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Methylation of the Wnt Signaling Antagonist, Wnt Inhibitory Factor 1 and Dickkopf-1 Genes in Acute Myeloid Leukemia at the Time of Diagnosis

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Abstract

Background: In acute myeloid leukemia (AML), a large number of tumor suppressor genes are silenced through DNA methylation such as CDKN2B and p73. Wnt inhibitory factor 1 (WIF1) and Dickkopf-1 (DKK-1) are negative regulator of the Wnt signaling pathway.
Objectives: In the present study, we studied the methylation status of WIF1 and DKK-1 genes in AML patients.
Patients and Methods: In this case-control study, blood samples from 120 AML patients and 25 healthy control subjects collected, isolated DNA was treated with sodium bisulphite and examined by methylation specific PCR (MS-PCR) 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 AML patients determined 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 French-American-British (FAB)-AML had the highest incidence of hypermethylation of WIF1 (P = 0.003) and DKK-1 (P = 0.005) genes.
Conclusions: 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 pathway are recommended.

Keywords: Acute Myeloid Leukemia, Wnt Inhibitory Factor 1, Dickkopf-1, DNA Methylation

1. Background

Acute myeloblastic 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.

In AML, a large number of tumor suppressor genes are silenced through DNA methylation such as CDKN2B, P73 and suppressor of cytokine signaling 1 [2]. Epigenetic disorders in contrast to genetic changes are reversible and a role of the DNA demethylating agents such as 5-aza-2'-deoxycytidine has been established in the treatment of hematopoietic malignancies [3-6].

Investigation of molecular genetic alterations affecting NPM1 (nucleophosmin1) and FLT3 (FMS-like tyrosine kinase 3) genes as well as WT1 (Wilms' tumor) assay are known as important prognostic factors in AML, in addition to age, white blood cells count and cytogenetic aberrations. In recent years, epigenetic disorders including methylation of tumor suppressor genes such as Wnt inhibitory factor 1 (WIF1) and Dickkopf-1 (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 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 has been described [10]. β-catenin is an intracellular regulator of transcription that is associated with cancers. Wnt controls the cytoplasmic level and stability of β-catenin [11]. In

absence of Wnt ligand and its protection 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), activates DV1 (disheveled) proteins [13]. Once Wnt signaling is suppressed by DKK-1, β-catenin is phosphorylated and subsequently targeted for ubiquitination and degradation [7]. Having accumulated in cytoplasm, β-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 its 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 [16].

2. Objectives

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 WIF1 and DKK-1 genes in de novo AML patients at diagnosis.

3. Patients and Methods

In this case-control study, at the beginning of the study, informed consent was obtained from all groups. Blood samples were drawn from 120 AML patients at diagnosis and from 25 healthy individuals as negative control group. 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 producer instructions. By this treatment unmethylated cytosine 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 for investigate the methylation of CpG islands. In this method we used 2 pairs of primers specified for checking the methylated or unmethylated residue.

The following methylated Dkk-1-specific primers were used: (F) 5′-CGGAATGTTTCGGGTTCGC-3′ and (R) 5′-CACGAAACCGTACCGATTCG-3′. The following unmethylated Dkk-1-specific primers were used: (F) 5′-GTTGGAATGTTTTGGGTTTGT-3′ and (R) 5′-CCACAAAACCATACCAATTCA-3′.

WIF1 MSP primers are as follows: 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′, 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, administered for each patient. In methylation testing we used 2 μL of DNA previously treated with bisulfite, 4 μL of dH_2O, 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 dH_2O, 12 μL of Master Mix, 0.5 μL of forward primer, 0.5 μL of reverse primer and 0.5 μL of $MgCl_2$. In the first step of MSP, reaction components put in pre-thermal condition including 99°C for 1 minute and 95°C for 3 minutes followed by 35 cycles including 99°C for 10 seconds, 95°C for 30 seconds, 58°C for 30 seconds (WIF1- UM Primer), 62°C for 30 seconds (WIF1 and DKK-1-M Primer) and 70°C for 5 minutes (extension).

In this study, we used EpiTect PCR control DNA kit (Qiagen, Germani, Inc cat No. 59695) containing unmethylated and completely methylated DNAs as negative and positive controls, respectively. Electrophoresis on 3% agarose gel done in order to MSP product identification (Figure 1). Fisher's exact two-sided tests, Mann-Whitney U-tests and SPSS-21 analytic software (SPSS Inc Chicago, IL) were used to statistical analysis of data. P value ≤ 0.05 were considered significant statistically.

4. Results

One hundred twenty studied AML patients included 78 (65%) males and 42 (35%) females. The age ranges of patients were 15 to 72 years old the averages of which were 45 $\pm$10 years. White blood cells and platelets counts were 600 - 145000 and 2000 - 280000 cells/μL and their mean values were 27818.5 $\pm$ 250 and 98633.3 $\pm$ 530 cells/μL respectively. WIF1 gene found 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%). None of control individuals showed methylation in WIF1 and DKK-1 genes. Correlation between hypermethyaltion of WIF1 and DKK-1 genes and laboratory and clinical symptoms of patients are indicated in Table 1.

Table 1. Correlation Between Hypermethylation of Wnt Inhibitory Factor 1 (WIF1) and Dickkopf-1 (DKK-1) Genes With Laboratory and Clinical Symptoms of AML Patients[a]

Characteristics	WIF1			DKK		
	M	U	P Value	M	U	P Value
Number of patients, No. (%)	42 (35)	78 (65)	-	34	86	-
Age, y[a]	45.4 ± 5.2	39.6 ± 3	0.319	46 ± 3.2	57 ± 5	0.692
Sex, %						0.577
Male	30	48	0.217	25	65	
Female	12	30		9	21	
WBC count, 10^9/L[a]	14.5 ± 2	29.6 ± 4	0.242	64.1 ± 7	13.4 ± 2.1	0.182
Platelet count, 10^9/L[a]	115.4 ± 21.3	102 ± 18	0.630	91 ± 15.3	121 ±2 8.5	0.408
Hb level, g/dL[a]	8.3 ± 1.5	8.7 ± 1.2	0.190	8.3 ± 1.5	8.7 ± 1.2	0.096
FAB type, No. (%)						
M0	8 (19)	2 (2.5)	0.003	7 (20.5)	3 (3.4)	0.005
M1	10 (23.8)	16 (20.5)	0.817	7 (20.5)	19 (22)	0.999
M2	10 (23.8)	22 (28.2)	0.525	6 (17.6)	26 (30.2)	0.178
M4	6 (14.2)	16 (20.5)	0.466	8 (11.7)	21 (20.9)	0.303
M5	4 (9.5)	14 (17.9)	0.288	4 (5.8)	18 (18.6)	0.094
M6	4 (7.1)	8 (11.5)	0.538	2 (5.8)	10 (11.6)	0.506
Outcome, No. (%)						
Complete remission	30 (71.4)	65 (83.3)	0.143	24 (70.5)	71 (82.5)	0.211
Death	3 (7.1)	2 (2.5)	0.137	1 (2.94)	4 (4.65)	0.999
Relapse	7 (16.6)	9 (11.5)	0.149	3 (8.82)	13 (15.11)	0.552

Abbreviations: AML, acute myeloblastic leukemia; FAB, French-American-British; Hb, Hemoglobin; M, Methylated; U, Unmethylated; WBC, White blood cell.
[a]Values are expressed as mean ± SD unless otherwise indicated.

In AML patients 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% (35 out of 120 patients) of patients showed methylated WIF1 and DKK-1 genes at the time of diagnosis (Table 1).

Aberrant methylations of these genes are 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 is no significant relationship between hypermethylation of WIF1 and DKK-1 genes with clinical parameters of patients including sex, age, white cell and platelet (Table 1).

Sixteen out of 120 patients developed relapse that 7 patients (16.6%) attributed to WIF1 gene and 3 patients (8.82%) for DKK-1 gene were hypermethylated. There is no any significant relationship between hypermehylation of both WIF1 and DKK-1 genes and relapse of patients. Also, information on the treatment of 109 patients (90.8%) were found, of these number, 95 patients (79.1%) had complete remission after induction chemotherapy. Of which 30 and 24 patients were hypermethylated in the WIF1 and DKK-1 genes. 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 is no significant relationship between hypermethylation in the WIF1 and DKK-1 genes among patients who developed whether methylation or not and complete remission after induction chemotherapy.

5. Discussion

The results of this study showed that hypermethylation of WIF1 and DKK-1 genes occur with a frequency of 35% (42 out of 120 subjects) and 28.3% (34 out of 120 patients) in AML patients at the time of diagnosis respectively.

While none of the normal blood samples revealed methylation. 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 roles of Wnt/β-catenin signaling in survival, proliferation and

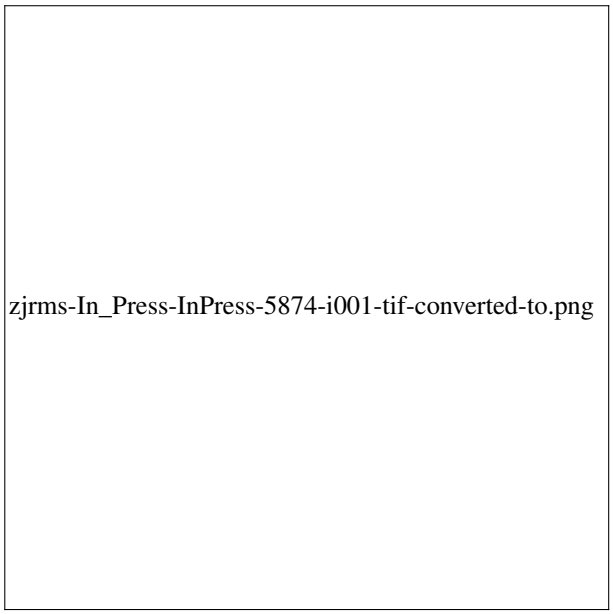

Figure 1. MSP analysis of A, WIF1; and B, DKK-1 in four AML patients. PC, positive contro; NC, negative control; P, patient; M, methylated; U, unmethylated and dH_2O, served as a blank control.

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. Those proteins bind to Wnt protein and thus inhibits 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].

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/β-catenin signaling antagonist DKK-1 is associated with poor survival in gastric cancer [24]. Epigenetic disorders, in contrast to genetic changes, are reversible and a role of the DNA demethylating agents such as 5-aza-2'-deoxycytidine has been established in the treatment of hematopoietic malignancies [3-6]. Cooper et al. 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 difference 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], 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 and DKK-1 genes in this study was 87 patients (72.5%) for WIF1 and 74 patients (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 DKK-1 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 reported by Hou et al. (26% and 30.1% respectively; total: 56.1%) [31]. These probably reflect 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 which inactivated following methylation. 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%) [31].

In addition, Hou et al. reported that DKK-1 methylation was also more common in FAB M0 subtype of AML (75%) and WIF1 methylation was preferentially found in AML M1 AND M3 (42.1% and 63.2% respectively) [31]. Our results showed that aberrant methylation of these genes toke 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%) and DKK-1 (70%) whereas those with M5 subtype had the lowest incidence WIF1 (22.2%) and DKK-1 (11.11%). Also, Hou et al. pointed out that DKK-1 hypermethylation frequently occurred concomitantly with hypermethylation of SFRP family but not WIF1 [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 may be associated to 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 $< 5\%$ blast cells in bone marrow and correction of blood cells count (neutrophil count $> 1000/\mu$L, platelet count $> 100000/\mu$L, hemoglobin > 10 g/dL while absence of blast cells in peripheral blood), furthermore bone marrow cellularity more than 20% should provide evidences on 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. pointed out that WIF1 methylation was an independent poor prognostic factor for event-free survival (EFS) and Valencia et al. showed AML patients with two or more methylated Wnt inhibitor genes had poorer relapse-free survival (RFS), but not overall survival (OS), in the subgroup of patients 60 years or younger with intermediate-risk cytogenetics by multivariate analysis [33, 34]. Large-scale studies with more AML patients are needed to clarify this point. In summary, our data shows that CpG island methylation of WIF1 and DKK-1 genes is a common event in AML patients and the study of other antagonists of Wnt signaling pathway are recommended.

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