Introduction

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 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 and p73. Wnt inhibitory factor 1 (WIF1) and Dickkopf-1 (DKK-1) are negative regulator of the Wnt signaling pathway. In the present study, we studied the methylation status of WIF1 and DKK-1 genes in AML patients.

Materials 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.

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 pathway are recommended.

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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]. 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.

Materials 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’-CGGAATGTGTTCCGGTTGCG-3’ and (R) 5’-CACGAAAACGTACCGATTGCG-3’. The following unmethylated Dkk-1-specific primers were used: (F) 5’-GCCGAGAACGGTACCGATTGCG-3’ and (R) 5’-CCACAAACACCATACCAAATC-3’.

WIF1 MSP primers are as follows: unmethylated (U) allele-specific primers (F) 5’-TGTT ATT TAG GTT GGG AGG TGA TGT-3’ and (R) 5’-AAC CTC CAC CCA CTTG AT 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 dH2O, 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 dH2O, 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 put in pre-thermal condition including 99°C for 1 min and 95°C for 3 min 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 min (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 (Fig. 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.

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±10 years. White blood cells and platelets counts were 600-145000 and 2000-280000 cells/µL and their mean values were 27818.5±250 and 98633.3±530 cells/µL respectively. WIFI 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 WIFI and DKK-1 genes. Correlation between hypermethylation of WIFI and DKK-1 genes and laboratory and clinical symptoms of patients are indicated in table 1.

In AML patients hypermethylation frequency of WIFI 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 WIFI 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 WIFI (*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 WIFI and DKK-1 genes with clinical parameters of patients including sex, age, white cell and platelet (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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>M</th>
<th>U</th>
<th>p-Value</th>
<th>M</th>
<th>U</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>42 (35)</td>
<td>78 (65)</td>
<td>-</td>
<td>34</td>
<td>86</td>
<td>-</td>
</tr>
<tr>
<td>Age (mean±SD) (y)</td>
<td>45.4±5.2</td>
<td>39.6±3</td>
<td>0.319</td>
<td>46±3.2</td>
<td>57±5</td>
<td>0.692</td>
</tr>
<tr>
<td>Sex (%)</td>
<td>Male</td>
<td>30</td>
<td>0.217</td>
<td>25</td>
<td>65</td>
<td>0.577</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>30</td>
<td>9</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>WBC count (10^3/L)</td>
<td>14±2</td>
<td>29.6±4</td>
<td>0.242</td>
<td>64.1±7</td>
<td>13.4±2.1</td>
<td>0.182</td>
</tr>
<tr>
<td>Platelet count (10^3/L)</td>
<td>115.4±21.3</td>
<td>102±18</td>
<td>0.630</td>
<td>91±15.3</td>
<td>121±28.5</td>
<td>0.408</td>
</tr>
<tr>
<td>Hb level (g/dL)</td>
<td>8.3±1.5</td>
<td>8.7±1.2</td>
<td>0.190</td>
<td>8.3±1.5</td>
<td>8.7±1.2</td>
<td>0.096</td>
</tr>
<tr>
<td>FAB type [N%]</td>
<td>M0</td>
<td>8 (19)</td>
<td>2 (2.5)</td>
<td>0.003</td>
<td>7 (20.5)</td>
<td>3 (3.4)</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>10 (23.8)</td>
<td>16 (20.5)</td>
<td>0.817</td>
<td>7 (20.5)</td>
<td>19 (22)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>10 (23.8)</td>
<td>22 (28.2)</td>
<td>0.525</td>
<td>6 (17.6)</td>
<td>26 (30.2)</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>6 (14.2)</td>
<td>16 (20.5)</td>
<td>0.466</td>
<td>8 (11.7)</td>
<td>21 (20.9)</td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>4 (9.5)</td>
<td>14 (17.9)</td>
<td>0.288</td>
<td>4 (5.8)</td>
<td>18 (18.6)</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>4 (7.1)</td>
<td>8 (11.5)</td>
<td>0.538</td>
<td>2 (5.8)</td>
<td>10 (11.6)</td>
</tr>
<tr>
<td></td>
<td>Complete remission</td>
<td>30 (71.4)</td>
<td>65 (83.3)</td>
<td>0.143</td>
<td>24 (70.5)</td>
<td>71 (82.5)</td>
</tr>
<tr>
<td>Outcome [N%]</td>
<td>Death</td>
<td>3 (7.1)</td>
<td>2 (2.5)</td>
<td>0.137</td>
<td>1 (2.94)</td>
<td>4 (4.65)</td>
</tr>
<tr>
<td></td>
<td>Relapse</td>
<td>7 (16.6)</td>
<td>9 (11.5)</td>
<td>0.149</td>
<td>3 (8.82)</td>
<td>13 (15.11)</td>
</tr>
</tbody>
</table>


Sixteen out of 120 patients developed relapse that 7 patients (16.6%) attributed to WIF1 gene and 3 patients (8.22%) for DKK-1 gene were hypermethylated. There is no any significant relationship between hypermethylation 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.

**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 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]. Ablerrant 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;
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 WIFI 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 WIFI 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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Conflict of Interest
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