cases were further classified into pro-B, pre-BI, pre-BII and immature/mature-B based on immunophenotypic results. **Results:** Among the WNT genes, WNT-7B ($p=0.026$), WNT-9A ($p=0.020$) and WNT-16B ($p=0.023$) were significantly over-expressed, whereas WNT-2B ($p=0.033$), WNT-5A ($p=0.016$), WNT-7A ($p<0.0001$) and WNT-10A ($p<0.0001$) were down-regulated in B-ALL. Among the T-ALL subtype, however, significant down-regulation of WNT-2B, WNT-5B, WNT-7A, WNT-10A and WNT-11 was evident. Comparison between B-ALL subtypes showed significant over-expression of WNT-7B, WNT-9A and WNT-5B in certain subtypes. **Conclusion:** Our results suggest contribution of the WNT genes in leukemogenesis of ALL.

Keywords: Acute Lymphoblastic Leukemia, Immunophenotype, RT-PCR, WNT

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children (1), but, in adults, it represents 20% of all leukemias (2). ALL is a rapidly progressive disease that involves proliferating immature lymphocytes including B- or T-cell progenitors (3). Pathogenic mechanisms of this lymphoid malignancy are poorly understosod. The mechanisms underlying the induction of ALL include aberrant expression of proto-oncogenes, chromosomal translocations and hyperdiploidy (4,5).

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Wnt proteins act on target cells via frizzled receptors (Fz-R). Based on receptor mediated signaling pathways activated, they are categorized into classical and non-classical members (activating canonical and non-canonical intracellular signaling pathways, respectively) and are involved in a variety of biological functions such as stem cell biology, self-renewal, lineage commitment and lymphocyte development (6-9).
Stabilized β-catenin in the canonical Wnt pathway enters the cell nucleus and consequently activates transcription of some genes including regulators of cell growth and proliferation, modulators of cell death pathways and cell–cell communication (10,11). However, it has also been reported that the non-canonical Wnt/Ca^{++} pathway may inhibit the canonical pathway by suppressing cyclin-D1 expression and negatively regulating growth and survival of progenitor thymocytes and B lymphocytes in a cell-autonomous manner (12-14).
Many reports have demonstrated aberrant expression and activation of some Wnt molecules and their signaling components in different types of solid tumors and hematological malignancies (6,9,15-17). A number of studies have demonstrated epigenetic dysregulation of some WNT, WNT receptor and WNT inhibitor genes and also aberrant Wnt signaling in leukemic cells from ALL patients (18-21).
Due to the importance of Wnt signaling in leukemogenesis, more studies need to be performed to investigate the expression pattern of these molecules and their involvement in tumor initiation or progression. In the present study, the expression pattern of 14 members of the WNT gene family was investigated for the first time in B-ALL and T-ALL patients. We have compared the expression of these genes between patients and normal subjects and also between different subtypes of B-ALL to elucidate their potential role in leukemogenesis of ALL.

MATERIALS AND METHODS

Patients and Controls. Patient samples were obtained from bone marrow (BM) and/or peripheral blood (PB) of 71 newly-diagnosed ALL patients at Hematology and Oncology Clinics of Vali-Asr and Ali-Asghar hospitals (affiliated with Tehran University of Medical Sciences). Diagnosis of patients was based on cytomorphologic and immunophenotypic features of BM leukemic cells. Major clinical and laboratory findings of the ALL patients are listed in Table 1. Sampling was performed at first presentation before therapeutic intervention. The mean age of the ALL patients was 9.6 years (range: 6 month-41 years). For determination of the WNT gene expression baseline, heparinized PB samples were collected from 36 normal healthy donors (with a mean age of 20.4 and a larg range of 4-37 years).
This study was approved by the ethics committee of Tehran University of Medical Sciences and informed consent was obtained from patients or their parents.
Isolation of Leukemic and Normal Mononuclear Cells. Normal and leukemic mononuclear cells were isolated from BM and PB using Histopaque (Sigma, St. Louis, USA) density-gradient centrifugation, as described (22). Isolated cells were washed twice with RPMI-1640 medium (GIBCO, Paisley, Scotland) prior to RNA extraction.
Immunophenotypic Analysis of ALL Patients. After separation, the mononuclear cells were stained with a panel of fluorescent-conjugated monoclonal antibodies (mAbs) (DAKO, Glostrup, Denmark) specific for myeloid lineage (CD14 and CD33), B cell

lineage (CD10, CD19 and CD20), T cell lineage (CD3 and CD5) and lineage non-specific (CD45, HLA-DR and Tdt) cell surface markers, as described [23]. Sample analysis and data acquisition was performed by Flomax flow-cytometry analysis software (Partec, Nuremberg, Germany). The criterion for surface marker positivity was expression by at least 20% of the leukemia blast cell population after subtraction of background staining with isotype-matched conjugated mAbs of irrelevant specifity (23). Based on specific immunophenotypic profiles, the B-ALL patients were further classified into pro-B (HLA-DR$^+$, CD19$^+$, CD10$^-$, CD20$^-$), pre-B I (HLA-DR$^+$, CD19$^+$, CD10$^+$, CD20$^-$), pre-B II (HLA-DR$^+$, CD19$^+$, CD10$^+$, CD20$^+$), and immature/mature-B (HLA-DR$^+$, CD19$^+$, CD10$^-$, CD20$^+$) sub-types (23).

Isolation of Normal B-cells by Nanomagnetic Beads. B cells were isolated from PBMC of 5 normal subjects using MACS negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, as described (24). Enrichment was assessed by flow cytometry (before enrichment: 8-20%, after enrichment: >90%).

RNA Extraction and cDNA Synthesis. Total RNA was extracted from leukemic cells and normal PBMC using RNA-Bee reagent (TEL Test Inc, Texas, USA) according to the manufacturer's instructions. RNA integrity and quantity were checked by electrophoresis and spectrophotometry, respectively. First-strand cDNA was synthesized using 2-3 µg of total RNA (incubated at 70°C for 5 minute to get single stranded RNA) in 20 µl reaction mixture consisting of 4 µl 5X reaction RT buffer, 2 µl 10 mM dNTPs (Roche, Mannheim, Germany), 1 µl 20 pmol/µl random hexamer (N6) (Roche, Mannheim, Germany) and 1 µl M-Mulv reverse transcriptase (200 U/µl) (Fermentas, Moscow, Russia). The mixture was incubated at 40°C for 45 minutes, followed by 90°C for 5 minutes. The integrity of the cDNA was confirmed by amplifying the β-actin gene.

PCR Aanalysis. PCR amplification was performed using WNT and β-actin (as a housekeeping gene) specific primers (25,26). Briefly, 25 µl PCR reaction mixture was prepared using 2.5 µl 10x PCR buffer (CinnaGen, Tehran, Iran), 1-3 µl of 25 mM MgCl2 (CinnaGen) for WNT genes and 3.5 µl for β-actin, 1.5 µl dNTPs (10 mM, Roche), 0.7 µl of each primer (10 pmol/µl), 0.2 µl Taq-DNA polymerase (5 unit/ µl, CinnaGen) and 1 µl cDNA. PCR products for each subject were finally visualized by simultaneously running on a 1.5% agarose gel containing ethidium-bromide (Sigma, St. Louis, USA). After electrophoresis, images were taken by the gel documentation system (UVP, LMS-20E, USA). WNT and β-actin band densities were determined by Labworks 4.0 software (UVP, Upland, USA) and the ratio of the WNT band density to that of the β-actin was calculated for all patients and normal controls and multiplied by 100 (WNT/β-actin × 100). These ratios were defined as relative WNT gene expression values. A number of established cell lines were used as positive controls for set-up and optimization of RT-PCR of all WNT genes included in this study (25).

Statistical Analysis. All calculations were performed using the SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). For parametric and nonparametric data, quantitative differences of WNT expression in patients and normal subjects were analyzed using the student's *t* and Mann-Whitney U tests, respectively. p values of less than 0.05 were considered significant.

RESULTS

Immunophenotyping of Leukemic Cells. From 71 Iranian ALL patients, 54 (76%) were identified as B-ALL, 16 (22.6%) as T-ALL and one patient (1.4%) was mixed lineage. Further classification of B-ALL patients included pro-B (n=6), pre-B I (n=30), pre-B II (n=15) and immature/mature-B (n=3) (Table 1).

WNT mRNA Expression in BMMC or PBMC of ALL Patients and PBMC of Normal Subjects. In this study, expression levels of 14 WNT gene transcripts including WNT-1, WNT-2B, WNT-3, WNT-3A, WNT-4, WNT-5A, WNT-5B, WNT-6, WNT-7A, WNT-7B, WNT-9A, WNT-10A, WNT-11 and WNT-16B were investigated in BM or PBMC of 71 patients and PBMC of 36 healthy controls. Representative expression pattern of these WNT genes in a number of ALL patients and normal subjects is illustrated in Figure 1.

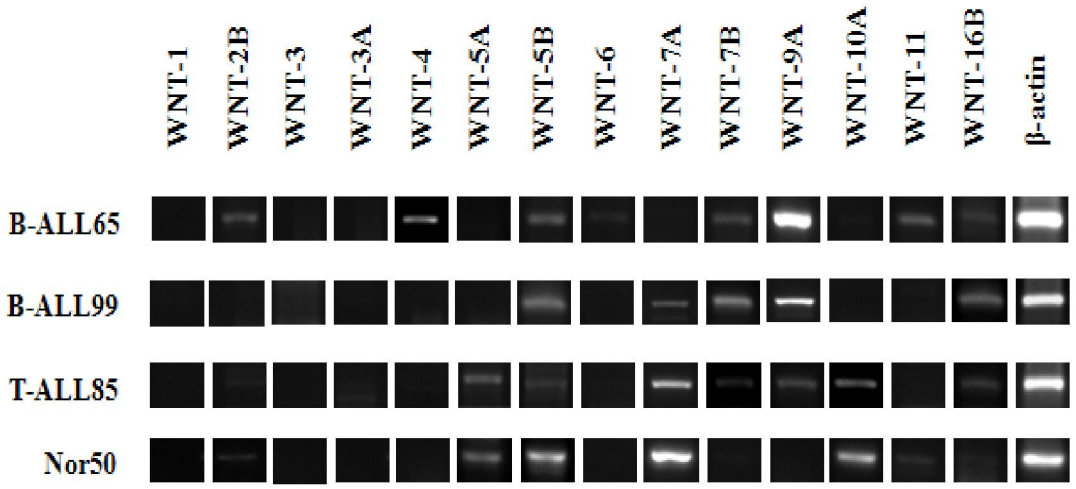

Figure 1. Representative RT-PCR results of WNT mRNA expression in B-ALL, T-ALL patients and healthy subjects.

Among the WNT genes, WNT-7B (p=0.026), WNT-9A (p=0.020) and WNT-16B (p=0.023) were significantly over-expressed and WNT-2B (p=0.033), WNT-5A (p=0.016), WNT-7A (p<0.0001) and WNT-10A (p<0.0001) were significantly down-regulated in B-ALL patients compared to normal subjects (Figure 2 and Table 2).

The results are expressed as the ratio of WNT to β-actin PCR product band density in ALL patients and normal subjects. Comparison of the relative expression levels of these WNT genes between B-ALL and normal controls demonstrate significant down-regulation of WNT-2B (p=0.033), WNT-5A (p=0.016), WNT-7A (p<0.0001) and WNT-10A (p<0.0001) and significant over-expression of WNT-7B (p=0.026), WNT-9A (p=0.02) and WNT-16B (p=0.023) genes. Also Comparison of the relative expression levels of these WNT genes between T-ALL and normal controls show significant down-regulation of WNT-2B (p=0.004), WNT-5B (p=0.047), WNT-7A (p<0.0001), WNT-10A (p<0.0001) and WNT-11 (p=0.001). Horizontal lines represent mean expression of each WNT gene in PBMC of normal subjects. Among the T-ALL patients,

however, down-regulation of WNT-2B (p=0.004), WNT-5B (p=0.047), WNT-7A (p<0.0001), WNT-10A (p<0.0001) and WNT-11 (p=0.001) was evident (Figure 2 and Table 2)**.** Comparison of the B-ALL and the T-ALL subtypes revealed no difference with the exception of WNT-11 which was over-expressed (p=0.041) in the B-ALL patients (Figure 2 and Table 2). Also comparison between B cell and non-B cell fractions from 5 normal samples with their PBMCs has shown no significant difference of any WNT mRNA expression (data not shown).

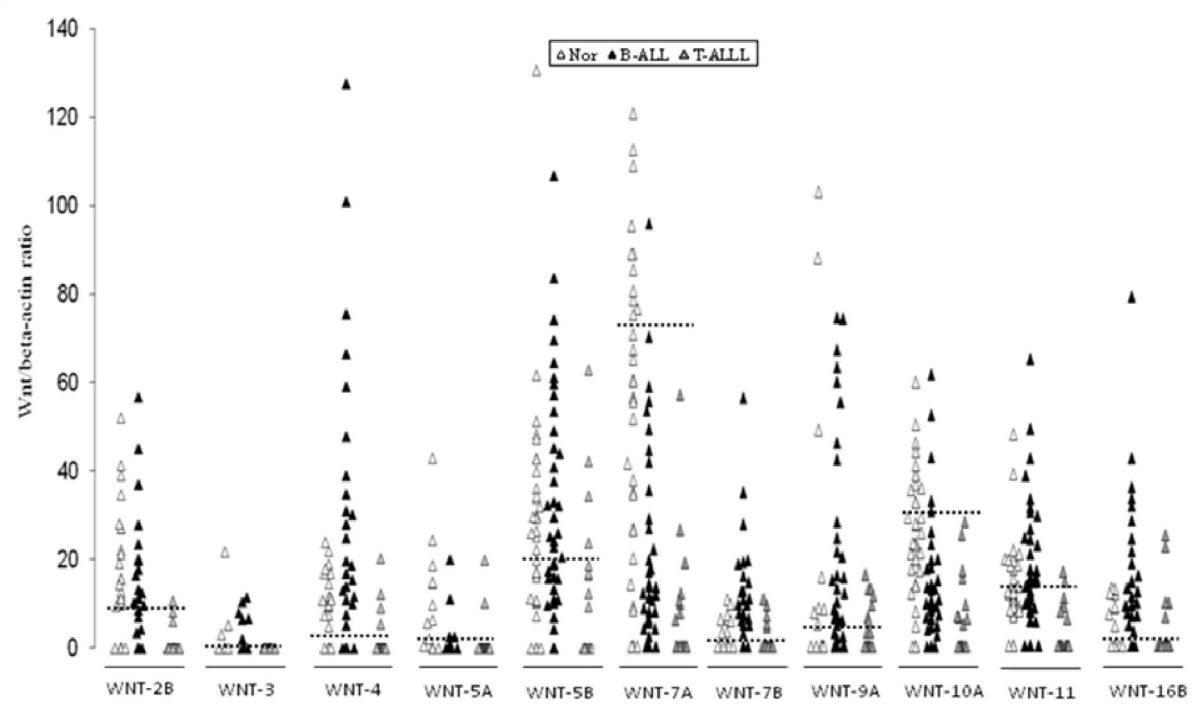

Figure 2. Relative expression of WNT mRNA in B-ALL, T-ALL patients and healthy subjects.

WNT mRNA expression in different subtypes of B-ALL patients. Comparison of B-ALL subgroups demonstrated significant over-expression of WNT-7B (p=0.003) and WNT-9A (p=0.044) in pre-B I versus pre-B II leukemic cells and WNT-5B in immature/mature-B versus pre-B I (p=0.023) and pre-B II (p=0.046) leukemic cells (Figure 3 and Table 2). Also, there were no substantial differences in WNT mRNA expression between FAB classification subgroups.

Correlation Analyses of WNT mRNA Expression with Some Clinical and Laboratory Characteristics of ALL Patients. Among the different clinical and laboratory findings of patients listed in Table 2, we only observed correlation between WNT-10A (r= -0.44, p=0.001) and WNT-11 (r= -0.466, p<0.0001) and white blood cell (WBC) count (Figure 4(A and B)), between WNT-11 (r= -0.45, p=0.005) and BM immature cell percentage (Figure 4(C)) and between WNT-7B (r= 0.411, p=0.004) and red blood cell (RBC) count (Figure 4(D)) only in B-ALL patients.

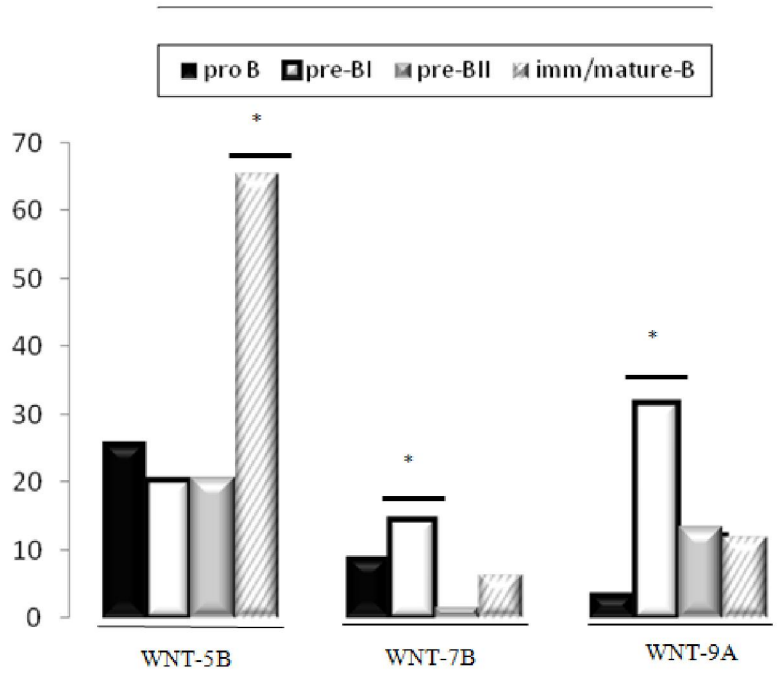

Figure 3. Relative expression levels of WNT-5B, WNT-7B and WNT-9A in leukemic cells of different B-ALL subtypes. The results demonstrate significant over-expression of WNT-7B (p=0.003) and WNT-9A (p=0.044) in pre-B I (n=30) versus pre-B II (n=15) leukemic cells and WNT-5B in immature/mature-B (n=3) versus pre-B I (p=0.023) and pre-B II (p=0.046) leukemic cells.

DISCUSSION

The Wnt signaling pathway is an evolutionally conserved mechanism that is crucial for development of T- and B-lymphocytes and has recently been implicated in the regulation of hematopoiesis (6,15,27,28). Aberrant activation of the Wnt/β-catenin signaling pathway can qualify to fit into the category of one of the hallmarks of cancer and hematological malignancies (6,17,29-31) and can be used as a therapeutic target for tumor suppression (32). Exogenous Wnt proteins supply proliferative and survival signals to ALL cells *in vitro* and are potential downstream targets of the Wnt/β-catenin signaling pathway (19). Also, it has been demonstrated that down-regulation of multiple Wnt signaling inhibitor genes in ALL cell lines and tumors due to promoter methylation, lead to the activation of Wnt signaling which is associated with poor prognosis in ALL (18,20,33,34). Thus, Wnt molecules could potentially play key roles in etiopathology of ALL. Since the leukemic cells of ALL patients are in the proliferative phase (3) and inhibition of Wnt signaling is known to reduce proliferation and mediate apoptosis of ALL cells (18,19,35), Wnt signaling pathways may play an important role in ALL tumorigenesis. There are few expression studies of individual WNTs in B-ALL patients which demonstrate over-expression of WNT-16B (36) and down-regulation of WNT-5A (12,37). Only one study has explored expression of 5 WNT mRNAs in 12 ALL patients in whom mRNA expression of WNT-2B (33%), WNT-5A (42%), WNT-10B

(58%), and WNT-16B (25%) was observed, but WNT-3A mRNA expression was not observed in any of the cases (19). In the present study, we showed elevated and reduced expression of various WNT mRNAs in 71 ALL patients consisting of T-ALL and B-ALL belonging to different immunophenotypic subtypes, as compared to 36 healthy age-matched subjects. We also compared expression levels of WNTs between children and adults to find any age-related differences. No significant difference was observed between these two groups, indicating a lack of association of WNT gene expression with age between patients and healthy controls (data not presented).

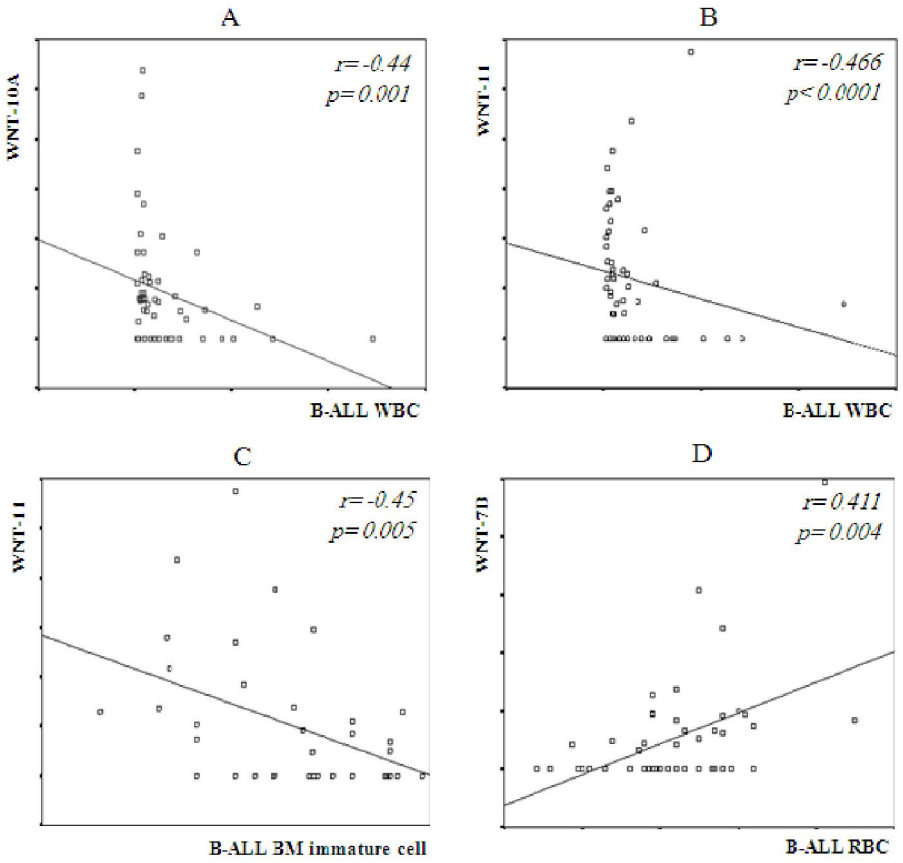

Figure 4. Correlation analysis of WNT mRNA expression with some laboratory findings of B-ALL patients. The results demonstrate correlation between expression of (A) WNT-10A (r= -0.44, p=0.001) and (B) WNT-11 (r= -0.466, p<0.0001) with peripheral blood WBC number, (C) WNT-11 (r= -0.45, p=0.005) with bone marrow immature cells percent and (D) WNT-7B (r= 0.411, p=0.004) with peripheral blood RBC number.

There are some important technical issues regarding our semi-quantitative RT-PCR assay which deserve special consideration. In this study, representation of WNT expression as a ratio to the housekeeping gene β-actin was considered to normalize the data and avoid variations due to differences in cDNA concentration or differences in amounts of PCR product loaded on the electrophoresis gel. In addition, simultaneous electrophoresis of WNT and β-actin PCR products for each subject on the same gel would minimize variations due to technical shortcomings. Although real-time PCR is

more demanding for quantitative measurement of gene expression, our semi-quantitative RT-PCR method has been shown to be a reliable and sensitive method for detection of a variety of tumor-associated markers (38-40). Unfortunately, we were not able to employ real-time PCR in this study due to instrumental limitations.

Some Wnt molecules (such as Wnt-5a, Wnt-5b and Wnt-11) can initiate the non-canonical signaling pathway with negative feed-back on the canonical pathway (41-43), thus acting as tumor suppressor molecules to inhibit leukemic cell growth and survival (12,44-46). Wnt-5a, through the non-canonical pathway, can suppress cyclin D1 and c-myc expression (through repression of β-catenin activation) and negatively regulate B-cell proliferation. Also, Wnt-5a down-regulation was observed in B-ALL and acute myeloid leukemia (12,25,42,47). On the other hand, high levels of endogenous β-catenin have also been detected in T-ALL and myeloid leukemias (48). Recent study showed WNT-5A promoter methylation and consequently low mRNA expression in leukemic cells and also demonstrated that increased Wnt-5a protein expression can inhibit malignant proliferation and arrest cell cycle in a leukemic cell line (21). Therefore, down-regulation of WNT-5A in B-ALL and that of WNT-5B and WNT-11 in T-ALL leukemic cells of our patients probably have a role in activation of the canonical signaling pathway and consequently in lymphoid leukemogenesis. Also, an inverse correlation of WNT-11 with PB WBC count and with BM immature cell percentage of our patients (Figure 4(B and C)) could confirm canonical pathway repression by Wnt-11 and magnify the role of the WNT signaling pathway in ALL patients.

Stimulation of non-canonical pathway by Wnt-7a has also been demonstrated in non-small cell lung cancer (49) and so it's down-regulation in leukemic cells of ALL patients possibly leads to activation of β-catenin in lymphoid progenitors.

WNT-2B and WNT-10A are significantly down-regulated in both T- and B-ALL samples. Wnt-2B could act as a growth factor in neural or retinal and hematopoietic progenitor cells and also in many tumors (50,51). Wnt-10a can stimulate human progenitor B-cell growth (6) and contrary to our data, it's up-regulation was demonstrated in human gastric cancers (52) and leukemic CLL cells (53,54). Thus, down-regulation of WNT-2B and WNT-10A in our ALL patients may be due to compensatory mechanisms for repression of the canonical pathway in leukemic cells. On the other hand, as *Mikels et al*. have shown, Wnt-5a can activate both the canonical and non-canonical signaling pathways depending on differential cellular Fz receptor expression pattern (13). So, according to the inverse correlation of WNT-10A mRNA with PB WBC count of our patients (Figure 4(A)), this molecule, like Wnt-5a, probably could activate the non-canonical pathway in leukemic cells and its down-regulation may stimulate β-catenin signaling pathway in ALL patients. In line with this, *Thiago et al.* have recently shown that Wnt-5A is not expressed in tumor cells from any of five B-ALL patients as well as two B-ALL cell lines investigated (55). They also demonstrated that addition of Wnt-5a protein induces β-catenin suppression leading to increased survival rate (with no effect on proliferation) of Nalm-16, but not the Nalm-6 B-ALL cell line.

We have previously reported elevated levels of WNT-7B, WNT9A, and WNT-16B mRNA in CLL patients (56) and in this study we have provided similar data for B-ALL patients. Similarly, WNT-7B can be up-regulated in multiple myeloma (57) and WNT-9A can be up-regulated in CLL tumor cells (53). Both WNT-7B and WNT-9A can activate the canonical signaling pathway (58,59). WNT-16B gene has already been shown to enhance cell proliferation and survival in pre-B ALL cells and its expression in these

cells are enhanced by the chromosomal translocation *t* (1;19) product (E2a-Pbx1). WNT-16 targeting by monoclonal antibodies or siRNA was found to reduce the signaling activity of Wnt molecules and to induce apoptosis in these cell lines (35,36,60). Thus, over-expression and activation of these genes seems to be of particular importance in B-cell malignancies.

Expression pattern of WNT genes in B- and T-ALL shows a very similar pattern with a difference in one gene,i.e. WNT-11 (Figure 2 and Table 2), suggesting a common tumorigenic function of the Wnt signaling pathway in these two subtypes of ALL. On the other hand, it was revealed that patients with T-ALL had a higher risk for relapse than B-ALL patients (61) and there is also an association between WNT-11 expression and T-ALL poor prognosis (62). Therefore, down-regulation of WNT-11 could have a role in disease progression and relapse in T-ALL patients probably via suppression of the non-canonical signaling pathway.

B-ALL subgroups demonstrated dissimilar expression of some WNT mRNAs. Significant over-expression of WNT-7B and WNT-9A in pre-B I versus pre-B II leukemic cells and WNT-5B in immature/mature-B versus pre-B I and pre-B II leukemic cells was observed (Figure 3 and Table 2). This preferential WNT gene expression in different subtypes of B-ALL may be related to the differentiation stage of the leukemic B-cell.

Investigation of protein expression, function and interaction with target receptors of Wnt molecules in different subtypes of ALL, may highlight many unresolved issues regarding these molecules and their roles in different stages of tumor development and pathogenesis of ALL. Finally, inhibition of these molecules or their signaling components by monoclonal antibodies or siRNA may prove to be a useful tool for identification of mechanisms underlying leukemogenesis or as immunotherapeutic intervention strategy.

ACKNOWLEDGMENTS

We would like to thank Professor Mahmood Jeddi-Tehrani and Dr. Hodjatallah Rabbani for critical consultations. This study was supported in part by a grant from Tehran University of Medical Sciences.

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