

# Expression Profile of Wnt Molecules in Leukemic Cells from Iranian Patients with Acute Myeloblastic Leukemia

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## ABSTRACT

**Background:** Wnt molecules play a key role in growth, proliferation and development of some embryonic and adult organs as well as hematopoietic stem cells. Wnt signaling pathways are aberrantly activated in many tumor types, including solid tumors and hematologic malignancies. **Objective:** To investigate the expression profile of a large number of Wnt genes in leukemic cells from Iranian patients with acute myeloblastic leukemia. **Methods:** RT-PCR method was used to determine the Wnt genes expression in bone marrow (BM) and/or peripheral blood (PB) samples from 16 patients with AML and PB samples of 36 normal subjects. **Results:** Among 14 Wnt molecules included in this study, Wnt-7A and Wnt-10A were significantly down-regulated ( $p = 0.002$  and  $p < 0.0001$ , respectively) and Wnt-3 was significantly over-expressed ( $p < 0.02$ ) in AML patients compared to normal subjects. No significant association was found between Wnt expression and FAB classification of the patients. **Conclusion:** Our results demonstrated for the first time aberrant expression of Wnt-7A, Wnt-10A and Wnt-3 genes in Iranian AML patients. This may be of relevance to the tumorigenesis process in this malignancy.

**Keywords:** Wnt,  $\beta$ -catenin, Acute Myeloblastic Leukemia, RT-PCR

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## INTRODUCTION

Acute myeloblastic leukemia (AML) is the most common type of acute leukemia in adults and its incidence increases with age (1). AML is characterized by the abnormal proliferation of myeloid precursor cells, decreased rate of self-destruction, resistance to apoptosis and an arrest in cellular differentiation (2, 3). The leukemic cells of these patients are highly heterogenous and the mechanisms of tumorigenesis are poorly understood.

Wnt molecules, derived from names of two genes *Drosophila wingless* and mouse *Int1*, are 19 cysteine-rich secreted glycoproteins serving as extra-cellular signaling molecules and play significant roles in normal and malignant developmental processes (4, 5). Wnt proteins act on target cells via frizzled receptors (Fz-R) based on receptor mediated signaling. The Wnt family is biologically categorized into two classes including Wnt-1 (classical Wnts) and Wnt-5A (non-classical Wnts), which usually activate canonical and non-canonical intracellular signaling pathways, respectively (4, 6-8). Canonical signaling pathway leads to increase in cytoplasmic level of  $\beta$ -catenin and activation of downstream signaling molecules (9). Non-canonical signaling pathway leads to intracellular  $Ca^{2+}$  flux (it activates  $Ca^{2+}$  dependent effector enzymes such as PKC and CamkII) or JNKs (jun amino-terminal kinase) activation which can suppress canonical signaling cascade (10, 11). Recently, Wnts have drawn attention as a set of factors operating in embryonic development, growth regulation of adult tissues and cancer formation (12). Some Wnts and their receptors have essential roles in hematopoietic stem cell survival as well as T and B cell development (4, 9, 12).

Different expression profiles of some Wnt genes and their related signaling molecules were reported in different types of solid tumors and hematological cancers (9, 13-16). In addition, it has been shown that Wnt-5A heterozygous mice are highly susceptible to develop B cell lymphoma and chronic myeloid leukemia (CML) (8). In AML patients, it is confirmed that  $\beta$ -catenin expression is rapidly lost upon myelomonocytic differentiation (17).

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 profile of a large number of the Wnt family members was investigated for the first time in a group of Iranian patients with AML.

## MATERIALS AND METHODS

**Patients and Normal Controls.** Twenty-three preservative-free heparinized fresh bone marrow (BM) and/or peripheral blood (PB) samples (7 paired BM and PB, 6 BM and 3 PB) were obtained from 16 newly diagnosed AML patients at 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. Diagnosis of AML was based on cytomorphologic (FAB criteria) and immunophenotypic features of BM and PB leukemic cells (data not shown). Major clinical and laboratory findings of the patients are listed in Table 1. Sampling was performed at first presentation before therapeutic intervention. The mean age of the patients was 11.3 years (range: 4 months - 33 years). For determination of the Wnt genes expression baseline, heparinized PB samples were collected from 36 normal healthy donors with a mean age of 20.4 years (range: 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.

**Table 1. Major clinical and hematological findings in AML patients**

Patient	Age (year)	Sex	FAB	WBC × (10 <sup>9</sup> /L)	RBC × (10 <sup>12</sup> /L)	HB (g/dl)	PLT × (10 <sup>9</sup> /L)
AML-1	7	M	NA	5.8	2.9	9.1	75
AML-2	23	F	M2	122.3	3.2	9.4	43
AML-4	2	M	NA	13.1	3.1	9.4	9.0
AML-6	3	M	M0	7.8	2.95	9.0	31
AML-7	4(m)*	F	M4-M5	157.8	2.2	7.0	37
AML-11	12	F	M2	10.7	2.1	7.0	31
AML-13	17	M	M5	85.4	3.1	8.8	22
AML-20	21	M	M4	18.5	3.1	9.3	17
AML-23	12	F	M3	1.0	3.6	9.8	34
AML-27	8	F	M1	3.2	2.6	7.9	<10
AML-30	12	M	M0	2.1	NA	8.6	104
AML-64	10	M	M1	1.3	2.7	7.2	21
AML-66	33	F	M3	NA	NA	NA	NA
AML-72	13	F	M3	8.7	2.3	7.6	45
AML-83	1	F	M3	226	3.15	9.5	15
AML-94	6	M	M2	187	2.91	6.2	39

Abbreviations: FAB: French-American-British staging system; WBC: white blood cell count; RBC: red blood cell count; HB: hemoglobin; PLT: platelet count; NA: Not available; M: male; F: female; (m)\*: month

**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 (18). Isolated cells were washed twice with RPMI-1640 medium (GIBCO, Paisley, Scotland) prior to RNA extraction.

**Cell Lines.** A number of cell lines were used as positive controls for RT-PCR optimization (19-23) (Table 2). All cell lines were provided by the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran). Pooled leukemic B-cells obtained from 5 chronic lymphocytic leukemia (CLL) patients were employed as positive control to detect Wnt-3, Wnt-5B and Wnt-16b (13). The cells were adapted to grow in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, Seromed Biochrome, Germany), L-glutamine (2 mM), Penicillin (100 U/ml) and streptomycin (100 µg/ml, all from Sigma, St. Louis, USA).

**Table 2. List of cell lines and samples employed as positive controls**

Cell line	NCBI code*	Species	Tissue	Wnt genes	Reference
MCF-7	C135	Human	Breast	Wnt-1, -3A	19
DU-145	C428	Human	Prostate	Wnt-2B, -7B, -9A, -11	20
LN-CAP	C439	Human	Prostate	Wnt-7A	20
A-431	C204	Human	Skin	Wnt-4	21
RAJI	C127	Human	Hematopoietic	Wnt-5A, -10A	22
C2C12	C521	Mouse	Muscle	Wnt-6	23
-----	-----	Pooled-CLL**	Hematopoietic	Wnt-3, -5B, -16	13

\* NCBI code: National Cell Bank of Iran code number given to each cell line

\*\*Pooled leukemic cells obtained from 5 B-CLL patients were used as positive control

**RNA Extraction and cDNA Synthesis.** Total RNA was extracted from leukemic cells and normal PBMCs using RNA-Bee reagent (TEL Test Inc, Texas, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using 2-3 µg of total 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.

**Polymerase Chain Reactions (PCR).** PCR amplification was performed using Wnts and β-actin (a housekeeping gene used as an internal control) specific primers (12, 24) (Table 3). Briefly, 25 µl PCR reaction mixture was prepared using 2.5 µl 10x PCR buffer (CinnaGen, Tehran, Iran), 1-3 µl of 25 mM MgCl<sub>2</sub> (CinnaGen) for Wnt genes and 3.5 µl for β-actin, 1.5 µl dNTPs (10 mM, Roche), 0.7 µl each primer (10 pmol/µl), 0.2 µl Taq-DNA polymerase (5U/µl; CinnaGen, Tehran, Iran), and 1 µl cDNA. PCR conditions for amplification of Wnt and β-actin genes are shown in Table 3.

**Table 3. List of primer sequences and PCR conditions employed**

Wnt gene	Primer sequences (5'to 3')	Amplicon sizes (bp)	PCR conditions	References
Wnt-1	<u>S:</u> TGCCTCTCTCAAAGGTGCGTACC <u>AS:</u> CCAGAAAAGTGGGGTCTTGGGCT	503	Cycle: 37 AT <sub>M</sub> : 61.5	*
Wnt-2B	<u>S:</u> AGACACGTCCTGGTGGTACATTGG <u>AS:</u> AAACCGCCGCACAGCCGTGCGAC	529	Cycle: 38 AT <sub>M</sub> : 60	*
Wnt-3	<u>S:</u> CTGGCTACCCAATTTGGTGGT <u>AS:</u> CATCTATGGTGGTGCAGTTCCA	225	Cycle: 37 AT <sub>M</sub> : 58.5	12
Wnt-3A	<u>S:</u> TACCCGATCTGGTGGTTCGCTGGCT <u>AS:</u> CGACTCCCTGGTAGCTTTGTCCAG	270	Cycle: 38 AT <sub>M</sub> : 60	*
Wnt-4	<u>S:</u> CCGCGAGCAACTGGCTGTACCTG <u>AS:</u> TGACCACTGGAAGCCCTGTGGGCT	398	Cycle: 37 AT <sub>M</sub> : 61.5	*
Wnt-5A	<u>S:</u> CCCTCGCCATGAAGAAGTCCATTG <u>AS:</u> CGTCTCGCGCTGCCATCTGCAT	413	Cycle: 38 AT <sub>M</sub> : 60	*
Wnt-5B	<u>S:</u> CGCCAACCTCTGGTGGTTCATTAGC <u>AS:</u> CGGTCTCTCGGCTGCCTATCTGCA	278	Cycle: 38 AT <sub>M</sub> : 60	*
Wnt-6	<u>S:</u> GCTCCAGCCACAGCAAGG <u>AS:</u> CAGCCTGCCCGCTCGTT	377	Cycle: 37 AT <sub>M</sub> : 58.5	12
Wnt-7A	<u>S:</u> CTACCTCCGGATCGGTGGCTTCTC <u>AS:</u> GTTCTCTCCAGGATCTTTCGGCC	529	Cycle: 38 AT <sub>M</sub> : 60	*
Wnt-7B	<u>S:</u> CAAGAGCTCCGAGTAGGGAGCCGT <u>AS:</u> TCCGGTCTCTAGAACCTTCTGTC	304	Cycle: 38 AT <sub>M</sub> : 60	*
Wnt-9A	<u>S:</u> AAGTACAGCAGCAAGTTCGTCAAGG <u>AS:</u> GCACTCCACATAGCAGCACCAAC	537	Cycle: 37 AT <sub>M</sub> : 61.5	12
Wnt-10A	<u>S:</u> ACACAGTGTGCTAACATTGCC <u>AS:</u> AGGCCTTCAGTTTGCCAG	296	Cycle: 38 AT <sub>M</sub> : 60	12
Wnt-11	<u>S:</u> TGGCATCAAGTGGCTGGCGCTGTC <u>AS:</u> CGACTCCCGGGTCCCTCTCTCCA	265	Cycle: 37 AT <sub>M</sub> : 61.5	*
Wnt-16	<u>S:</u> CCCAAGGAAACTGGATGTGGTTGG <u>AS:</u> ATCAACTTGGCGACAGCCTGCCTT	571	Cycle: 38 AT <sub>M</sub> : 60	*
β-actin	<u>S:</u> CCTTCTGGGCATGGAGTCCTG <u>AS:</u> GGAGCAATGATCTTGATCTTC	203	Cycle: 29 AT <sub>M</sub> : 55	24

Abbreviations: S= Sense; AS= Anti-sense; bp= base pair; AT<sub>M</sub>= Annealing Temperature °C

\* = These primers were designed in this study

PCR products were finally visualized by running 1.5% agarose gel electrophoresis containing ethidium-bromide (10µg/ml; Sigma, St Louis, USA). Wnt and β-actin PCR products of each sample were simultaneously run in a single gel to minimize variation between gels. After electrophoresis, images were taken by gel documentation system (UVP, LMS-20E, USA). Wnt and β-actin band densities were determined by Lab works 4.0 software (UVP, Upland, USA), and the ratios of Wnt/β-actin were calculated for each sample. To obtain values higher than one, these ratios were multiplied by 100 and defined as relative expression of Wnt genes.

**Statistical Analyses.** All calculations were performed using the SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Quantitative differences of Wnt band densities in patients and normal subjects were analysed using the Mann-Whitney U test. The Exact Chi-Square test was applied to determine the qualitative differences. P-values of less than 0.05 were considered significant.

## RESULTS

The ratio of each Wnt PCR product to  $\beta$ -actin band densities were calculated for each sample and compared with those obtained for normal subjects. To determine the baseline expression level of each Wnt gene in normal samples, PBMC from 36 normal subjects were tested. The mean ratio of each Wnt to  $\beta$ -actin band densities was calculated for normal samples to obtain an arbitrary cut-off value for qualitative analyses of the Wnt gene expression in patients' samples. Values greater or less than the baseline level were taken to imply either over-expression or down-regulation of a given Wnt gene in the tested samples. Among 14 Wnt molecules tested, Wnt-3 was significantly over-expressed (Figure 1) ( $p = 0.013$ ), whereas Wnt-7A and Wnt-10A were significantly down-regulated (Figures 2 and 3) ( $p = 0.002$  and  $p < 0.0001$ , respectively) in leukemic cells of AML patients (Figure 4 and Table 4). A significant correlation was observed between Wnt-3 expression and leukocyte count ( $r = 0.697$ ,  $p = 0.004$ ) as well as Wnt-11 expression and hepatomegaly ( $r = - 0.652$ ,  $p = 0.028$ ) in our patients. The expression profile of the remaining Wnt genes was similar in patients and normal samples (Table 4). No other significant associations were found among Wnt genes expression and FAB classification or other laboratory and clinical findings of the patients (data not presented).

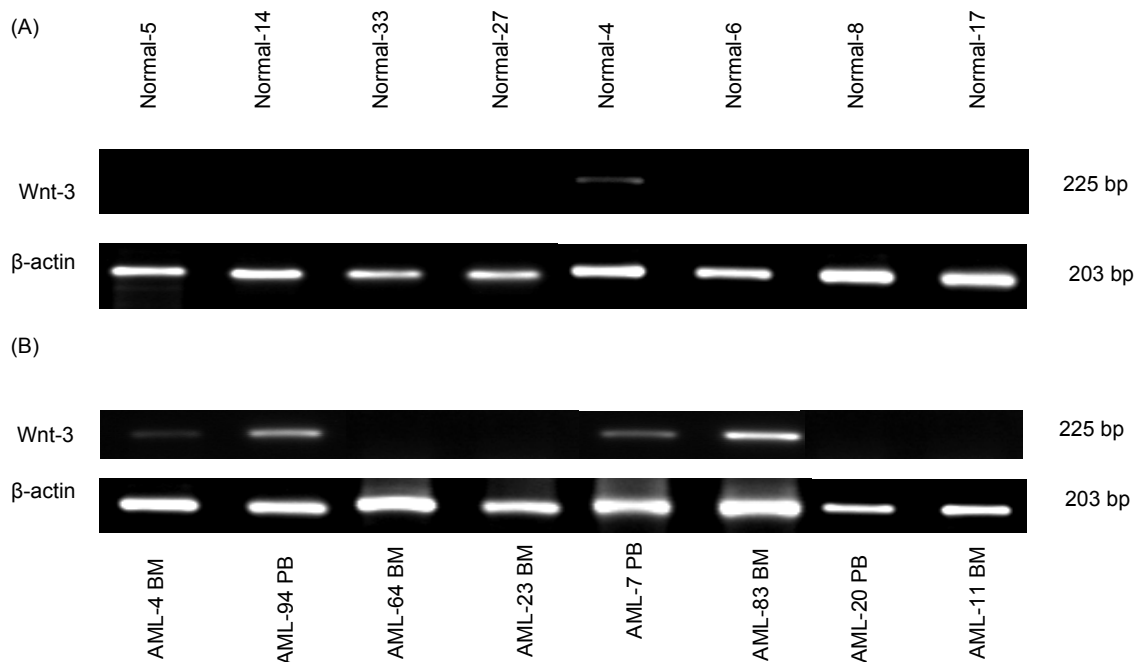
**Table 4. Frequency and ratio of Wnt gene expression in AML patients and normal subjects**

Gene	Mean Ratio*		Frequency (%)**		P-value	
	Normal	AML	Normal	AML	Qualitative	Quantitative
Wnt-1	0	0	0% (0/36)	0% (0/16)	1	1
Wnt-2B	12.1	8.4	38.9%(14/36)	31.3% (5/16)	0.28	0.299
Wnt-3	0.6	3.6	2.8% (1/36)	25% (4/16)	0.046	0.013
Wnt-3A	0	3.2	0% (0/36)	6.2% (1/16)	0.31	0.134
Wnt-4	5.9	7.5	44.5%(16/36)	56.2% (9/16)	0.19	0.402
Wnt-5A	3.5	0	19.5% (7/36)	0% (0/16)	0.058	0.061
Wnt-5B	26.3	21.4	44.5%(16/36)	31.3% (5/16)	0.15	0.217
Wnt-6	0	0.8	0% (0/36)	6.2% (1/16)	0.31	0.134
Wnt-7A	87.8	23.3	55.6%(20/36)	6.2% (1/16)	0.001	0.002
Wnt-7B	3	7.9	25% (9/36)	37.5% (6/16)	0.16	0.146
Wnt-9A	14.6	3.2	16.7% (6/36)	6.2% (1/16)	0.21	0.814
Wnt-10A	42.1	9.8	41.7%(15/36)	6.2% (1/16)	0.004	<0.0001
Wnt-11	23.7	33.1	41.7%(15/36)	50% (8/16)	0.24	0.921
Wnt-16b	3.8	4.3	22.2% (8/36)	18.8% (3/16)	0.45	0.76

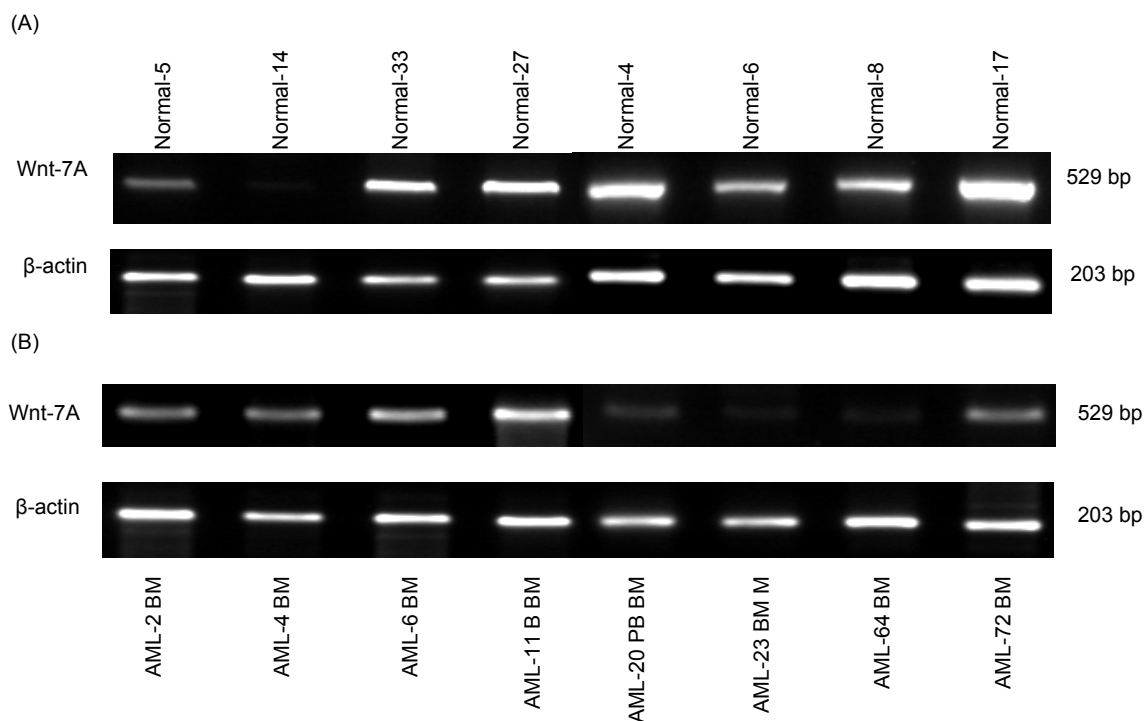
\*The ratio of expression of each Wnt gene was calculated by dividing the PCR amplicon band density of the selected Wnt gene to that of the  $\beta$ -actin of the same subject.

\*\*Frequency denotes percentage of normal subjects or patients displaying a ratio of Wnt to  $\beta$ -actin band density greater than the arbitrary cut-off level assigned for normal subjects.

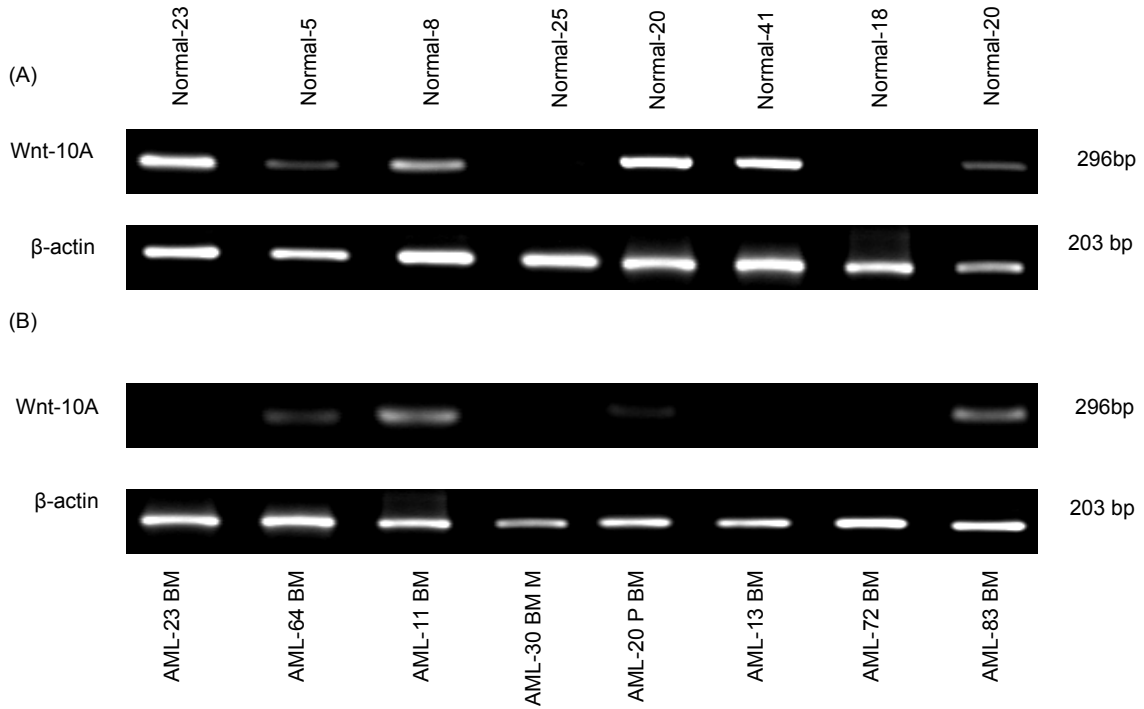
Expression profile of Wnt molecules in AML



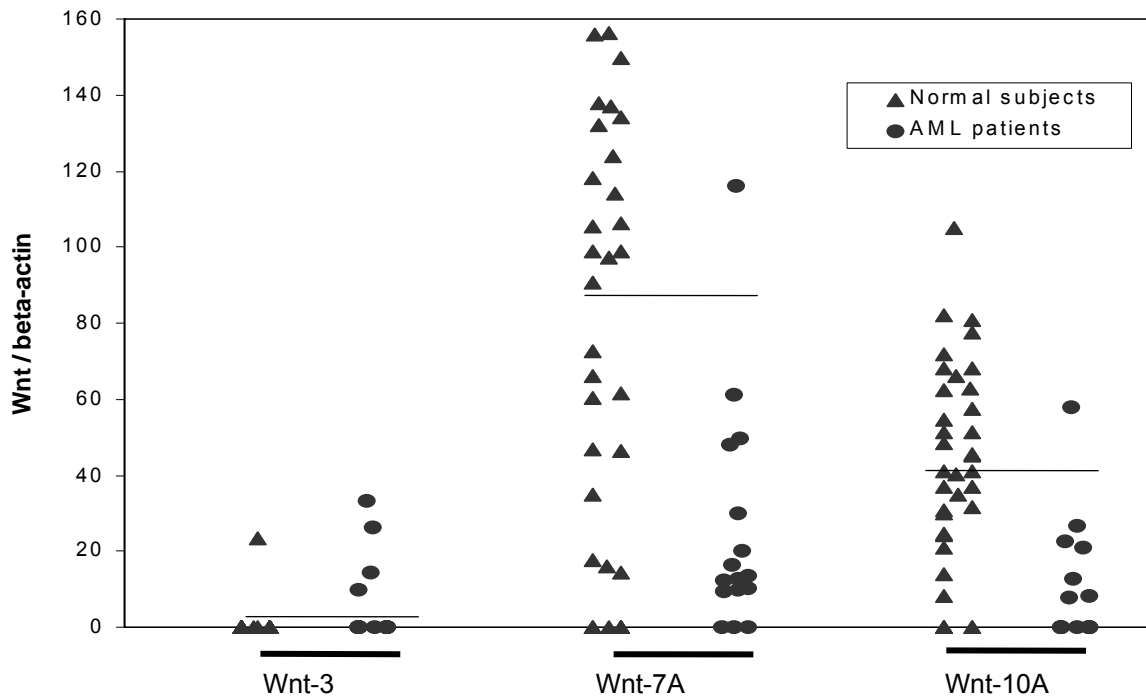
**Figure 1.** Representative RT-PCR results obtained for Wnt-3 mRNA expression in eight normal subjects (A) and AML patients (B). The results obtained for β-actin are shown for each sample.



**Figure 2.** Representative RT-PCR results obtained for Wnt-7A mRNA expression in eight normal subjects (A) and AML patients (B). The results obtained for β-actin are shown for each sample.



**Figure 3.** Representative RT-PCR results obtained for Wnt-10A mRNA expression in eight normal subjects (A) and AML patients (B). The results obtained for β-actin are shown for each sample.



**Figure 4.** Relative expression of Wnt-3, Wnt-7A and Wnt-10A mRNA in PBMCs of normal subjects (n=36) and leukemic cells of AML patients (n=16). Horizontal lines show mean expression of each Wnt gene in normal subjects.

## DISCUSSION

Wnt signaling is an important regulator of developmental pathways, thus abnormalities in these signals may contribute to tumor formation and leukemogenesis (25-28). Due to their involvement in different signaling pathways, some Wnt molecules can induce cell proliferation via canonical pathway, while others may repress the effect of their own induced proliferation via non-canonical pathway. Different effects of Wnt molecules on target cells depend on receptor context which may explain controversial effects of a single Wnt molecule under different conditions (11, 29).

Since limited studies have previously been performed on Wnt gene expression in AML, we investigated the expression profile of 14 members of Wnt gene family in a number of Iranian AML patients. Presentation of Wnt expression as a ratio of the expression of a housekeeping gene,  $\beta$ -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 gels. Further caution was taken by simultaneous electrophoresis of Wnt and  $\beta$ -actin PCR products of each subject on the same gel. This would minimize variations due to technical shortcomings.

The results demonstrated significant over-expression of Wnt-3 in leukemic cells ( $p < 0.02$ ) (Figure 4 and Table 4), which is in agreement with the correlation between Wnt-3 expression and leukocytosis ( $r = 0.697$ ,  $p = 0.004$ ) in our patients. There are several studies showing over-expression of  $\beta$ -catenin signaling pathway in AML patients (28, 30, 31). Since exposure to Wnt-3 leads to activation of canonical signaling pathway followed by stabilization and accumulation of  $\beta$ -catenin, therefore our results are in agreement with those of others (28, 30, 31) regarding activation of canonical signaling pathway and probably induction of leukemic cells proliferation in AML patients. In addition to AML, Wnt-3 is over-expressed in a subset of other malignancies such as CLL (13), breast cancer and also tumor cell lines such as A549 cells (lung cancer) and MKN45 cells (gastric cancer) (32). Up-regulation of Wnt-3 in various tumor types indicates that the  $\beta$ -catenin signaling pathway might have a crucial role in tumor pathogenesis and oncogenesis.

We have also demonstrated that Wnt-7A and Wnt-10A were significantly down-regulated in our AML patients ( $p = 0.002$  and  $p < 0.0001$ , respectively). Down-regulation of these genes may lead to the activation of the canonical signaling pathway. There is no data about suppressive effects of these two Wnt molecules, but probably non-canonical signaling pathway can be initiated after exposure of leukemic cells to Wnt-7A and Wnt-10A (like Wnt-5A and Wnt-11; (33)) and these effects are highly associated to cellular receptor context (11). Contrary to our findings, over-expressed levels of Wnt-7A and Wnt-10A have been reported in several cell lines derived from solid tumors (34), as well as fresh leukemic cells from patients with B-CLL (13), respectively. Different expression patterns of these molecules in various types of cancer may be due to the heterogeneity of cancers and also different mechanisms involved in tumorigenesis. Additional studies should be performed to resolve these controversial issues.

In this study, we also found decreased levels of Wnt-5A in AML patients compared to normal subjects, though the difference did not reach statistical significance, perhaps due to the small sample size ( $p = 0.061$ ). Down-regulation of Wnt-5A has been reported in B-ALL and AML patients, in concordance with over-expression of cyclin-D1 and c-myc (8, 29). Furthermore, up-regulation of Wnt-5A has been observed in some human solid tumors (8). Other controversial findings have also been reported for Wnt-1 and



Wnt-2B. Although we observed a similar representation of these two molecules in our AML patients and normal subjects (Table 4), up-regulated levels of both molecules has recently been reported in AML patients (30). Different parameters such as age, ethnicity and methodological aspects may contribute to these controversial findings. The mean age of patients in Simon's study (30) was 53.7 years compared to 11.3 years in our study. Considering the high prevalence of childhood AML in Asian populations compared to Western patients (35), the discrepancy may be related to heterogeneity and differences between childhood and adult AML and also different ethnic origins.

Investigation of protein expression of Wnt molecules in different types of cancer, their functions and also interaction with their receptors may highlight many unresolved issues regarding these molecules and their roles in different stages of tumor development. Transfection of Wnt-7A and Wnt-10A in AML cells or cell lines not expressing these two members of the Wnt family and also silencing of Wnt-3 in leukemic cells over-expressing this molecule may provide further insight into their mechanisms of action in AML initiation and/or pathogenesis.

In summary, in this study the expression profile of several Wnt genes in Iranian AML patients and normal controls has been shown. Three members of the Wnt gene family were found to be either significantly over-expressed (Wnt-3) or down-regulated (Wnt-7A and Wnt-10-A) in these patients. Modulation of these molecules may prove to be a useful tool for identification of mechanisms underlying AML leukemogenesis as well as immunotherapeutic interventions.

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