Concurrent Evaluation of the Expression and Methylation of Secreted Frizzled-Related Protein 2 along with Beta-Catenin Expression in Patients with non-M3 Acute Myeloid Leukemia

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Abstract

Background: Wnt signaling is a critical pathway for the development of acute myeloid leukemia (AML). Some studies have evaluated the expression or methylation of secreted frizzled-related protein 2 (SFRP2) as an antagonist and beta-catenin (β-catenin) as a critical mediator of this pathway. Since we found no comprehensive study on these genes in Iran, we aimed to investigate the status of both SFRP2 expression and methylation, and also β-catenin expression, in conjunction with clinical characteristics, in Iranian patients with de novo non-M3 AML.

Methods: The methylation and expression of SFRP2 were determined in 188 patients with primary non-M3 AML and 60 healthy controls who were referred to Shariati Hospital, Tehran, Iran, between January 2017 and February 2019. The methylation-specific polymerase chain reaction (PCR) and real-time quantitative PCR were used, respectively. The expression of β-catenin was explored via real-time quantitative PCR. Statistical analysis was performed using the Mann–Whitney U test (SPSS software, version 23). A P value of less than 0.05 (2-tailed) was considered significant.

Results: SFRP2 mRNA showed a significant decline in the AML group compared with the controls (P<0.001). The hypermethylation of the SFRP2 promoter occurred in 25.5% (48/188) of the cases. SFRP2 expression exhibited a negative correlation with the white blood cell count (P=0.003). The expression of β-catenin increased significantly in the patients in comparison with the controls (P<0.001), and a significant difference was observed between the patients who achieved complete remission and those who did not (P=0.046).

Conclusion: The findings of this study showed that alterations in SFRP2 and β-catenin expression can be used as a potential biomarker for differentiating patients with new non-M3 AML from the controls. Additionally, an evaluation of β-catenin expression may be valuable in predicting complete remission in patients with non-M3 AML.


Keywords ● SFRP2 protein ● Humans ● Leukemia ● Myeloid ● Acute ● beta Catenin ● Wnt signaling pathway

What’s Known

- Wnt signaling is critical for the development of many malignancies, including acute myeloid leukemia. Research has shown the importance of the expression or methylation of secreted frizzled-related protein 2 as an antagonist and beta-catenin as a critical mediator of this pathway.

What’s New

- We investigated the status of both secreted frizzled-related protein 2 expression and methylation simultaneously, and their correlation with beta-catenin expression in Iranian patients. We showed that evaluating beta-catenin expression might be valuable in predicting complete remission in patients with non-M3 acute myeloid leukemia.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous blood cancer characterized by the clonal disorders of hematopoietic progenitor...
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Genetic and epigenetic abnormalities are considered to be a critical player in the pathogenesis of AML. Aberrant promoter hypermethylation is one of many epigenetic aberrations, that contribute to leukemogenesis.

In recent years, a considerable number of signaling pathways have been recognized, and indicated as important factors for the regulation of cellular processes. The Wnt signaling pathway is one of them with a key role in hematopoietic cell fate. Based on numerous studies, the abnormal activation of the Wnt signaling pathway is indicated as the pathogenesis of AML given its critical roles in differentiation, proliferation, cell adhesion, and migration. Many instances of Wnt signaling dysregulation have been detected in various cancers, including AML. Wnt signaling comprises the canonical pathway (beta-catenin [beta-catenin]-dependent) and noncanonical pathways (beta-catenin-independent).

In the absence of Wnt signaling, the phosphorylated form of beta-catenin is degraded by ubiquitination, and the cytoplasmic levels of protein remain low. With the activation of the Wnt pathway, the phosphorylation and degradation of beta-catenin are inhibited, leading to its accumulation in the cytoplasm, and transport into the nucleus. Nuclear non-phosphorylated beta-catenin is the downstream effector of canonical Wnt signaling, and mediates the expression of several genes, including cyclin D1 and c-Myc.

Several families of Wnt signaling antagonists such as secreted frizzled-related proteins (SFRPs), Dickkopf (Dkk) proteins, and Wnt inhibitory factor 1 act as modulators of the Wnt signaling cascade through the inhibition of Wnt proteins. In humans, SFRPs consist of five members, and have been implicated as the largest family among Wnt antagonists. The aberrant methylation of SFRP genes, which was associated with abnormal Wnt signaling activation, was demonstrated in AML. Four out of five SFRP genes (SFRP1, SFRP2, SFRP4, and SFRP5) contain dense CpG islands around their promoter regions. Previous studies have reported that SFRP genes, except for SFRP3 are silenced by promoter hypermethylation in various malignancies, including AML. Moreover, it has been indicated that Wnt signaling can also be activated by mutant fms-like tyrosine kinase 3 (FLT3). In total, these findings show that Wnt signaling aberration involves multiple mechanisms, and is a common dysregulated pathway in various cancers. Furthermore, some studies have proposed that the hypermethylation of the SFRP promoter is an adverse risk factor for survival in patients with AML.

Accordingly, in the present study, we aimed to investigate the status of SFRP2 expression and methylation simultaneously, and explore their clinical significance besides their correlation with the beta-catenin expression as the most important mediator of the canonical Wnt signaling pathway, in Iranian patients with de novo non-M3 AML.

Patients and Methods

Patients and Samples

In this cross-sectional study, written informed consent was obtained before bone marrow specimens were collected from 188 patients with de novo non-M3 AML (98 male and 90 female patients), who were referred to the Hematology-Oncology and Stem Cell Transplantation Research Center of Shariati Hospital (Tehran, Iran) between January 2017 and February 2019. Based on previous studies and via the Cochran formula, the sample size was determined. The diagnosis and classification of patients with de novo AML were made according to the criteria of the French-American-British (FAB) classification systems and the World Health Organization (WHO) (blast$\geq$20%). Patients who had a history of other malignancies, myelodysplastic syndromes, and treatment with cytostatic drugs (e.g., steroids) were excluded from the study. The main clinical and laboratory features of the patients are summarized in table 1. Sixty age- and sex-matched healthy controls with no current morbidity or history of serious diseases were included in the study. All the patients received standard 3+7 induction chemotherapy, comprising idarubicin (Pfizer, Australia; 12 mg/m$^2$) for three days plus cytarabine (Abbvie, Australia; 100 mg/m$^2$) for seven days. The project was approved by the Ethics Committee of Tehran University of Medical Sciences (code: IR.TUMS.REC.1395.2313).

DNA Extraction, Bisulfite Modification, and Methylation-Specific Polymerase Chain Reaction (MSP)

Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). DNA was bisulfite converted using the EpiTect Kit (QIAGEN, Hilden, Germany) between January 2017 and February 2019. DNA was bisulfite converted using the EpiTect Kit (QIAGEN, Hilden, Germany). DNA was bisulfite converted using the EpiTect Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. MSP was carried out using SFRP2 promoter methylation and non-methylation-specific primer pairs (table 2) designed in previous studies. MSP was carried out for each sample in a final reaction volume of 20 µL, containing 0.4 µM of primers, 10 µL of Taq DNA Polymerase Master Mix RED (10 µL) (Ampliqon, Stenhuggervej, Denmark), and 50 ng of bisulfite-treated DNA.
MSP was performed on the Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) conditions were as follows: initial denaturation for three minutes at 94 °C, followed by 40 cycles of 30 seconds at 94 °C, 30 seconds at 64 °C, 30 seconds at 72 °C, and final elongation for seven minutes at 72 °C. Unmethylated and completely methylated DNAs, contained in the EpiTect PCR Control DNA Kit (QIAGEN, Hilden, Germany), were used as positive controls. Electrophoresis on a 2.5% agarose gel was done for the identification of the MSP product.

**Flow Cytometric Analysis**

Normal and leukemic mononuclear cells were isolated from bone marrow using Histopaque (Sigma, St Louis, USA) density-gradient centrifugation. After separation, the mononuclear cells were stained with a panel of fluorescent-conjugated monoclonal antibodies (Dako, Glostrup, Denmark), including CD45,
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CD34, CD38, CD13, CD33, CD14, CD64, CD10, HLA-DR, CD19, CD20, and isotype controls, in accordance with the manufacturer’s instructions as described previously. The Flomax software (Version: 2.6; PARTEC, Nuremberg, Germany) was used for data analysis.

**RNA Extraction and Reverse Transcription and Real-time Quantitative Polymerase Chain Reaction**

Total RNA was isolated from mononuclear cells using the TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed into complementary DNA using the PrimeScript RT Reagent Kit (Takara Bio, Tokyo, Japan). Real-time quantitative PCR was carried out for each sample in a final reaction volume of 20 μL, consisting of 0.4 μM of primers, 10 μL of SYBR Premix Ex Taq II, 0.4 μL of 50x ROX (Takara Bio, Tokyo, Japan), and 50 ng of complementary DNA. The primer sequences for *β-catenin* and *SFRP2* (target genes), and *ABL1* (reference gene) are listed in (table 2).

Real-time quantitative PCR was performed on StepOnePlus (Applied Biosystems, Foster City, CA, USA). The PCR program was carried out at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds, 59 °C for 20 seconds, and 72 °C for 25 seconds. The mRNA expression level of the *SFRP2* gene was calculated relative to the expression of the reference gene using the $2^{-\Delta\Delta C_t}$ formula.

**Gene Mutation Detection**

*FLT3-ITD* and *NPM1* mutations were detected by fragment analysis as reported previously.

**Statistical Analysis**

The Mann–Whitney *U* test and the Kruskal–Wallis test were applied for the comparison between two and multiple groups, respectively. The Spearman correlation coefficient was utilized to study the correlations between different values. The SPSS software, version 23, (SPSS, Chicago, IL, USA) and GraphPad Prism, version 5, (GraphPad Software, Inc., La Jolla, CA, USA) were employed for the analyses. The receiver operating characteristic curve (ROC) and the area under the ROC curve (AUC) were drawn upon to assess the diagnostic value of *SFRP2* and *β-catenin* expression in differentiating patients with non-M3 AML from healthy controls. A *P* value of less than 0.05 (2-tailed) was considered to be statistically significant.

**Results**

**Expression and Methylation of SFRP2 in Patients with non-M3 Acute Myeloid Leukemia**

The level of *SFRP2* expression was investigated in 188 patients with non-M3 AML and 60 normal controls. The *SFRP2* level showed a significant decline in the AML group compared with the control group (figure 1A). Additionally, the methylation of *SFRP2* promoter regions was analyzed (figure 2). The *SFRP2* promoter was fully methylated in 25.5% (48/188) of the patients, partially methylated in 38.3% (72/188), and unmethylated in 36.2% (68/188).

![Figure 1](image_url)

*Figure 1:* Relative expression levels of secreted frizzled-related protein 2 (*SFRP2*) (A) and *β-catenin* (*β-catenin*) are illustrated between patients with non-M3 acute myeloid leukemia (AML) compared with healthy controls. The solid line represents the median of $2^{-\Delta\Delta C_t}$ of the expression of each gene. The *SFRP2* mRNA level decreased, and the *β-catenin* mRNA level increased significantly in patients with non-M3 AML compared with controls.
SFRP2 methylation was found in none of the 60 (0%) controls. Collectively, the methylation of the SFRP2 gene occurred in 63.8% (120/188) of the patients with AML.

Value of SFRP2 and β-catenin Expression for Differentiating Patients with Non-M3 Acute Myeloid Leukemia from Controls Using the Receiver Operating Characteristic Curve

The expression of SFRP2 and β-catenin was evaluated using the ROC curve to determine whether it was useful as a potential diagnostic marker for de novo AML. It was concluded that the level of SFRP2 and β-catenin expression could be helpful as a potential diagnostic biomarker for differentiating patients with non-M3 AML from controls with an AUC of 0.861 (95% CI: 0.785 to 0.936; P<0.001) for SFRP2, and an AUC of 0.88 (95% CI: 0.79 to 0.97; P<0.001) for β-catenin (figure 3). At a cutoff value of 5.85% for SFRP2, the sensitivity and the specificity were 75% and 88%, respectively. The cutoff value of β-catenin was 0.3%, and the sensitivity and the specificity were 98% and 70%, respectively.

Impact of Different SFRP2 Expression and Methylation on the Clinical and Laboratory Characteristics of Acute Myeloid Leukemia

The expression of SFRP2 was evaluated based on a cutoff value of 5.85%. Low SFRP2 (≤5.85‰) and high SFRP2 (>5.85‰) expression levels, as well as the methylation status of the SFRP2 gene, were evaluated in two groups: methylated and unmethylated. The results revealed no significant differences in the hemoglobin level, the platelet count, the percentage of CD34+, the percentage of blasts in bone marrow, and gene mutations between the two groups of high and low SFRP2 expression levels. However, there was a negative correlation between the white blood cell count (WBC) and SFRP2 expression (P=0.003). A low SFRP2 expression level was found more frequently in male subjects (P=0.001). SFRP2 expression was different between AML subtypes (P=0.001). Additionally, a significant difference in terms of SFRP2 expression was detected between the two age groups of younger than 60 and at least 60 years old (P=0.009) (table 1). After the evaluation of methylation status, no significant difference was found in gender, age, WBC, the hemoglobin level, the platelet count, the percentage of blasts in bone marrow, the percentage of CD34+, the FAB classification, and the gene mutations between the methylated and unmethylated groups (table 1).
Correlation between the Expression and Methylation of SFRP2 in Patients with Acute Myeloid Leukemia

The relationship between the expression and methylation of SFRP2 was analyzed. The results demonstrated no significant difference concerning SFRP2 expression between the methylated and unmethylated SFRP2 groups (P=0.635).

Correlation between SFRP2 Expression and Methylation and β-catenin Expression

The results showed that the β-catenin expression level rose significantly in the AML group in comparison with the control group (P<0.0001) (figure 1B). The expression of β-catenin was not significantly different between the methylated and unmethylated groups. No correlation was found between β-catenin expression and SFRP2 expression. Furthermore, β-catenin expression was significantly different between the patients who achieved complete remission and those who did not (P=0.046).

Discussion

The critical role of the Wnt signaling pathway in the cellular processes of the differentiation and proliferation of hematopoietic progenitors has been determined. The dysregulation of this pathway plays a role in leukemogenesis. The epigenetic inactivation of the Wnt pathway inhibitors has been indicated as an important explanation for the Wnt pathway activation. SFRP2 hypermethylation has been implicated in various cancers such as breast cancer,9 gliomas,12 gastric cancer,13 and pituitary adenomas.14 In this study, we explored SFRP2 methylation and expression simultaneously, and evaluated their correlation with B-catenin expression and clinical features in Iranian patients with non-M3 AML. We assessed the results in conjunction with clinical characteristics and treatment responses. In our study, the percentage of the aberrant methylation of at least one SFRP2 gene was 63.8% (120/188), while no methylation was reported in the controls. Previous studies have reported various frequencies of SFRP2 hypermethylation events in patients with AML (17%-66%). Nonetheless, our result (25.5%) is comparable with that reported by Shen and others25 (25.4%) and Guo and others26 (27.3%). Therefore, the methylation of these genes may contribute to the onset of AML.

We could not evaluate our patients’ karyotype in our study; nevertheless, SFRP2 methylation has been reported in adults with core-binding factor leukemia with poor prognosis16 as well as in patients with intermediate-karyotype leukemia younger than 60 years of age.6

We found no correlation between SFRP2 hypermethylation and the age or gender of the patients. Some researchers have shown that the aberrant SFRPs promoter methylation is correlated significantly with an increase in age16,26 and male gender.21,26

Our results also demonstrated no correlation between the methylation and expression of SFRP2. This result is consistent with that reported by Guo and others.26,27 Still, interestingly, they detected a negative correlation between SFRP1 expression and methylation in patients with AML. In contrast with our results, in a study by Jost and others, there was an association between SFRP2 hypermethylation and transcriptional downregulation.19

In contrast to Guo and others,27 we assessed SFRP2 expression in all the study patients after diagnosis not after achieving complete remission. Guo and colleagues suggested that SFRP2 expression could be potentially used as a biomarker for disease monitoring in patients with cyogenetically normal AML after complete remission.27 Consequently, our results could not be used for disease monitoring. In the same line with Guo and others,27 however, we concluded that SFRP2 expression could be drawn upon as a diagnostic biomarker.

In our study, complete remission after 7+3 induction chemotherapy was achieved in 132 out of 188 patients (70.2%). Nonetheless, we did not detect any significant association between the hypermethylation of SFRP2 and the percentage of complete remission. In accordance with our result, some studies have found no prognostic factor for aberrant methylation in the SFRP2 promoter alone, and they have shown that the concurrent methylation of Wnt antagonists is needed.6,26,28 Griffiths and others pointed out that patients suffering from AML with SFRP2 and SFRP5 methylation at the time of diagnosis had an increased risk of relapse, and SFRP2 methylation was associated with a higher risk for death.19 Valencia and others reported that the methylation of SFRPs and DKKs was associated with an adverse prognosis in young patients suffering from AML with intermediate-risk cytogenetics.6 Unfortunately, we only evaluated the hypermethylation of SFRP2 and not that of other mediators, and our information about prognosis is not complete yet. These results indicate that the aberration of several mediators simultaneously is important for the prediction of prognosis in patients.

Interestingly, in contrast to Guo and others,27 we found a negative correlation between WBC
and SFRP2 expression. Our results revealed low SFRP2 expression levels more frequently in our male patients. SFRP2 expression was different between AML subtypes, and we found a significant difference vis-à-vis SFRP2 expression between the two age groups of younger than 60 and at least 60 years old. In general, different results have been obtained in various studies, which may be due to racial differences, environmental conditions, and different methods of testing and sampling.

As has been indicated, β-catenin plays a role as the main mediator of the Wnt signaling pathway, and its accumulation in the cytoplasm/nucleus is critical for the activation of this pathway. The overexpression of β-catenin has been previously reported in blast crisis-CML CD34+ progenitors. Wang and others analyzed the relationship between the expression levels of β-catenin and the methylation status of Wnt antagonist genes in gastric cancer, and detected no association between them, which is in concordance with our findings. It appears that as an important Wnt antagonist, SFRP2 hypermethylation alone cannot affect the Wnt signaling pathway and ultimately β-catenin expression. Ysebaert and others indicated that β-catenin expression did not affect complete remission but could be considered as an independent prognostic factor for both poor event-free survival and shortened overall survival.

We showed that β-catenin expression levels increased significantly in the non-M3 AML group by comparison with the control group. In contrast to the study by Ysebaert and others, we found a significant difference in the rates of complete remission, but we did not evaluate event-free survival and overall survival in our research. The overexpression of β-catenin is regarded as an independent adverse prognostic factor. Some studies have suggested that β-catenin inhibition could be a treatment option at least in some subtypes, but it cannot be a universal target in all patients with AML. These data can be utilized in designing personalized treatment procedures. So, indubitably, further research in this field seems necessary.

Conclusion

Our results showed that alterations in SFRP2 expression could be used as a potential biomarker for differentiating between patients with de novo non-M3 AML and controls. However, its use as a prognostic factor alone requires complete information on relapse and overall survival. Moreover, an evaluation of β-catenin expression may be valuable in predicting the achievement of complete remission in patients with non-M3 AML. We suggest that further studies be performed on β-catenin at the level of gene and protein expression in patients with AML.

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Conflict of Interest: None declared.

References

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