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Dysregulation of the WNT Signaling Pathway Through Methylation of Wnt Inhibitory Factor 1 and Dickkopf-1 Genes among AML Patients at the Time of Diagnosis

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Abstract

Background: In acute myeloblastic leukemia, a large number of tumor suppressor genes are silenced through DNA methylation such as CDKN2B & p73. Wnt inhibitory factor 1 (WIF1) and Dickkopf-3 (DKK-1) are negative regulators of Wnt signaling pathway. In the present study, we evaluated the methylation status of WIF1 and DKK-1 genes in acute myeloblastic leukemia patients.

Patients and Methods: Blood samples were taken from 120 AML patients and 25 healthy control subjects. DNA was isolated, treated with sodium bisulphite, and examined using methylation-specific polymerase chain reaction (MSP) with primers specific for methylated and unmethylated sequences of the WIF1 and DKK-1 genes.

Results: The frequency of aberrant hypermethylation of WIF1 and DKK-1 genes in acute myeloblastic leukemia patients were determined to be 35% (42/120) and 28.3% (34/120), respectively. In addition, for all subjects in control group, methylation of WIF1 and DKK-1 genes were negative. Patients with M0 subtype of FAB-AML had the highest incidence of hypermethylation of WIF1 (P = 0.003) and DKK-1 (P = 0.005) genes.

Conclusion: The present study showed that, like many solid tumors, WIF1 and DKK-1 genes methylation also occurs in acute myeloblastic leukemia. The study of other antagonists of Wnt signaling pathways are recommended.

Key words: AML, Wnt inhibitory factor 1, dickkopf, DNA methylation.

Introduction

Acute myeloblastic leukemia (AML) is a clonal hematopoietic disorder characterized by uncontrolled self-renewal of hematopoietic stem cells, maturation arrest at myeloblast level, and peripheral blood and bone marrow infiltration of blast cells [1]. It has been demonstrated that pathogenesis of AML is associated with some disorders including genetic changes and chromosomal translocations. Advancements in molecular research have improved our understanding of the leukemogenesis in AML. In AML, a large number of tumor suppressor genes are silenced through DNA methylation such as CDKN2B, p73 and suppresion of cytokine signaling [2]. Epigenetic disorders, in contrast to genetic changes, are reversible and the role of the DNA demethylating agents such as AZA and 5-aza-2´-deoxycytidine has been established in treatment of hematopoietic malignancies [3-6]. In addition to age, white blood cells count and cytogenetic aberrations, 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 recent years, epigenetic disorders including methylation of tumor suppressor genes such as Wnt inhibitory factor 1 (WIF1) and Dickkopf-3 (DKK-1 (genes have also been shown to play a role in AML pathogenesis [7]. These alterations may lead to differentiation and apoptosis arrest in leukemic blasts as well as an increase in proliferation and self-renewal [8]. WIF1 and DKK-1 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 [9]. Chronic myeloid leukemia was the first malignancy in which the important role of Wnt signaling pathway was described [10]. β-catenin is an intracellular regulator of transcription which is associated with cancers. Wnt controls the cytoplasmic level and stability of β-catenin [11]. In 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 [12]. But when the ligand adheres to its receptor (frizzled receptor), it activates DV1 (dishevelled) proteins [13]. Once Wnt signaling is suppressed by DKK-1, beta-catenin is phosphorylated and subsequently targeted for ubiquitination and degradation [7]. Having accumulated in cytoplasm, beta-catenin migrates to nucleus where it causes expression of some genes involved in cell proliferation and differentiation [9,14]. It has recently been demonstrated that both chromosomal alterations and FLT-3 mutations associated with AML pathogenesis affect Wnt signaling pathway [15]. Methylation of WIF1 and DKK-1 genes leads to loss of their inhibitory effect on Wnt pathway. Then cytoplasmic and nuclear levels of beta-catenin enhance that as a transcription factor and make some genes associated in cell cycle regulation like MYC, COX and Cyclin D to be expressed [16]. Since the methylation of these genes may play a role in initiation and leukemogenesis of AML, in the present study we investigated the methylation status of WIF1 and DKK-1 genes among de novo AML patients at diagnosis.

Patients and Methods

Blood samples were drawn from 120 AML patients at diagnosis and from 25 healthy individuals as the negative control group. All patients were divided to FAB (French-American-British) classification groups. The clinical parameters consisting of white blood cell count, platelet count, age, hemoglobin level, and the 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 17. In the next step extracted DNA underwent bisulfite conversion using Epitect Bisulfite Kits (Qiagen) following the producer's instructions. By this treatment unmethylated cytosine was converted to uracil where methylated cytosine stayed intact. Then the methylation status of WIF1 and DKK-1 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 2 pairs of primers specified for checking the methylated or unmethylated residue are used.

The methylated Dkk-1–specific primers forward, 5'- CGGAATGTTTCGGGTTCGC-3' and reverse, 5'-CACGAAACCGTACCGATTCG-3' as well as the unmethylated Dkk-1–specific primers forward, 5'- GTTGGAATGTTTTGGGTTTGT-3' and reverse, 5'-CCACAAAACCATACCAATTCA-3' were utilized.

WIF1 MSP primers were as follow: unmethylated (U) allele-specific primers (F) 5'-TGGT ATT TAG GTT GGG AGG TGA TGT-3' and (R) 5'-AAC CTC CAC CCA CAA TAC CAA-3, and methylated (M) allele-specific primers (F) 5'-ATT TAG GTC GGG AGG CGA CGC-3' and (R) 5'-GAC CTC CGC CCG CAA TAC CAA-3'.

Four MSP reactions using methylated and unmethylated primers related to WIF1 and DKK-1 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 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 were put in pre-thermal condition including 99 Cº for 1 minute and 95 Cº for 3 minutes followed by 35 cycles including 99 Cº for 10 seconds, 95 Cº for 30 seconds, 58 Cº for 30 seconds (WIF1- UM Primer), 62 Cº for 30 seconds (WIF1 and DKK-1-M Primer) and 70 Cº 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 3% Agarose gel was performed for MSP product identification. Fisher's exact two-sided test, Mann–Whitney U-test and SPSS analytic software (version 21, SPSS Inc Chicago, IL) were used for statistical analysis

of data. P values less than 0.05 were considered statistically significant.

Results

Out of 120 studied AML patients 78 (65%) were males and 42 (35 %) were females. The age range of patients was 15 to 72 years and the average age was 45 years. White blood cells and platelets counts were 600-145000 and 2000-280000 cell per microliter and their mean values were 27818.5 and 98633.3 cells per microliter, respectively. WIF1 gene found to be hemi-methylated in 45 patients (37.5%), completely methylated in 42 patients (35%), and completely unmethylated in 37 patients (30.8%); while DKK-1 gene was hemi-methylated in 40 of patients (33.3%), completely methylated in 34 patients (28.3%), and completely unmethylated in 46 patients (38.3%) (Figure 1). None of control

Table 1. Correlation between hypermethylation of Wnt inhibitory factor 1(WIF1) and Dickkopf (DKK) genes and laboratory and clinical symptoms among AML patients.

	WIF1			*DKK*		
Characteristics	*M*	*U*	*P*	*M*	*U*	*P*
Number of Patients, (%)	42 (35)	78 (65)		34 (28.3)	86 (71.6)	
Age, median(range) years	45.4 (23-60)	39.6 (15-60)	.319	46 (24-70)	57 (15-72)	.692
Sex, %			.217			.577
Male	30	48		25	65	
Female	12	30		9	21	
WBC count, 10^9/L, median	14.5	29.6	.242	64.1	13.4	.182
Platelet count, 10^9/L, median	115.4	102	.630	91	121	.408
Hb level, g/dL, median	8.3	8.7	.190	8.3	8.7	.096
FAB type, n (%)						
M0	8 (19)	2 (2.5)	.003	7 (20.5)	3 (3.4)	.005
M1	10 (23.8)	16 (20.5)	.817	7 (20.5)	19 (22)	.999
M2	10 (23.8)	22 (28.2)	.525	6 (17.6)	26 (30.2)	.178
M4	6 (14.2)	16 (20.5)	.466	4 (11.7)	18 (20.9)	.303
M5	4 (9.5)	14 (17.9)	.288	2 (5.8)	16 (18.6)	.094
M6	3 (7.1)	9 (11.5)	.538	2 (5.8)	10 (11.6)	.506
Outcome, n (%)						
Complete remission	30 (71.4)	65 (83.3)	.143	24 (70.5)	71 (82.5)	.211
Death	3 (7.1)	2 (2.5)	.137	1 (2.94)	4 (4.65)	.999
Relapse	7 (16.6)	9 (11.5)	.149	3 (8.82)	13 (15.11)	.552

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

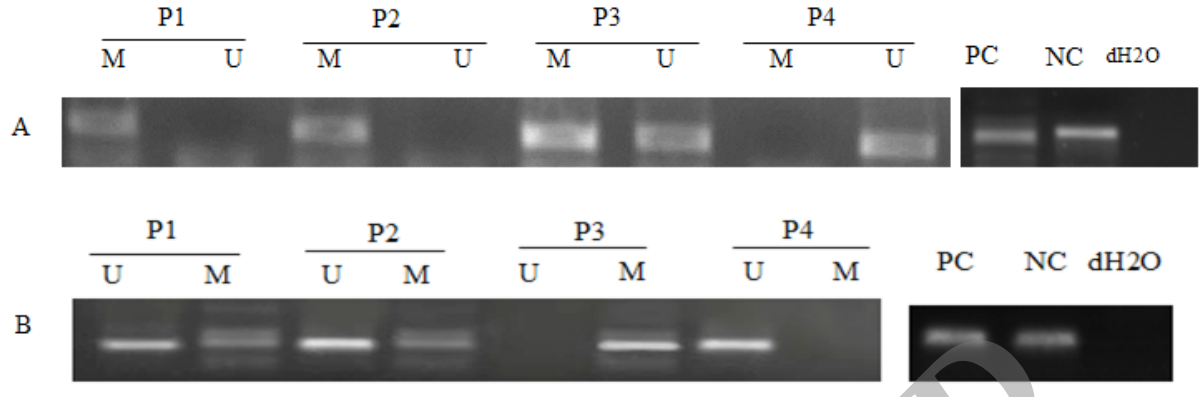

Figure 1. MSP Analysis of WIF1 (A) and DKK-1 (B) in Four AML patients. PC= positive control, NC= negative control, P= patient، M= methylated، U= unmethylated. dH2O served as a blank control.

individuals showed methylation in WIF1 and DKK-1 genes. Correlation between hypermethylation of WIF1 and DKK-1 genes and laboratory and clinical symptoms of patients are shown in table 1.

Among AML patients the hypermethylation frequency of WIF1 and DKK-1 genes were 35% (42 out of 120 patients) and 28.3% (34 out of 120 patients), respectively. Also 29.1% of patients (35 out of 120) showed methylated WIF1 and DKK-1 genes at the time of diagnosis (Table 1).

Aberrant methylation of these genes was found in all FAB classifications of AML. Hypermethylation of WIF1 (P = 0.003) and DKK-1 (P = 0.005) genes were associated with FAB-M0 subtype of AML (Table 1). There was no significant relationship between hypermethylation of WIF1 and DKK-1 genes with clinical parameters of patients including sex, age, white cell count and platelet count (Table 1).

Sixteen out of 120 patients developed relapse with 7 patients (16.6 %) showing WIF1 gene and 3 patients (8.82 %) showing DKK-1 gene hypermethylation. There was no significant relationship between hypermethylation of both WIF1 and DKK-1 genes and the relapse among patients (P = 0.149 and P = 0.552, respectively). Also, Information on the treatment of 109 patients (90.8 %) were found, with 95 patients (79.1 %) having complete remission after induction chemotherapy. Among patients with complete remission 30 and 24 patients were hypermethylated in the WIF1 and DKK-1 genes, (P = 0.143, P = 0.211, respectively). Thirty three patients (27.5 %) were refractory to induction chemotherapy, of these 14 and 9 patients had hypermethylation in the WIF1 and DKK-1 genes, respectively. There was no significant relationship between hypermethylation of WIF1 and DKK-1 genes among patients who developed methylation and complete remission after induction chemotherapy.

Discussion

Wnt/β-catenin signaling pathway has been implicated in many cellular procedures including proliferation, morphology, motions, destiny determination of cells and organ development [18]. Understanding the role 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 [18-20]. WIF1 and DKK-1 are tumor suppressor proteins that modulate the Wnt/β-catenin signaling pathway. These proteins bind to Wnt protein and thus inhibit its binding to Wnt-receptor. The result is inactivation of Wnt signaling pathway. Hence there may be an association between methylation of Wnt signaling antagonists genes and the activation of this pathway in solid tumors and leukemia [19,20]. Aberrant methylation

of tumor suppressor genes is a more specific and common genetic events in human cancers [21,22].

In this study, we investigated the methylation status of WIF1 and DKK-1 genes in newly diagnosed AML patients. The results of this study showed that hypermethylation of WIF1 and DKK-1 genes occurs with a frequency of 35% and 28.3 %) respectively, in AML patients at the time of diagnosis, while none of the normal blood samples revealed methylation. The hypermethylation of other inhibitors of Wnt signaling pathway has been shown in some malignancies, such as SFRP genes methylation in AML [23]. Yu et al. demonstrated that promoter methylation of the Wnt/b-Catenin signaling antagonist DKK-1 is associated with poor survival in gastric cancer [24]. Epigenetic disorders, in contrast to genetic changes, are reversible and the role of DNA demethylating agents such as AZA and 5-aza-2´-deoxycytidine has been established in treatment of hematopoietic malignancies [3-6]. Simon et al. have suggested that recombinant SFRP may be a novel therapeutic strategy for cancers with suppressed SFRP expression [25].

The Dkk-1 gene, located on chromosome 11p15.1, is suppressed in a multitude of human cancer cell lines and in numerous kinds of human cancers such as non-small cell lung carcinomas [26,27], human renal clear cell carcinoma [26] and acute lymphoblastic leukemia [28], which also makes it a candidate tumor suppressor gene. The percentage of patients with aberrant methylation of at least one WIF1 or DKK-1 gene in this study was 72.5 % for WIF1 and 61.6 % for DKK-1. Therefore, methylation of these genes may be involved in the onset of AML and it may also have a role in its pathogenesis by dysregulation of the WNT signaling pathway. A likely relation between impaired survival and DKK1 promoter hypermethylation has been suggested by Suzuki et al. [29].

The frequencies of hypermethylation of WIF1 and DKK-1 (35% and 28.3%, respectively; total: 63.3%) in this study were higher than those (32 % and 16 %, respectively; total: 48 %) reported by Griffiths et al. [30] and Hou et al. [31] (26 % and 30.1 %, respectively; total: 56.1 %). This probably reflects the difference in patient selection and ethnic diversity. Like previous studies, our study also showed that WIF1 and DKK-1 genes are epigenetic modulation targets in AML patients. Hou et al. showed that patients with FAB M0 subtype of AML had the highest incidence (100 %) of hypermethylation of Wnt inhibitors, whereas those with M4/M5 subtype had the lowest incidence (47.3%, P = 0.00006) 31. In addition, Hou et al. reported that DKK-1 methylation was also more common in FAB M0 subtype of AML (75 %, P = 0.0104) and WIF1 methylation was preferentially found in AML M1 and M3 (42.1 %, P = 0.0035 and 63.2 %, P = 0.0005, respectively) 31. Our results showed that aberrant methylation of these genes takes place in all FAB-AML subgroups including M0, M1, M2, M4, M5 and M6. Patients with FAB M0 subtype of AML had the highest incidence of hypermethylation of WIF1 (80 %, P = 0.003) and DKK-1 (70 %, P = 0.005); whereas those with M5 subtype had the lowest incidence of WIF1 (22.2 %, P = 0.288) and DKK-1 (11.11 %, P = 0.094) hypermethylation. Also Hou et al. pointed out that DKK-1 hypermethylation frequently occurs concomitantly with hypermethylation of SFRP family (P < 0.0001), but not Wif-1 (P = 0.7645) [31]. In this study, we did not observe any significant association between hypermethylation of these genes and conventional prognostic factors in AML like age and WBC count. Also no significant relationship was observed between methylation of these genes and other clinical parameters like sex, platelet count and hemoglobin, while that association might be seen with an increased sample size.

Among 120 patients, complete remission after induction chemotherapy was observed in 95 patients (79.1 %). Complete remission is defined with less than 5% blast cells in bone marrow and correction of blood cells count (neutrophil count more than 1000 cells per microliter, platelet count more than 100000 cells per microliter, hemoglobin more than 10 gr/dL and the absence of blast cells in peripheral blood), furthermore bone marrow cellularity of more than 20% should provide evidence of hematopoiesis of three cell lineages [32].

In the present study, no significant association was observed between hypermethylation of WIF1 and DKK-1 and complete remission after induction chemotherapy and the response to treatment was identical in patients with and without hypermethylation. However, Chim et al. [33] pointed out that WIF1 methylation was an independent poor prognostic factor for DFS, and Valencia et al. [34] showed that AML patients with two or more methylated Wnt inhibitor genes had poorer RFS,

but not OS, in the subgroup of patients 60 years or younger with intermediate-risk cytogenetics. Large-scale studies with more AML patients are needed to clarify this point.

Conclusion

The present study showed that, like many solid tumors, WIF1 and DKK-1 genes methylation also occurs in AML. The study of other antagonists of Wnt signaling pathways are recommended.

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