Iranian Journal of Blood & Cancer

Journal Home Page: www.ijbc.ir

ORIGINAL ARTICLE

Methylation Status of SOX17 and RUNX3 Genes in Acute Leukemia

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ARTICLE INFO

Article History:
Received: 01.06.2015
Accepted: 13.09.2015

Keywords:
Acute myeloid leukemia
Acute lymphoblastic leukemia
RUNX3
SOX17
DNA Methylation
Prognosis

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ABSTRACT

Background: Several studies have examined the presence of DNA methylation of CpG islands in leukemia. Methylation of SOX17 and RUNX3 genes may play a role in leukemogenesis through silencing tumor suppressor genes. We investigated the methylation status of SOX17 and RUNX3 genes in patients with acute leukemia.
Methods: In this case-control study, peripheral blood samples from 100 AML and 100 ALL patients and 100 healthy controls were collected. Isolated DNA was treated with sodium bisulfite and methylation status was examined by methylation specific PCR (MS-PCR) with primers specific for methylated and unmethylated sequences of SOX17 and RUNX3 genes.
Results: The frequency of hypermethylation of SOX17 and RUNX3 genes were 36% and 28%I in patients with acute myeloid leukemia (AML), and 21% and 22% in patients with acute lymphoblastic leukemia (ALL), respectively. Aberrant methylation of these genes was found in all FAB classifications of AML and ALL. Hypermethylation of SOX17 (P=0.055) and RUNX3 (P=0.003) genes were associated with FAB-M0 and M1 subtypes of AML, respectively. Also, aberrant methylation of RUNX3 gene was associated with FAB-L1 subtype of ALL (P=0.053). There was not any significant association between hypermethylation of SOX17 and RUNX3 genes and clinical parameters of patients with leukemia including sex, age, WBC, and platelet counts.
Conclusion: Hypermethylation of SOX17 and RUNX3 genes was seen in patients with acute leukemia. Moreover, no significant association was observed between hypermethylation of SOX17 and RUNX3 and induction of remission.

Please cite this article as: Ghasemi A, Ghotaslou A, Ghaffari K, Mohammadi M. Methylation Status of SOX17 and RUNX3 Genes in Acute Leukemia. IJBC 2015; 7(5): 213-219.

Introduction

DNA hypermethylation of promoter-associated CpG islands of tumor suppressor and DNA repair genes has been the most studied epigenetic alteration in human neoplasia.[1] In acute lymphoblastic leukemia (ALL), promoter hypermethylation is reported to be associated with a poor prognosis.[2] In acute myeloid leukemia (AML), many tumor suppressor genes are silenced through DNA methylation such as CDKN2B, P73 and suppressor of cytokine signaling.[3] Epigenetic disorders, in contrast to genetic changes are reversible and the role of DNA demethylating agents such as azacitidine and 5-azadeoxycytidine (decitabine) has been established in the treatment of hematopoietic malignancies.[4-7] DNA methylation patterns are often altered in cancer cells with increased level of the DNA methyltransferase.[8-10] Moreover, widespread genomic hypomethylation and simultaneous regional increase in DNA methylation patterns have been reported.[11] Molecular genetic alterations affecting NPM1 (nucleophosmin1) and FLT3 genes as well as WT1 (Wilms' Tumor) are among known important prognostic factors in AML.

In recent years, epigenetic disorders including methylation of tumor suppressor genes such as Wnt (Wingless and integration site) gene and its antagonist, Dickkopf-1 (DKK-1) has been shown to play some role in AML pathogenesis.[12] These alterations may lead to differentiation and apoptosis arrest in leukemic blasts as well as increase in proliferation and self-renewal.[13] Epigenetic aberrations in Wnt pathway are critical for the initiation of a variety of epithelial cancers and it has been demonstrated that abnormalities of this pathway are also common in hematopoietic malignancies.[14-16] In normal cells, Wnt signaling and β-catenin localization are tightly controlled by a number of intracellularly secreted inhibitory proteins including Dickkopf 1, 2 (DKK1,2), serine/ threonine kinase 11 (LKB1), Ras association domain-containing protein 1, runt-related transcription factor 3 (RUNX3), secreted frizzled related proteins 1, 2, 4, 5 (sFRP1, 2, 4, 5), SRY-box containing gene 17 (SOX17), and WNT inhibitory factor 1 (WIF1).[14,17] In some malignancies like colorectal cancers, head and neck tumors and gastric cancers, aberrant Wnt signaling pathway has been shown to cause uncontrolled cell proliferation.[18] β-catenin is an intracellular regulator of transcription that is associated mainly with epithelial cancers. Wnt controls the cytoplasmic level and stability of β-catenin.[19]

In the absence of Wnt ligand and its protective role, β-catenin level decreases due to destruction by Casein Kinase 1 and Glycogen Synthase Kinase 3b enzymes.[20] When the ligand adheres to its receptor (frizzled receptor), activates Dv1 (disheveled) proteins.[21] Having accumulated in cytoplasm, β-catenin migrates to nucleus where it causes expression of some genes involved in cell proliferation and differentiation.[18]

It has recently been demonstrated that both chromosomal alterations and FLT-3 mutations associated with AML pathogenesis, affect the Wnt signaling pathway.[22] SRY-related (Sox) transcription factors contain a HMG DNA-binding domain that regulate stem cell identity and function in multiple tissues.[23] Sox17 activates endodermal target genes and is required for the formation of endoderm and vascular endothelium.[24-26] Sox17 also plays an important role in the maintenance of fetal and neonatal hematopoietic stem cells.[27] Reduction of mature blood cell formation in zebrafish by RUNX3 depletion suggested a role for RUNX3 in hematopoiesis.[28] The role of RUNX3 in tumorigenesis and its potential involvement in hematopoiesis suggests a role for this transcription factor in hematological malignancies. However, genetic alterations of RUNX3 have not been reported in acute myeloid leukemia.[29]

Methylation of SOX17 and RUNX3 genes leads to loss of their inhibitory effect on Wnt pathway. Then cytoplasmic and nuclear levels of β-catenin enhances that as a transcription factor makes some genes associated in cell cycle regulation like MYC, COX and Cyclin D to be expressed.[30] we aimed to investigate the methylation status of SOX17 and RUNX3 genes in de novo non-M3 patients with AML and ALL at diagnosis.

Patients and Methods

One hundred patients with non-M3 AML and 100 patients with ALL and also 100 healthy controls were enrolled. At the beginning of the study, informed consent was obtained from all groups.

All patients were divided in FAB classification groups. The clinical parameters consist of white blood cell count, platelet, age, hemoglobin, and rate of recovery following induction chemotherapy extracted from patients medical records. Mononuclear cells of drawn samples including leukemic blast cells were isolated by concentration gradient sedimentation using Ficoll-hypaque followed by DNA extraction by saturated salt standard method.[17] In the next step extracted DNA underwent bisulfite conversion with the Epitect Bisulfite kit (Qiagen, Germani, Inc cat no. 59695) using the maunfacturer's instructions. By this treatment unmethylated cytosine converted to uracil where methylated cytosine stayed intact. Then the methylation status of SOX17 and RUNX3 genes was investigated using MSP (Methylation specific PCR) technique. MSP is a type of PCR used to investigate the methylation of CpG islands. In this method we used 2 pairs of primers specified for checking the methylated or unmethylated residue. These primers are shown in table 1, accompanied by product values.

Four MSP reactions using methylated and unmethylated primers related to SOX17 and RUNX3 were administered for each patient. In methylation testing we used 2 µl of DNA previously treated with Bisulfite, 4 µl of dH20, 12 µl of Master mix, 0.5 µl of forward primer and 0.5 µl of reverse primer while in order to investigate the unmethylated status. We used 2 µl of DNA, 7.5 µl of

Table1: SOX17 and RUNX3 gene primers sequence, annealing temperature and product size for MSP assays

Primer	Sequence (5' to 3')	Annealing temperature	Product Size (bp)
SOX17- MF	CAAAAACGAATCCCGTATCCGACG	62	79
SOX17- MR	TTGCGTTAGTCGTTTGCGTTC		
SOX17-UF	CAAACCAAAAACAAATCCCATATCCAACA	60	91
SOX17- UR	GATTTTGTTGTGTTAGTTGTTTGTGTTTG		
RUNX3-MF	GGCGGTCGTCGGGTTAGCGAGGTTTC	62	87
RUNX3- MR	CCCGAACCTCAAAACGCAAAAAACGACG		
RUNX3-UF	GTGGGTGGTTGTTGGGTTAGTGAGGTTTT	60	92
RUNX3-UR	AACCCAAACCTCAAAACACAAAAAACAACA		

M: Methylated, U: Unmethylated, F: Forward, R: Reverse

Figure 2: Karyotype study of the patient showed 45, XY, -7 (monosomy 7)

dH20, 12 µl of master mix, 0.5 µl of forward primer, 0.5 µl of reverse primer and 0.5 µl of MgCl2. In the first step of MSP, reaction components put in pre-thermal conditions including 99ºC for 1 minute and 95ºC for 3 minutes followed by 35 cycles including 99ºC for 10 seconds, 95ºC for 30 seconds, 60ºC for 30 seconds (SOX17 and RUNX3-UM Primer), 62ºC for 30 seconds (SOX17 and RUNX3-M Primer) and 70ºC for 5 minutes (extension). In this study, we used EpiTect PCR control DNA kit (Qiagen Inc cat no. 59695) containing unmethylated and completely methylated DNAs as negative and positive controls, respectively. Electrophoresis on 4.5% agarose gel was done in order to identify MSP products (figures 1 and 2). Fisher's exact two-sided tests, Mann-Whitney U test were used as approapriated. Data were analyzed using SPSS software, version 21(version 21, SPSS Inc Chicago, IL). P-value less than 0.05 were considered significant.

Results

The AML group included 70 (70%) men and 30 (30%) women and the ALL group included 60 (60 %) men and 40 (40 %) women, respectively. Mean±SD age of patients with AML and ALL was 43.5±10 years (range: 15-75 years) and 43.5±10 years (range: 13-62 years), respectively. WBC and platelet counts in patients with AML were 0.45-375×10⁹/L and 0.015-280×10⁹/L (mean values were 15.2±0.16×10⁹/L and 95±0.65×10⁹/L), respectively and in ALL patients WBC and platelet counts were 0.320-150×10⁹/L and 30-320×10⁹/L, respectively (mean values were 11±0.120 and 80±0.330×10⁹/L), respectively.

SOX17 gene found to be hemi-methylated in 30 (30%) patients with AML and 32 (32%) patients with ALL, completely methylated in 36 (36%) patients with AML and 21(21%) patients with ALL and completely unmethylated in 34 (34%) patients with AML and 47 (47%)patients with ALL , while RUNX3 gene was hemi-methylated in 42 (42%) patients with AML and 46 (46%) patients with ALL, completely methylated in 28 (28%) patients with AML and 22 (22%) patients with ALL and completely unmethylated in 30 (30%) patients with AML and 32 (32%)patients with ALL. Methylation in SOX17 and RUNX3 genes was not seen in the control group. Correlation between hypermethyaltion of SOX17 and RUNX3 genes and clinical and laboratory features of leukemia patients are shown in tables 2 and 3, respectively. In patients with AML, frequency of hypermethylation of SOX17 and RUNX3 genes were 36% and 28% and in patients with ALL, it was 21% and 22%, respectively.

Patients with AML with hypermethylation of RUNX3 genes had higher hemoglobin than those without hypermethylation (P=0.065). Aberrant methylation of these genes was found in all FAB classifications of AML and ALL. Hypermethylation of SOX17 (P=0.055) and RUNX3 (P=0.003) genes were associated with FAB-M0 and -M1 subtype of AML, respectively (table 2). Also, aberrant methylation of RUNX3 gene was associated with

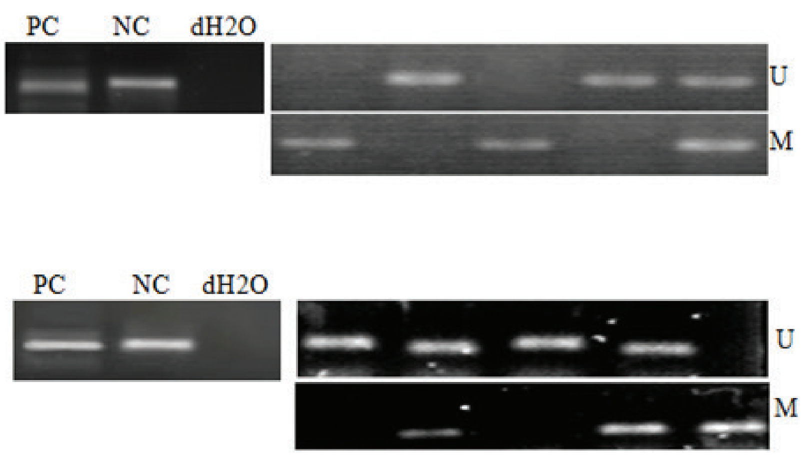

Figure 1: MSP analysis of SOX17 and RUNX3 genes in AML patients and normal control. PC: Positive control; NC: Negative control; P: Patient; M: Methylated; U: Unmethylated. dH2O served as a blank control.

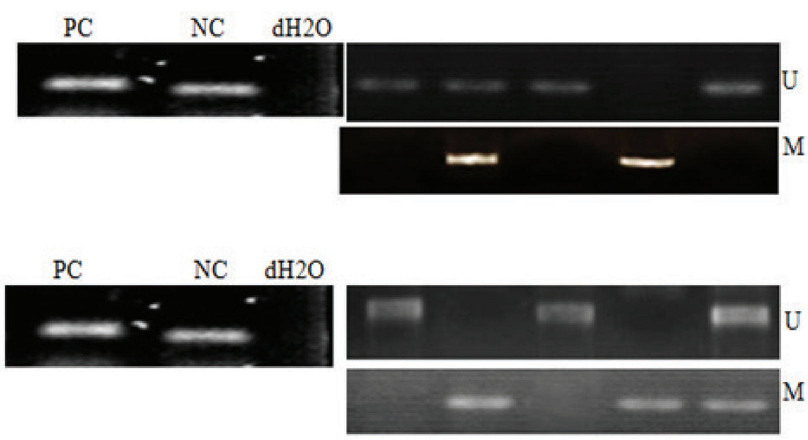

Figure 2: MSP analysis of SOX17 and RUNX3 genes in ALL patients and normal control. PC: Positive control; NC: Negative control; P: Patient; M: Methylated; U: Unmethylated. dH2O served as a blank control.

Table 2: Correlation between hypermethylation of SOX17 and RUNX3 genes and laboratory and clinical symptoms of AML patients.

	SOX17			RUNX3		
Characteristics	**M**	**U**	**P**	**M**	**U**	**P**
Number of Patients, (%)	36 (36)	64 (64)		28 (28)	72 (72)	
Age, median (range) years	39.6±3 (21-65)	35.1±6 (18-62)	0.415	42±5 (23-70)	48±8 (15-72)	0.432
Sex, %			0.175			0.629
Male	22	48		21	49	
Female	14	16		7	23	
WBC count (10^9/L, median)	15.2±3	25.4±7	0.311	34.1±5	30.2±2	0.265
Platelet count (10^9/L, median)	120.6±10	212±8	0.524	98±4	135±6	0.321
Hb, g/dL (median)	8.6±0.8	9.1±0.5	0.201	8.9±0.2	8.3±0.6	0.065
FAB type, n (%)						
M0	4 (11.1)	1 (2.7)	0.055	3 (10.7)	2 (2.7)	0.132
M1	6 (16.6)	8 (12.5)	0.563	9 (32.1)	5 (6.9)	0.003
M2	10 (27.7)	21 (32.8)	0.658	6 (21.4)	25 (34.7)	0.235
M4	8 (22.2)	16 (25)	0.812	6 (21.4)	18 (25)	0.798
M5	6 (16.6)	14 (21.8)	0.610	3 (10.7)	17 (23.6)	0.175
M6	2 (5.5)	4 (6.2)	0.999	1 (3.5)	5 (6.9)	0.999
Outcome, n (%)						
Complete remission	22 (61.1)	45 (70.3)	0.381	18 (64.2)	49 (68)	0.814
Death	4 (11.1)	7 (10.9)	0.999	3 (10.7)	8 (11.1)	0.999
Relapse	10 (27.7)	12 (18.75)	0.322	7 (25)	15 (20.8)	0.788

AML: Acute Myeloblastic leukemia, Hb: Hemoglobin, WBC: White blood cell, FAB: French-American-British, M: Methylated, U: Unmethylated

Table 3: Correlation between hypermethylation of SOX17 and RUNX3 genes and laboratory and clinical symptoms of ALL patients.

	SOX17			RUNX3		
Characteristics	**M**	**U**	**P**	**M**	**U**	**P**
Number of Patients, (%)	21 (21)	79 (79)		22 (22)	78 (78)	
Age, median (range) years	27.6±5 (19-55)	30±3 (13-62)	0.325	22±2 (17-55)	25±6(15-60)	0.183
Sex, %			0.317			0.999
Male	15	45		13	47	
Female	6	34		9	31	
WBC count (10^9/L, median)	9.5±2	15±2.5	0.632	14.3±2	18±3	0.331
Platelet count (10^9/L, median)	110.5±8	200±5	0.424	89±6	125±4	0.543
Hb g/dL(median)	8.9±0.6	10.5±1	0.187	9.2±0.2	9.8±0.8	0.139
FAB type, n (%)						
L1	4 (19)	12 (15.1)	0.739	6 (27.7)	10 (12.8)	0.053
L2	9 (42.8)	38 (48.1)	0.807	8 (36.3)	39 (50)	0.335
L3	8 (38)	29 (36.7)	0.999	8 (36.3)	29 (37.2)	0.999
Outcome, n (%)						
Complete remission	15 (71.4)	68 (86)	0.187	17 (77.2)	66 (84.6)	0.520
Death	2 (9.5)	3 (3.8)	0.282	2 (9.1)	3 (3.8)	0.303
Relapse	4 (19.1)	8 (10.2)	0.271	3 (13.7)	9 (11.5)	0.723

ALL: Acute Lymphoblastic leukemia, Hb: Hemoglobin, WBC: White blood cell, FAB: French-American-British, M: Methylated, U: Unmethylated

FAB-L1 subtype of ALL (P=0.053, table 3).

There was no significant association between hypermethylation of SOX17 and RUNX3 genes and clinical parameters of patients with leukemia including sex, age, WBC, and platelet counts (tables 2 and 3).

Twenty two out of 100 patients with AML developed relapse in whom 10 and 12 patients were hypermethylated for SOX17 and RUNX3 genes, respectively. There was no significant association between hypermehylation of both SOX17 and RUNX3 genes and relapse of patients with AML (P=0.322 and P=0.788, respectively). 67 (67%) patients with AML developed complete remission after induction chemotherapy; of whom 22 and 18 were hypermethylated for SOX17 and RUNX3 genes (P=0.381 and P=0.814, respectively). There was no significant association between hypermethylation in the SOX17 and RUNX3 genes and achievement of induction remission in patients with AML (table 2). Twelve out of 100 patients with ALL developed relapse whom 4 patients were hypermethylated for SOX17 and 3 for RUNX3 genes. There was no significant association between hypermehylation of both SOX17 and RUNX3 genes and relapse of patients with ALL (P=0.271 and P=0.723, respectively).

Demographic and clinical features of 93 patients with ALL were available of whom 83 (93%) patients achieved complete remission of induction; 15 and 17 were hypermethylated in the SOX17 and RUNX3 genes, respectively (P=0.187 and P=0.520, respectively). There was no significant association between hypermethylation in the SOX17 and RUNX3 genes and remission rate in patients with ALL (table 3).

Discussion

In this study we investigated the methylation status of SOX17 and RUNX3 genes in newly diagnosed patients with AML and ALL. The results of this study showed that hypermethylation of SOX17 and RUNX3 genes occurred with a frequency of 36% and 28% in patients with AML and 21% and 22% in patients with ALL, respectively. Understanding the roles of Wnt/β-catenin signaling in survival, proliferation and differentiation of hematopoietic stem cells resulted in developing the hypothesis that this signaling pathway may be involved in leukemogenesis.[31-33]

More recently, inactivation of RUNX3 was reported in a wide range of other cancer types.[34] There is evidence that RUNX3 is inactivated by gene silencing or protein mislocalization in more than 80% of gastric cancers.[35,36]

Frequent SOX17 gene methylation has been detected in colon, liver, and breast cancers.[37-39] SOX17 belongs to the high-mobility group (HMG)-box transcription factor superfamily, which is homologous to the sex-determining gene SRY.[40] SOX17 has been reported to promote degradation of β-catenin/TCF via a GSK3β-independent mechanism in Wnt signaling pathway and has been recognized as an important antagonist and inhibitor of the canonical Wnt signaling pathway.[41,42] Hypermethylation of other inhibitors of Wnt signaling pathway has been found in some malignancies such as SFRP, WIF1 and DKK-1 gene methylation in AML.[43,44] Yu and colleagues demonstrated that promoter methylation of the Wnt/β-Catenin signaling antagonist DKK-1 is associated with poor survival in gastric cancer.[45]

The percentage of patients with AML with aberrant methylation was 66% and 70% for SOX17 and RUNX3 and in patients with ALL, 53% for SOX17 and 68 % for RUNX3. The frequency of hypermethylation of SOX17 and RUNX3 in patients with AML in this study was higher than those reported by Griffiths and co-workers (29% and 27 %, respectively; total: 56%).[44] These probably reflect the difference in patient selection and ethnic diversity. SOX17 and RUNX3 genes are epigenetic targets in AML patients which are inactivated through methylation processes.[44,46]

Interestingly, methylation-associated RUNX3 silencing was detected in half of the ALL and CML cell lines, suggesting that RUNX3 methylation occurs in certain types of hematological malignancies.[46] Moreover, Cheng and colleagues pointed out that unlike in AML, RUNX3 was epigenetically silenced by promoter methylation in t(12;21)-positive cells. Whether RUNX3 is also transcriptionally repressed by TEL-RUNX1 awaits further investigation.[46]

Our results showed that aberrant methylation of SOX17 and RUNX3 occurred in all FAB-AML and -ALL subtypes. Patients with FAB-M0 and -M1 subtype had the highest incidence of hypermethylation of SOX17 (80 %, P=0.055) and RUNX3 (65 %, P=0.003), respectively; whereas those with M6 subtype had the lowest incidence of SOX17 (33.4 %, P=0.999) and RUNX3 (16. %, P=0.999) hypermethylation, respectively. Likewise, patients with FAB-L1 subtype of ALL had the highest incidence of hypermethylation of SOX17 (25%, P=0.739) and RUNX3 (37.5 %, P=0.053), respectively; whereas those with L2 subtype had the lowest incidence of SOX17 (20%, P=0.8) and RUNX3 (17%, P=0.3), respectively. In this study, we did not observe any significant association between hypermethylation of these genes and prognostic factors.

Griffiths and co-workers reported that methylation of SOX17 was associated with a trend toward increased risk of relapse and methylation of sFRP4 was associated with an increased risk for death.[44] In our study, induction of remission was observed in 67% and 83% in patients with AML and ALL, respectively. In our study, no significant association was observed between hypermethylation of SOX17 and RUNX3 and induction of remission.

Conclusion

We found that CpG island methylation of SOX17 and RUNX3 genes is a common event in patients with AML and ALL. Patients with FAB-M0 and -M1 subtype and FAB-L1 subtype of ALL had the highest incidence of hypermethylation of SOX17 and RUNX3. Moreover, no significant association was observed between hypermethylation of SOX17 and RUNX3 and induction of remission.

Conflict of Interest: None declared.

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