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Comparison of Secreted Frizzled-Related Protein -4 & -5 Promoter Methylation in Patients with Acute Myeloblastic Leukemia and Healthy Individuals

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ABSTRACT

Background: DNA methylation patterns are often changed in cancer cells. Many of the tumor inhibitor genes are silenced by methylation, such as CDKN2B, p73, and the suppressor of cytokine signaling in patients with acute myeloblastic leukemia (AML). Secreted frizzled-related protein -4 and -5 (SFRP4, 5) are negative regulators of the Wnt signaling pathway. We aimed to evaluate the methylation status of SFRP4 and SFRP5 genes in patients with AML.
Methods: Blood samples were isolated from 60 patients with AML and 30 healthy controls. DNA was exploited, treated with sodium bisulfite, and tested utilizing methylation-specific polymerase chain reaction with specific primers for methylated and unmethylated sequences of the SFRP4 and SFRP5 genes.
Results: The frequency of unfit hypermethylation of SFRP4 and SFRP5 genes in patients with AML was characterized to be 50% (30/60) and 40% (24/60), respectively. Moreover, for all the subjects in the control group, methylation of SFRP4 and SFRP5 genes was negative. The spread of SFRP4 and SFRP5 promoter methylation in patients with AML was higher than the control population.
Conclusion: Hypermethylation was seen in SFRP4 and SFRP5 genes in patients with AML.

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Introduction

Acute myeloblastic leukemia (AML) is a heterogeneous group of hematological malignancies characterized by uncontrolled self-renewal of hematopoietic stem cells, maturation arrest at myeloblast level, peripheral blood and bone marrow infiltration of blast cells.[1] Progress in molecular studies has improved our understanding of leukemogenesis in AML. In addition to age, white blood cells count and cytogenetic aberrations; molecular genetic modifications affecting nucleophosmin-1 (NPM1), FLT3 genes and wilms tumor (WT1) are identified as important prognostic factors in patients with AML.[2] DNA methylation is the most important epigenetic marker which involves the addition of the methyl group to the cytosine residue of CpG (CpG Island) located within the promoter region of gene-regulating cell proliferation, apoptosis, and DNA repair.[3] Improper promoter methylation leading to functional inactivation of tumor suppressor genes is a well-recognized mechanism capable of driving carcinogenesis.[4] In AML, many tumor suppressor genes are silenced by DNA methylation, such as CDKN2B, P73, and suppressor of cytokine signaling. Epigenetic disturbances, in contrast to genetic modifications are reversible and hence, the role of DNA demethylating agents such as azacitidine and decitabine in treating hematopoietic malignancies will be more attractive.[5] In recent years, epigenetic modifications, as well as methylation of tumor suppressor genes such as the SFRP family genes have a role in the pathogenesis of AML.[1, 6] SFRPs are the extracellular antagonists of Wnt signaling that sequester Wnt molecules at the cell surface membrane[7] and by this are recognized as sensitive regulators of the canonical Wnt signaling pathway.[8] The signaling pathway of Wnt contributes to the regulation of cell differentiation and proliferation.[9] In normal cells, Wnt signaling and β-catenin localization are tightly controlled by a number of intracellular secreted inhibitory proteins, including Dickkopf -1, -2 (DKK-1, -2), serine/ threonine kinase-11 (LKB1), Ras association domain-containing protein 1, runt-related transcription factor 3 (RUNX3), (SFRP-1, -2, -4, -5), SRY-box containing gene 17 (SOX17), and WNT inhibitory factor 1 (WIF1).[10-12] Aberrant activation of Wnt/β-catenin signaling is thought to be involved in tumorigenesis.[13] Considering the aberrant promoter methylation of these genes in leukemogenesis, we aimed to study the methylation status of SFRP-4 and SFRP-5 genes among de novo patients with AML.

Materials and Methods

After obtaining written informed consent, blood samples were taken from 60 patients with AML at diagnosis and 30 healthy controls. The patients were divided according to FAB (French-American-British) classification system. The clinical and laboratory parameters including age, CBC, complete remission, death, and relapse were elicited from patients' medical records.

Mononuclear cells of isolated samples including leukemic blast cells were separated by concentration gradient sedimentation applying Ficoll-hypaque, subsequently DNA was extracted using a saturated salt standard procedure.[12] In the next phase, exploited DNA experienced bisulfite conversion using Epitect Bisulfite Kits (Qiagen). By this treatment, unmethylated cytosine was transformed to uracil while methylated cytosine remained intact. The methylation situation of SFRP-4 and SFRP-5 genes was studied using Methylation-specific polymerase chain reaction (MSP-PCR). MSP is a kind of PCR applied to investigate the methylation state of the CpG islands. In this method, two pairs of primers specified for checking the methylated or unmethylated residues are utilized. The methylated SFRP4–specific primers forward, 5'-AGTTTACGTTAGGGGAGGTGTC-3' and reverse, 5'- CTCCAATCGACAACAAAACG-3' as well as the unmethylated SFRP4–specific primers forward, 5'-GAGTTTATGTTAGGGGAGGTGTT-3' and reverse, 5'- AAACTCCAATCAACAACAAAACAA-3' were used. SFRP5 MSP primers were as follow: unmethylated (U) allele-specific primers (F) 5'-TGGTGTTGGGTGGGATGTTTG-3' and (R) 5'- CAACCCAAACCTCACCATACAC-3, and methylated (M) allele-specific primers (F) 5'-TGGCGTTGGGCGGGACGTTC-3' and (R) 5'-AACCCGAACCTCGCCGTACG-3'. In methylation examination, we used 2 µl of DNA which previously had been treated with Bisulfite, 4 µl of dH20, 12 µl of master mix, 0.5 µl of a forward primer and 0.5 µl of reverse primer while in order to survey the unmethylated status, we used 2 µl of DNA, 7.5 µl of dH20, 12 µl of master mix, 0.5 µl of forwarding primer, 0.5 µl of reverse primer and 0.5 µl of MgCl2. At the first level of MSP, reaction components were put in pre-thermal condition, including 96 ºC for 1 minute and 95 ºC for 3 minutes, followed by 30 cycles, including 96 ºC for 10 seconds, 95 ºC for 60 seconds, 58 ºC for 30 seconds (unmethylated Primers), 60 ºC for 30 seconds (methylated Primers) and 72 ºC for 3 minutes (extension). In the current study, we used the EpiTect PCR control DNA kit (Qiagen) containing unmethylated and thoroughly methylated DNAs as negative and positive controls, respectively. Electrophoresis on the 2.5 % Agarose gel was performed for MSP product recognition (figure 1).

Fisher's exact two-sided test, Mann–Whitney U-test and SPSS analytic software (version 21, SPSS Inc, Chicago, IL) were utilized for statistical analysis of the data. P<0.05 was considered statistically significant.

Results

There were 43 (71.7%) men and 17 (28.3%) women in the studied AML patients (age range: 45-69 years). SFRP4 gene was found to be hemi-methylated, entirely methylated and completely unmethylated in 18 (30%), 12 (20%), and 30 (50%) patients; whereas SFRP5 gene was hemi-methylated, completely methylated and thoroughly unmethylated in 16 (26.7%), 8 (13.3%) and 36 (60%) patients (figure 1). None of the participants in the control group had methylation in SFRP4 and SFRP5 genes. The number of patients with hypermethylation of SFRP4 and SFRP5 genes were 20% and 13.3%, respectively. Also, 18.4 %of the patients (11 out of 60) showed methylated SFRP4 and SFRP5 genes at the time of diagnosis (table 1). Improper methylation of these genes was found in all FAB subtypes of AML. There was no association between hypermethylation status of SFRP4 and SFRP5 genes with FAB subtypes of AML and clinical and laboratory

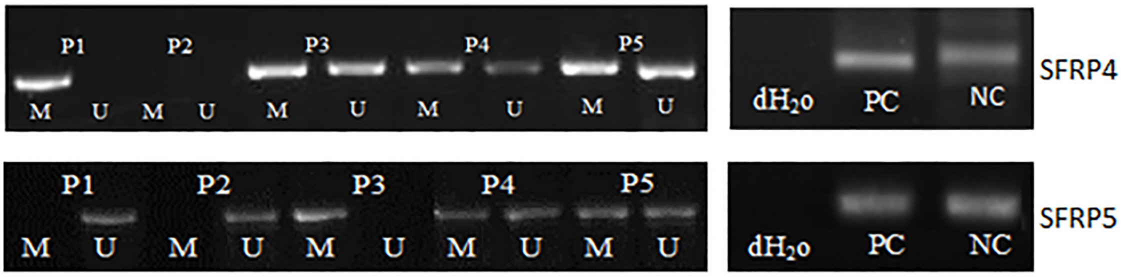

Figure 1: MSP analysis of SFRP4 and SFRP5 genes in AML patients and normal control. dH2O served as a blank control. PC: Positive control; NC: Negative control; P: Patient; M: Methylated; U: Unmethylated.

Table 1: Association between methylation of SFRP4 and SFRP5 genes and clinical indications of AML patients.

Characteristics	SFRP4			SFRP5		
	M	U	P	M	U	P
Number of Patients, (%)	30 (50)	30 (50)		24 (40)	36 (60)	
Age, median[26]years	59.1 (51-69)	56.8 (49-64)	0.247	54.3 (45-66)	53 (51-62)	0.513
Sex, %			0.567			0.773
Male	23	20		18	25	
Female	7	10		6	11	
WBC count (mm3), mean±SD	31716±7312	26587±3563	0.203	35137±8109	30155±5579	0.424
Platelet count (mm3), mean±SD	221706±17534	236321±10345	0.521	1964743±20321	2086071±10211	0.317
Hb g/dL, mean±SD	15.6±1.5	15.3±1.3	0.399	14.9±1.8	15.5±2.1	0.386
FAB type, n (%)						
M0/M1	2 (6.7)	1 (3.3)	0.554	1 (4.1)	2 (5.5)	0.809
M2	11 (36.6)	13 (43.4)	0.598	12 (50)	14 (38.9)	0.395
M4	8 (26.7)	10 (33.3)	0.573	6 (25)	10 (27.8)	0.812
M5	7 (23.3)	6 (20)	0.754	4 (16.8)	9 (25)	0.443
M6	2 (6.7)	0	0.150	1 (4.1)	1 (2.8)	0.801
Outcome, n (%)						
Complete remission	21 (70)	25 (83.4)	0.784	22 (91.6)	24 (66.6)	0.910
Death	3 (10)	2 (6.6)	0.640	2 (8.3)	3 (8.3)	0.333
Relapse	5 (16.6)	6 (20)	0.739	4 (16.6)	2 (5.5)	0.160

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

parameters including age, sex, WBC and platelet count (table 1). 11 out of 60 patients developed relapse, of which 5 patients showed SFRP4 and 4 patients displayed SFRP5 gene hypermethylation.

There was no correlation between hypermethylation in SFRP-4 and -5 genes and relapse (P=0.739 and P=0.160, respectively). 46 (76.7 %) patients achieved remission after induction chemotherapy. Among patients with complete remission, 21 (70 %) and 22 (91.6 %) patients were hypermethylated in the SFRP4 and SFRP5 genes, respectively (P=0.784, P=0.910). 3 (5%) patients did not achieve remission, of these 1 and 2 patients had hypermethylation in the SFRP4 and SFRP5 genes, respectively. There was no association between hypermethylation of SFRP4 and SFRP5 genes and remission status after induction chemotherapy.

Discussion

Epigenetic alterations, such as DNA methylation have emerged as additional and equally important mechanisms besides genetic alterations.[14] Understanding the developing role of the Wnt pathway in the permanence, multiplication, and differentiation of hematopoietic stem cells have led to the different hypotheses that this signaling pathway may be involved in leukemogenesis.[15] This critical developmental pathway is dysregulated in several human tumors including breast cancer,[16] acute leukemia,[17] human hepatocellular carcinoma[18] and B-cell chronic lymphocytic leukemia.[14] Aberrant promoter methylation leading to the inactivation of tumor suppressor genes is a well-known mechanism capable of driving carcinogenesis.[19] Wnt/β-catenin signaling pathway has been involved in a large number of pathways such as cell proliferation, cell morphology, destiny designation of cells and organ development.[18] SFRP-4 and -5 are tumor suppressor proteins that modulate the Wnt/ β-catenin signaling pathway. These proteins bind to Wnt protein and consequently prevent its binding to the Wnt-receptor.

In the present study, we investigated the methylation status of SFRP4 and SFRP5 genes in newly diagnosed patients with AML. The results of this study showed hypermethylation of SFRP4 and SFRP5 genes in 30% and 24% of AML patients, respectively. None of the subjects in the control group revealed any methylation. Wnt signaling activates pathways that play an important role in proliferation and differentiation.[20] Hypermethylation of Wnt signaling pathway inhibitors such as SFRP-1, SFRP-2,[1] Wnt inhibitory factor 1 (WIF1) and dickkopf-1 (DKK-1)[21] genes has also been shown in AML. Therefore, methylation of these genes may be involved in the initiation of AML and it may also have a role in its pathogenesis by dysregulation of the WNT signaling pathway. Yu and colleagues demonstrated that SFRP gene methylation may be involved in acute leukemia progression, with a possible epigenetic mechanism influencing Wnt signaling.[17] Simon et al. have recommended that recombinant SFRP might be a new treatment strategy for cancers with suppressed SFRP expression.[22] Epigenetic changes, in contrast to genetic modifications, are reversible and the role of DNA demethylating agents such as AZA and 5-aza-2′-deoxycytidine has been recognized in the treatment of hematopoietic disorders.[5, 23] The percentage of patients with an aberrant methylation of at least one SFRP4 or SFRP5 gene in this study was 20% for SFRP4 and 13.3% for SFRP5. silencing of SFRPs by CpG island methylation is another possible mechanism contributing to the aberrant activation of the Wnt signaling pathway in CLL suggested by Liu et al.[14] The frequency of hypermethylation of SFRP4 or SFRP5 genes in our study was lower than those reported by Griffiths et al.[24] and higher than those reported by Jian-Zhen Shen et al. (6.8% and 11.9%, respectively; total: 18.7%).[17] This is probably due to the differences in patient selection and ethnic diversity. Our results showed that aberrant methylation of these genes were observed in all FAB subtypes (M0 through M6). Hou et al. pointed out that DKK-1 hypermethylation frequently occurs concomitantly with hypermethylation of the SFRP family, but not Wif-1.[25]

In this study, we did not observe any significant association between hypermethylation of these genes and clinical, laboratory or conventional prognostic factors in AML such as age and sex, WBC, platelet count or hemoglobin.

Conclusion

The present study showed that hypermethylation in SFRP-4 and -5 genes occurs in AML similar to solid tumors. Assessment of other antagonists of Wnt signaling pathways are recommended to further explore the status of DNA methylation, cell differentiation and proliferation in different kinds of malignancies.

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Conflict of Interest: None declared

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