Promoter Methylation of p16 and EDNRB Gene in Leukemia Patients in Taiwan

Pei-Ching Hsiao¹, Min-Chang Liu², Li-Mien Chen³, Ching-Yi Tsai¹,⁵, Yu-Ting Wang⁴, Jung Chen⁶, and Li-Sung Hsu⁴

¹Department of Internal Medicine
Chung Shan Medical University Hospital
Taichung
²Department of Pediatrics
Chi-Mei Foundation Hospital
Liouying, Tainan
³Division of Hematology/Oncology, and Department of Internal Medicine
Armed Forces Taichung General Hospital
Taiping, Taichung
⁴Institute of Biochemistry and Biotechnology
Chung Shan Medical University
Taichung
⁵Department of Laboratory Examine
Armed Forces Taichung General Hospital
Taiping, Taichung
and
⁶Department of Biomedical Sciences
Chung Shan Medical University
Taichung, Taiwan, Republic of China

Abstract

Both epigenetic and genetic alternations are involved in cancer formation. In this study, we have identified the methylation frequency of p16 and endothelin receptor type B (EDNRB) of 26 leukemia patients and 8 randomly selected normal blood donors in Taiwan. Promoter methylation of p16 was detected in 85% of acute lymphocytic leukemia (ALL), 83% in acute myeloid leukemia (AML) whereas no methylation was detected in chronic myeloid leukemia (CML) in blast crisis. Hypermethylation of EDNRB was observed in 92% of ALL, 75% AML and 100% in CML in blast crisis. No aberrant methylation of p16 and EDNRB was found in 8 normal blood donors. Taken together, aberrant methylation of p16 and EDNRB was highly prevalent in leukemia patients in Taiwan.

Key Words: p16, EDNRB, methylation, leukemia

Introduction

Cancer formation is a multistep process in which defects in a wide range of cancer genes accumulate (1). Eventually every cancer receives an enormous complexity of altered gene functions, including activation of proto-oncogene as well as silences of genes with tumor-suppressing function (18). Genetic alternations including mutation, deletion, and DNA amplification have been shown to play an important role in tumorigenesis (19); however, the genetic abnormalities found in cancers will not provide the whole picture of genomic alternations. Epigenetic alternation of the DNA such as methylation of CpG island in promoter region or histone modification do not alter sequence code. Instead, they participated in
the regulation of gene expression that is now recognized as an additional method to be involved in tumorigenesis (20, 24, 26). Methylation of cytosine residue at CpG dinucleotides in mammalian genomes is found to have significant effect on gene expression (2, 9).

The \( p16 \) protein is encoded by the \( CDKN2 \) gene and functions as an inhibitor of cyclin dependent kinase 4 and 6 (\( CDK4/6 \)) (28). Hypermethylation of the \( p16 \) tumour suppressor gene and subsequent transcriptional silencing has been implicated as an additional mechanism of \( p16 \) gene inactivation in diverse types of cancer including gastric cancer, lung cancer, colon cancer, thyroid carcinoma, and hepatic carcinoma (7, 10, 12, 29, 30). Methylation of \( p16 \) gene correlated with decrease expression in human gastric cancer (29), and hypermethylation of \( p16 \) is in the early stage of hepatic cell carcinomas and associated with hepatitis B virus infection (30).

The \textit{endothelin receptor type B (EDNRB)} gene plays an important role in vaso-constriction (3). Evidence has been shown that the 5’ flanking region of \( EDNRB \) contains numerous CpG dinucleotide repeat and the methylation of these CpG sites can regulate gene expression (8). Recently, using the arbitrarily primed PCR (AP-PCR) technique, it has been found that the 5’ region of \( EDNRB \) is found to be hypermethylated in cancer as compared with normal blood cells (WBC) (35). Pao et al. demonstrated that the \( EDNRB \) is unmethylated in normal bladder and prostate tissue whereas \( EDNRB \) is hypermethylated in tumor compared to normal tissue (25). Silencing of \( EDNRB \) gene expression mediated through promoter hypermethylation also has been identified in nasopharyngeal carcinoma and melanoma (8, 23). Promoter methylation of \( EDNRB \) was found in hepatocellular carcinoma and lung cancer in Taiwan region (4, 17). The high frequency of promoter hypermethylation suggested that down-regulation of \( EDNRB \) gene may involve in human tumorigenesis.

Leukemia was developed from unbalanced haematopoetic cells proliferation and death. Now, many genetic and epigenetic alternations were found to play an important role in leukemia tumorigenesis. Elevated DNA methyltransferase expression level was shown in acute myeloid leukemia. Promoter hypermethylation of several tumor suppressor genes such as \( p16, p15, E-cadherin \) were also found in different type of leukemias (11, 13). In this study, we demonstrated the aberrant methylation of \( EDNRB \) and \( p16 \) gene in leukemia samples. High frequency of hypermethylation of \( p16 \) and \( EDNRB \) was found in 80% and 88% of total samples, respectively. Our result suggested that promoter methylation of these two genes plays an important role in leukemia tumorigenesis.

### Materials and Methods

#### Preparation of Genomic DNA from Leukemia Patients

Peripheral blood samples were aspirated from 26 patients present with leukemia at Chung Shan Medical University Hospital. Eight normal control peripheral blood samples also enrolled in this study. Genomic DNA was isolated using TriZOL Reagent (Invitrogen) according to the manufacturer’s recommendation.

#### Bisulfite Modification of Genomic DNA

Bisulfite modification of genomic DNA was performed (16). Briefly, DNA (1 \( \mu \)g) in a volume of 50 \( \mu l \) was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. For samples with nanogram quantities of human DNA, 1 \( \mu \)g of salmon sperm DNA (Sigma) was added as carrier before modification. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 \( \mu l \) of 3M sodium bisulfite (Sigma) at pH 5, both freshly prepared, were added and mixed, and samples were incubated at 50°C for 16 hrs. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega, Madison, WI, USA) and eluted into 50 \( \mu l \) of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in Tris-EDTA buffer and used immediately or stored at -20°C.

#### Methylation Specific PCR Amplification

Methylation-specific primers and PCR reactions were performed as previously described. Briefly, PCR reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of Taq DNA polymerase (BRL). Amplification was carried out in a temperature cycler for 35 cycles, 30 sec at 95°C, annealing temperature (4, 17), and 30 sec at 72°C, followed by a final 4 min extension at 72°C. Controls without DNA were performed for each set of PCRs. Each PCR (10 \( \mu l \)) was directly loaded onto 3% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

#### Statistical Analysis

Chi-square test was used to analyze the association between promoter methylation status of \( EDNRB \) or \( p16 \) and clinicopathological features.

### Results

#### Promoter Hypermethylation of \( p16 \) and \( EDNRB \) in Leukemia Patients

To determine the aberrant promoter methylation of \( p16 \) and \( EDNRB \) gene in leukemia patients, we have
Methylation of P16 and EDNRB in Leukemia

performed methylation-specific PCR. Promoter hypermethylation of p16 gene and EDNRB was found in 20 (80%, 20/25) and 23 (88%, 23/26) of the samples, respectively (Fig. 1 and Fig. 2). No hypermethylation was detected in 8 normal control samples. Our data demonstrated that significant higher frequency of aberrant methylation of p16 and EDNRB was observed in cancer patients compared to normal donors (P < 0.0001).

Table 1. Correlation between methylation of p16 and EDNRB with clinical characteristics in leukemia patients

<table>
<thead>
<tr>
<th></th>
<th>p16 methylation</th>
<th>p</th>
<th>EDNRB methylation</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total samples</td>
<td>80% (20/25)</td>
<td>0.23</td>
<td>88% (23/26)</td>
<td>0.51</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytic leukemia</td>
<td>86% (12/14) 0.28*</td>
<td>92% (12/13) 0.44*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>85% (11/13)</td>
<td></td>
<td>92% (11/12)</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>100% (1/1)</td>
<td></td>
<td>100% (1/1)</td>
<td></td>
</tr>
<tr>
<td>Myeloid leukemia</td>
<td>67% (6/9)</td>
<td>82% (9/11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>83% (5/6)</td>
<td>75% (6/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>33% (1/3)</td>
<td>100% (3/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>100% (2/2)</td>
<td>100% (2/2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>87% (13/15)</td>
<td>81% (13/16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>100% (10/10)</td>
<td>70% (7/10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.42</td>
<td></td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>&lt; 25</td>
<td>86% (12/14)</td>
<td>80% (12/15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 25</td>
<td>73% (8/11)</td>
<td>100% (11/11)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P indicated P-value
*compared the percentage of lymphocytic leukemia and myeloid leukemia

Clinicopathological Correlations with Promoter Hypermethylation

The association between aberrant methylation and clinicopathological characteristics of patients was summarized in Table 1. Promoter methylation of p16 was found in 85% (11 of 13) of acute lymphocytic leukemia (ALL), 83% of acute myeloid leukemia
(AML) and one atypical chronic myeloid leukemia (CML) whereas no methylation was detected in two CML in blast crisis. Hypermethylation of EDNRB was observed in 92% (11 of 12) of ALL, 75% (6 of 8) in AML and 100% (3 of 3) CML including atypical and blast crisis. Nearly 77% of leukemia samples harbored concurrent methylation of both p16 and EDNRB. Moreover, the age of three unmethylation samples of EDNRB were under 25. However, aberrant methylation of EDNRB and p16 was not related to tumor type, gender, and age.

**Discussion**

Epigenetic alternations mainly occur in the promoter methylation of CpG islands which rendered tumor suppressor genes to be silenced plays an important role in haematological tumorigenesis (27). In this study, we demonstrated that the aberrant methylation of p16 and EDNRB genes was highly prevalent in leukemia in Taiwan region. Promoter methylation of p16 gene was a common event in a wide range of tumors and was a good prognosis factor of specific tumors such as gastric cancer (33) and large B-cell lymphomas (31). Aberrant methylation of p16 was also found in several types of leukemia (15). No methylation of p16 was found in AML and only 6% was found in ALL in China region (6). Chim et al. have shown that aberrant methylation of p16 gene was detected in 14.3% of chronic lymphocytic leukemia and there was no association between age, sex and overall survival in Chinese patients (5). In this report, our results demonstrated that over 80% leukemia patients harbored p16 hypermethylation which reflected distinct methylation frequency of p16 in different region.

Recently promoter methylation of EDNRB gene has been shown in several human tumors (4, 17). The prevalence of EDNRB methylation (88%) in leukemia was higher, compared to other human tumors (32, 36, 37). This difference may arise from the tissue-specific methylation status. The correlation between EDNRB methylation and clinic pathological characteristics has been shown in several reports (32, 36, 37). Aberrant promoter methylation of EDNRB gene was found both in normal and tumor tissues of prostate cancer and medulloblastoma (21, 22). Recently, promoter hypermethylation of lung and hepatocellular carcinoma has also been shown not to be associated with any clinical features. In contrast, Woodson et al. found that EDNRB methylation correlates with the stage of prostate cancer but not with the tumor grade by using different primer sequences (36). Similarly, aberrant methylation of EDNRB if found to correlate with the pathological stage and Gleason score of primary prostate cancers (37). In this study, our results also indicated that promoter methylation in leukemia patients was not correlated to clinical characteristics. Moreover, consistent with previous report demonstrated that promoter hypermethylation of p73, p57, and p15 was significantly higher in adult ALL than in children ALL (14), our result demonstrated that promoter methylation of EDNRB was slightly higher in older than in young age. In contrast, methylation of MDR1 has inverse correlation with age in ALL (34). These reports suggested that methylation frequency in the elder group of leukemia patients was in a gene-specific manner; however, the exact mechanism of age in hypermethylation still remained to be elucidated. In conclusion, aberrant methylation of p16 and EDNRB appears to be a common event during leukemia tumorigenesis, but, the methylation status was not correlated with any clinical features. Finally, the functional consequences of down regulation of EDNRB in leukemia are still unclear.

**Acknowledgments**

This work was supported by Grant CMSU 92-OM-B-028 and CMSU 92-OM-B-020.

**References**

12. Gazzeri, S., Gouyer, V., Vourch, C., Brambilla, C. and Brambilla,