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# Study of SFRP1 and SFRP2 methylation status in patients with de novo Acute Myeloblastic Leukemia

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**ABSTRACT**

**Intoduction:** Acute myeloid leukemia (AML) is a heterogeneous group of hematologic malignancies with abundant changeability in the pathogenesis. DNA methylation of CpG islands within the promoters of specific genes may play roles in tumor initiation and progression. Secreted frizzled-related proteins (SFRPs) are negative regulator of the Wnt signaling pathway. In the present study, we examined the methylation status of SFRP1 and SFRP2 genes in patients with AML.

**Methods:** Isolated DNA from peripheral blood of 43 AML patients and 25 healthy subjects as a control group was treated with sodium bisulfite was treated with sodium bisulfite and analyzed by methylation-specific polymerase chain reaction (MSP) with primers specific for methylated and unmethylated promoter sequences of the SFRP1 & -2 genes. We used Mann-Whitney u-tests to investigate the correlation between SFRP1 and SFRP2 genes hypermethylation and clinical parameters.

**Results:** The frequency of aberrant hypermethylation of SFRP1 and SFRP2 genes in patients with AML was determined 30.2% (13/43) and 20.9% (9/43), respectively. In addition, for all subjects in control group, methylation of SFRP1 and SFRP2 genes were negative. Patients with M0 subtype of FAB-AML had the highest incidence of hypermethylation of SFRP1 ($P$ = 0.028) and SFRP2 ($P$ = 0.004).

**Conclusion:** The present study showed that, like many solid tumors, methylation of SFRP genes also occurs in AML. Therefore, the methylation of these genes may play a role in the initiation of leukmogenesis

**Keywords:** AML, SFRP, DNA Methylation



## INTRODUCTION

Acute myeloid leukemia (AML) is a clonal hematopoietic disorder characterized by uncontrolled self-renewal of hematopoietic stem cells, maturation arrest at myeloblast level, peripheral blood and bone marrow infiltration of blast cells. [1]It is demonstrated that pathogenesis of AML is associated with some disorders including genetic changes and chromosomal translocations. Developments in molecular researches have improved our understanding of the leukemogenesis in AML. Investigation of molecular genetic alterations affecting NPM1 (nucleophosmin1) and FLT3 genes as well as WT1 (Wilms' Tumor) assay are known as important prognostic factors in AML, in addition to age, white blood cell counts and cytogenetic aberrations. In recent years, epigenetic disorders including methylation of tumor suppressor genes like SFRPs (Secreted frizzled related proteins) family genes have also been shown to play a role in AML pathogenesis. These alterations may lead to differentiation and apoptosis arrest in leukemic blasts as well as increase in proliferation and self-renewal. [2]

Secreted frizzled-related proteins (SFRPs) are Wnt antagonists that suppress this signaling pathway in healthy individuals. Wnt signaling pathway contributes to regulation of cell proliferation and differentiation. In some malignancies like colorectal cancers, head and neck tumors and gastric cancer, aberrant Wnt signaling pathway has been shown to cause uncontrolled cell proliferation. [3] Chronic myeloid leukemia was the first malignancy in which the important role of Wnt signaling pathway has been described. [4] B-catenin is an intracellular regulator of transcription that is associated with cancers. Wnt controls the cytoplasmic level and stability of β-catenin. [5] In absence of Wnt ligand and its protectional role, β-catenin level decreases due to destruction by Casein Kinase 1 and Glycogen Synthase Kinase 3b enzymes. [6] But when the ligand adheres to its receptor (frizzled receptor), activates DV1 (dishevelled) proteins. [7] Having accumulated in cytoplasm, β-catenin migrates to nucleus where it causes expression of some genes involved in cell proliferation and differentiation. [3,8] It has recently been demonstrated that both chromosomal alterations and FLT-3 mutations associated with AML pathogenesis affect Wnt signaling pathway [9]. Methylation of SFRP genes lose their inhibitory effect on wnt singnaling pathway leading to elevated cytoplasmic and nuclear levels of β-cathenin and subsequently β-cathenin as a transcription factor promotes expression of Myc and cyclin D genes that are involved in cell cycle regulation. Since the methylation of these genes may play a role in initiation and leukemogenesis of AML, in present study we investigated the methylation status of SFRP1 and SFRP2 genes in newly diagnosed AML patients who were admitted to the Hematology, Oncology and Bone Marrow Transplantation Research Center in Shariati Hospital, Tehran.

## MATERIAL AND METHODS

Peripheral blood samples were drawn from 25 healthy individuals (negative control group) and 43 patients with newly diagnosed AML who were admitted to the Hematology, Oncology and Bone Marrow Transplantation Research Center in Shariati Hospital, Tehran. All patients were classified according to the FAB classification. The clinical parameters consisted of white blood cell count; platelet, age, hemoglobin and rate of recovery following induction chemotherapy were 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 [11]. In the next step, extracted DNA underwent bisulfite conversion with the Epi Tect Bisulfite kit (Qiagen) using producer instructions. By this treatment, unmethylated cytosine converted to uracil where methylated cytosine stayed intact. Then, the methylation status of SFRP1 and SFRP2 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 given in Table-1 accompanied with product values. The sequences of these primers are designed in previous studies. [12, 13]

Four MSP reactions using methylated and unmethylated primers related to SFRP1 and SFRP2 were administered for each patient .In methylation testing, we used 2 µl of bisulfite-treated DNA, 4.5 µl of dH20 , 12.5 µl of Master mix , 0.5 µl of forward primer and 0.5 µl of reverse primer, while we used 2 µl of DNA , 8.5 µl of dH20 , 12.5 µl of master mix , 0.5 µl of forward primer , 0.5 µl of reverse primer and 1 µl of MgCl2 to investigate the unmethylated status. In the first step of MSP, reaction components put in pre-thermal condition including 98 Cº for 1 minute and 96 Cº for 3 minutes followed by 40 cycles including 99 Cº for 10 seconds, 97 Cº for 20 seconds, 54 Cº for 30 seconds (SFRP1- UM Primer), 64 Cº for 30 seconds (SFRP1, 2-M Primer) and 72 Cº for 7 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. Meanwhile, ddH2O served as a blank control. Electrophoresis on a 2.5% agarose gel was done for MSP product identification. All patients received 7+3 remission chemotherapy regimen. Fisher's exact two-sided tests, Mann–Whitney U-tests and SPSS analytic software version 21(version

21, SPSS Inc Chicago, IL) were used for statistical analysis of data. P value less than 0.05 was considered statistically significant.

## RESULTS

A total of 43 patients, 31 (72.1%) males and 12 (27.9%) females, were included in this study. Average age was 45.5 years old ranging from 15-72 years. White blood cells and platelet counts were 600-145000 and 2000-280000 cells per microliter and their mean values were 27818.5 and 98633.3 cells per microliter, respectively.

SFRP1 gene was hemi-methylated in 13 of patients (30.2%), completely methylated in 13 patients (30.2%) and completely unmethylated in 17 patients (39.5%), while SFRP2 gene was hemi-methylated in 16 of patients (37.2%), completely methylated in 9 patients (20.9%) and completely unmethylated in 18 patients (41.8%) (Figure 1). None of control individuals showed methylation in SFRP1 and SFRP2 genes. Correlation between hypermethylation of SFRP1and SFRP2 genes and laboratory and clinical symptoms of patients are indicated in Table 2.

In AML patients, hypermethylation frequency of SFRP1 and SFRP2 genes were 30.23% ( 13 out of 43 patients) and 20.9% (9 out of 43 patients), respectively.

Aberrant methylation of these genes was found in all FAB classifications of AML, except subtype M6. Hypermethylation of SFRP1 (*P* = 0.028) and SFRP2 (P = 0.004) genes were associated with FAB-M0 subtype of AML (Table 2). There was no significant relationship between hypermethylation of genes (SFRP1 and SFRP2) and clinical parameters of patients including sex, age, white cells and platelet counts (Table 2).

The causes of relapse developed in 6 of 43 subjects were attributed to hypermethylated SFRP1 and SFRP2 genes in 2 (4.6%) and 1 (2.3%) patient, respectively. There was no significant relationship between hypermehylation of both SFRP 1 and SFRP 2 genes and relapse of patients. Also, 30 (69.76%) out of 38 (88.37%) patients had complete remission

**Table 1**. SFRP-1 and SFRP-2 gene primers sequences, annealing temperature and product size for MSP assays.

| Primer | Sequence | Annealing tem | Product Size (bp) |
|---|---|---|---|
| **SFRP-1 MF** | TGTAGTTTTCGGAGTTAGT GTCGCGC | 62 | 126 |
| **SFRP-1 MR** | CCTACGATCGAAAACGACG CGAACG | | |
| **SFRP-1 UF** | GTTTTGTAGTTTTTGGAGT TAGTGTTGTGT | 54 | 135 |
| **SFRP-1 UR** | CTCAACCTACAATCAAAAA CAACACAAACA | | |
| **SFRP-2 MF** | GGGTCGGAGTTTTTCGGAG TTGCGC | 62 | 138 |
| **SFRP-2 MR** | CCGCTCTCTTCGCTAAATA CGACTCG | | |
| **SFRP-2 UF** | TTTTGGGTTGGAGTTTTTT GGAGTTGTGT | 64 | 145 |
| **SFRP-2 UR** | AACCCACTCTCTTCACTAA ATACAACTCA | | |

**M**: methylated, **U**: unmethylated, **F**: forward, **R**: reverse

## DISCUSSION

Wnt/β-catenin signaling pathway has been implicated in many cellular procedures including proliferation, morphology, motions, destiny determination of cells and organ development [14]. 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. [14-16] SFRP is a tumor suppressor protein that modulates the Wnt/β-catenin signaling pathway. This protein binds to Wnt protein and thus inhibits its binding to Wnt-frizzled receptor. The result is inactivation of Wnt signaling pathway. Hence, there may be an association between methylation of Wnt signaling antagonist genes and the activation of this pathway in solid tumors and leukemia. [15-17] Aberrant methylation of tumor suppressor genes is a more specific and common genetic events in human cancers .[18, 19]

**Table 2.** Correlation between hypermethylation of SFRP-1 & -2 genes and laboratory and clinical symptoms of AML patients

| | SFRP -1 | | | SFRP -2 | | |
|---|---|---|---|---|---|---|
| **Characteristics** | M | U | P | M | U | P |
| **Number of Patients, %** | 13 (30.2) | 30 (69.7) | | 9 (20.9) | 34 (79.1) | |
| **Age, median (range)yeas** | 45.4 (23-60) | 39.6 (15-60) | .319 | 46 (24-70) | 57 (15-72) | .692 |
| **Sex, %** | | | .651 | | | .692 |
| **Male** | 10 | 21 | | 6 | 25 | |
| **Female** | 3 | 9 | | 3 | 9 | |
| **WBC count, $10^9$/L, median** | 15.7 | 31.7 | .242 | 66.1 | 14.4 | .182 |
| **Platelet count, $10^9$/L, median** | 105.2 | 95.6 | .630 | 89 | 118 | .408 |
| **Hb level, g/dL, median** | 8.9 | 9.9 | .190 | 8.9 | 9.9 | .096 |
| **FAB type, n (%)** | | | | | | |
| **M0** | 2 (15.3) | 0 | .028 | 2 (22.2) | 0 | .004 |
| **M1** | 2 (15.3) | 5 (16.6) | .919 | 0 | 7 (20.5) | .646 |
| **M2** | 4 (30.7) | 8 (26.6) | .789 | 3 (33.3) | 9 (26.4) | .223 |
| **M4** | 2 (15.3) | 8 (26.6) | .433 | 2 (22.2) | 8 (23.5) | .936 |
| **M5** | 2 (15.3) | 4 (13.3) | .863 | 2 (22.2) | 8 (23.5) | .936 |
| **M6** | 0 | 2 (6.66) | .352 | 0 | 2 (5.8) | .468 |
| **Unclassified** | 1 (7.6) | 3 (10) | .816 | 0 | 4 (11.7) | .291 |
| **Outcome, n (%)** | | | | | | |
| **Complete remission** | 9 (69.2) | 19 (63.3) | .717 | 5 (55.5) | 25 (73.5) | .308 |
| **Failure** | 3 (23) | 7 (23.3) | .968 | 2 (22.2) | 8 (23.5) | .142 |
| **Death** | 1 (7.6) | 1 (3.3) | .544 | 1 (11.1) | 1 (2.9) | .312 |
| **Relapse** | 2 (15.3) | 4 (13.3) | .863 | 1 (11.1) | 5 (14.7) | .788 |

**AML**: acute myeloblastic leukemia, **Hb**: hemoglobin, **WBC**: white blood cell, **FAB**: French-American-British, **M**: methylated, **U**: unmethylated.

In present study, we investigated the methylation status of SFRP1 and SFRP2 genes in newly diagnosed AML patients. The results of this study showed that hypermethylation of SFRP1 & SFRP2 genes occurs with a frequency of 30.23% (13 out of 43 subjects) and 20.9% (9 out of 43 patients) in AML patients at the time of diagnosis, respectively, while none of the normal samples showed methylation. Also, 13 out of 43 (32.2%) patients showed simultaneous methylation of both SFRP1 and SFRP2 genes at the time of diagnosis.

Veeck et al demonstrated that epigenetic changes of SFRP1 through methylation were even linked to poor prognosis in patients with breast cancer. [20,21] Simon et al. suggested that recombinant SFRP1 might be a novel therapeutic strategy for cancers with suppressed SFRP1 expression. [22] Moreover, SFRP2 methylation as one of the epigenetic targets occurs in cancers such as colon cancer [23], esophagus cancer [13], bladder cancer [9], gastric cancer [24,25], liver cancer [26] and lung cancer. [12]

Like previous studies, our study also showed that SFRP1 and -2 genes are epigenetic modulation targets in AML patient. The percentage of patients with aberrant methylation of at least one SFRP gene in this study was 26 patients (60.5%) for SFRP1 and 25 patients (58.2%) for SFRP2. Therefore; methylation of these genes may be involved in the onset of AML.

In this study, the hypermethylation frequencies of SFRP1 and SFRP2 (30.2% and 20.9, respectively; total 51.1 %) were lower than those (41% & 31%, respectively; total: 72%) reported by Valencia et al [27] and higher than those (29% and 19%, respectively; total: 48%) reported by Jost et al [3], but were similar to those (31.6 % and 19.3 %, respectively; total: 50.9%) reported by H-A Hou et Al. [28] These possibly reflect the dissimilarity in patient selection and ethnic diversity.

Methylation of SFRP genes has also been shown in hematologic malignancies, so as Pehlivan et al, showed the activation of Wnt signaling pathway through methylation of SFRP1 leads to drug resistance in patients with chronic myeloid

leukemia treated with imatinib msylate by suppression of imatinib mesylate effect over BCR-ABL signaling pathway .[29]

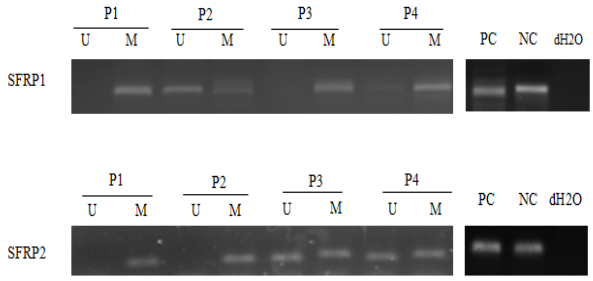


Figure 1. Methylation Analysis of SFRP1 and SFRP2 in Four AML patients. PC= positive control, NC= negative control, P= patient، M= methylated، U= unmethylated. dH2O served as a blank control.

Hong et al., showed that methylation of SPRP genes in patients with MDS is associated with poor prognosis and less survivability. [17] To investigate the prognostic relevance of aberrant methylation of SFRP1 and SFRP2 genes in AML patients, we concentrated on the clinical outcome of patients, including relapse, complete remission, failure and death. Among 43 patients, complete remission after induction chemotherapy was observed in 30 patients (69.76%). However, we did not observe any significant association between hypermethylation of these genes and clinical outcome of patients. Jost et al and H-A Hou et al also did not find any prognostic factor for aberrant methylation in SFRP promoters[3,28] whereas Griffiths et al., showed that *SFRP2* and *SFRP5* methylations were associated with poorer disease-free survival and OS in cytogenetically normal AML[30] Valencia et al reported that methylation of *SFRPs* and *DKKs* was associated with a poorer prognosis only in young adult patients with intermediate-risk cytogenetics. [27]

Furthermore we could not find significant differences between AML patients with and without SFRP hypermetylation in conventional prognostic factors such as age, sex, WBC & platelet count, and hemoglobin concentration, Though significant differences may be observed when increased sample size.

Cheng et al pointed out that SFRP promoter methylation was significantly correlated with increased age (median age: 12 years vs. 3.5 years; P= 0.0001) and male sex in pediatric patients with AML. [31] Moreover, H-A Hou et al showed that male patients had a higher incidence of hypermethylation of Wnt inhibitors than females [28]. Our present study also showed that male patients had a higher incidence of hypermethylation of SFRP1 and SFRP2 than females (76.9% *vs.* 23% for SFRP1 and 66.6% *vs.* 33.3% for SFRP2).

H-A Hou et al., showed that patients with FAB M0 subtype of AML had the highest incidence of hypermethylation of Wnt inhibitors, whereas those with M4/M5 subtype had the lowest incidence (28). Our results also showed that aberrant methylation of these genes took place in all FAB-AML subgroups including M0, M1, M2, M4 and M5, except subgroup M6.

The highest hypermethylation rate of SFRP1 (100%, P=0.028) & SFRP2 (100%, P=0.004) were observed in M4 and M1 subgroups respectively, Compared with M0 subgroup of AML that has the lowest rate of hypermethylation of SFRP1 (25%, P= 0.433) and SFRP2 (0%, P=0.936), respectively.

## CONCLUSION

The present study has showed that the methylation of SFRP1 and SFRP2 genes contingently takes place in all FAB subgroups of AML except subgroup M6 in patients with AML at the time of diagnosis. In this study, we did not observe an association between methylation of relevant genes and clinical findings in AML patients such as age, sex, WBC, platelet counts and complete remission after induction therapy. Therefore, methylation of these genes and other molecular events are involved in AML. However, it is recommended to conduct more studies to determine the role of hypermethylation of these genes in the pathogenesis of AML and other hematologic malignancies.

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