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

Pei-Ching Hsiao[1], Min-Chang Liu[2], Li-Mien Chen[3], Ching-Yi Tsai[4, 5], Yu-Ting Wang[6], Jung Chen[6], and Li-Sung Hsu[4]

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

Corresponding author: Dr. Li-Sung Hsu, Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 402, Taiwan, ROC. Tel: +886-4-24730022 ext. 11685, Fax: +886-4-23248195, E-mail: lshsu405@yahoo.com.tw or lsh316@csmu.edu.tw
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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 *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 μg) in a volume of 50 μl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. For samples with nanogram quantities of human DNA, 1 μg of salmon sperm DNA (Sigma) was added as carrier before modification. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 μl 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 μl 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 μl) 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 methyaltion 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

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

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

P indicated *P*-value

*compared the percentage of lymphocytic leukemia and myeloid leukemia

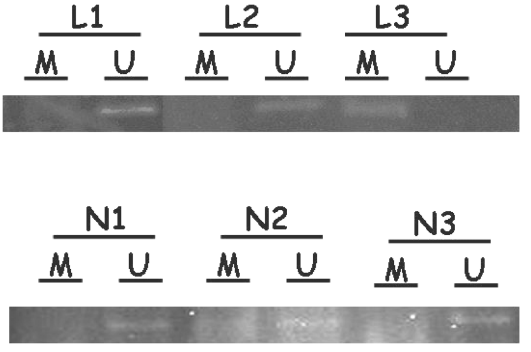

Fig. 1. Methylation-specific PCR analysis *p16* of leukemia samples. Genomic DNA derived from leukemia samples underwent MS-PCR using primer specific for *p16*. PCR products were separated on 3% agarose gel, stained with ethidium bromide and visualized under UV illumination. U represents amplification of unmethylation alleles and M represents methylated alleles. The numbers shown were sample identification numbers. N indicated the normal control.

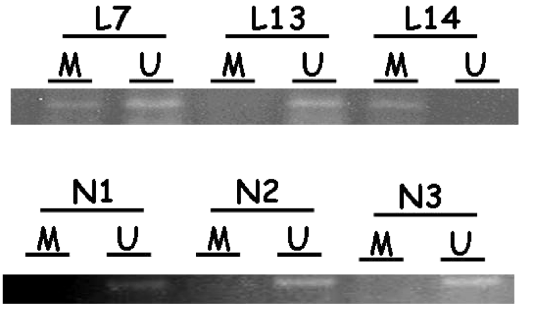

Fig. 2. Methylation-specific PCR analysis of *EDNRB* of leukemia patients. Genomic DNA derived from HCC samples underwent MS-PCR using primer specific for *EDNRB*. PCR products were separated on 3% agarose gel, stained with ethidium bromide and visualized under UV illumination. U represents amplification of unmethylation alleles and M represents methylated alleles. The numbers shown were sample identification numbers. N indicated the normal control.

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 ($P < 0.0001$).

Clinicopathological Correlations with Promoter Hypermethylation

The association between aberrant methylation and clinicopathlogical 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 import 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.

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