Aberrant methylation of *EDNRB* and *p16* genes in hepatocellular carcinoma (HCC) in Taiwan

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Abstract. Epigenetic alternation via the promoter hypermethylation of putative tumor suppressor genes has been implicated in the development of hepatocellular carcinoma (HCC). In this study, we investigated the epigenetic changes in two candidate tumor suppressor genes, endothelin receptor type B (*EDNRB*) and *p16*, and their relation to the expression of these two genes in HCC. Methylation-specific polymerase chain reaction (MS-PCR) was performed to analyze the promoter methylation status of the *EDNRB* and *p16* genes in tumors and paired non-tumor liver portions of 34 HCC patients. The mRNA expression was assessed by reverse transcription-PCR assay. Hypermethylation of the *EDNRB* and *p16* genes was detected in 29.4% (10/34) and 32.3% (11/34) of HCC patients, respectively. Moreover, the reduction of mRNA expression was correlated to the promoter hypermethylation of the *EDNRB* and *p16* genes. In conclusion, aberrant methylation of *EDNRB* and *p16* genes is highly prevalent in HCC. It suggested that epigenetic alteration of the *EDNRB* and *p16* genes may play an important role in the pathogenesis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent disease in many areas of the world, particularly the Far East and sub-Saharan Africa, where it is strongly associated with HBV and HCV infection and chronic exposure to aflatoxin B1-contaminated food (1).

Accumulating evidence supports the involvement of a multi-step process including inactivation of tumor suppressor genes and activation of proto-oncogenes in the development of HCC (2,3). In addition, epigenetic alternation via the promoter hypermethylation of putative tumor suppressor genes has been implicated in the development of HCC (4,5).

The endothelin receptor type B (*EDNRB*) gene encodes a protein that belongs to the superfamily of G-protein coupled receptor mediated endothelin-induced development and transformation of the neural crest cell-specific lineage (6) and is involved in Hirschsprung's disease (7,8). Recently, using arbitrarily primed polymerase chain reaction (AP-PCR), it has been found that the 5' region of *EDNRB* is hypermethylated in cancer as compared to normal blood cells (WBCs) (9). Furthermore, *EDNRB* is unmethylated in normal bladder and prostate tissues but is hypermethylated in tumors (9). Treatment with 5-aza-2'-deoxycytidine (5-Aza-dC), a DNA demethylation agent (10), induces expression of *EDNRB* in T24 cancer cells (9). Promoter hypermethylation-mediated silencing of *EDNRB* expression has also been identified in nasopharyngeal carcinoma and prostate cancer (11,12). Smith *et al* demonstrated that expression of *EDNRB* was lower in four primary small cell lung carcinomas than in normal bronchial epithelium (13). The high frequency of promoter hypermethylation in cancers supports the suggestion that down-regulation of this gene may be involved in human tumorigenesis.

The *p16* gene encodes a cyclin-dependent protein kinase (CDK) inhibitor. The protein binds to CDK4/6, inhibiting CDK4/6-mediated activities and, thus, regulating cell cycle arrest in the G1 phase (14). Inactivation of *p16* and the resultant defective cell cycle control have been observed in various types of tumorigenesis (15-17). Point mutation and loss of heterozygosity of *p16* was suggested to be involved in the pathogenesis of HCC (18). In addition, the promoter hypermethylation of *p16* leading to its inactivation has been demonstrated in HCC patients (19,20) and was found in the early stage of HBV infection (21).

Epigenetic alterations of tumor suppressor genes have been suggested to play an important role in hepatocarcinogenesis (22). To investigate the roles of *EDNRB* and *p16* in HCC, we performed methylation-specific PCR (MS-PCR) (23) to determine the promoter methylation status of *p16* and *EDNRB* in human HCC. Our observations support that promoter hypermethylation-mediated silencing of *EDNRB* and *p16*

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Table I. Correlation between methylation status and clinocopathological features of HCC patients.

	Methylation of *EDNRB* (%)	p-value	Methylation of *p16* (%)	p-value
Gender				
Male	4/15 (26.7)		4/15 (26.7)	
Female	6/19 (31.5)	0.96	7/19 (36.8)	0.53
Age				
<50	1/9 (11)		1/9 (11)	
>50	10/25 (40)	0.11	9/25 (36)	0.16
Stage				
I and II	8/25 (32.0)		8/25 (32.0)	
III and IV	2/9 (22.2)	0.58	3/9 (33.3)	0.94
Virus infection				
HBV	6/20 (30.0)		6/20 (30.0)	
HCV	2/11 (18.2)[a]	0.47	4/11 (36.4)	0.72[a]
Non-B/non-C	2/3 (66.7)[a]	0.21	1/3 (33.3)	0.91[a]

[a]p-value compared to HBV group.

expression is likely to contribute towards the carcinogenesis and development of HCC.

Materials and methods

HCC sample collection. Tumor tissues and corresponding non-tumor liver portions from 34 HCC patients were obtained after surgical resection from Taipei Veterans General Hospital. All samples were immediately fresh-frozen in liquid nitrogen and stored at -70˚C until use. The 34 pairs of HCC specimens from 15 men and 19 women (ranging in age from 26 to 75), included two stage I lesions, 23 stage II lesions, six stage III lesions, and three stage IV lesions. Serology testing indicated that 20 patients were HBV-infected, 11 cases were HCV-positive, and three were free from hepatitis virus infection.

Sodium bisulfite modification of genomic DNA. Genomic DNA derived from HCC samples was purified using the QIAamp DNA mini kit (Qiagen, Valencia, CA). One μg of genomic DNA was subjected to bisulfite modification as previously described (23). The modified DNA was purified using the Gene-Spin™ 1-4-3 DNA extraction kit (Protech Technology, Taipei, Taiwan) according to the manufacturer's protocol, followed by ethanol precipitation, and eluted into 50 μl of distilled water. The final preparation was stored at -20˚C until use. The promoter methylation status of *EDNRB* and *p16* was determined by methylation-specific PCR (MS-PCR). Bisulfite-modified DNA was amplified by primer sets specific for unmethylated and methylated *EDNRB* and *p16B* sequences, respectively (11,23). Negative control (water instead of DNA) was included in each experiment. The PCR products were separated by 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

RT-PCR amplification of EDNRB and p16 from HCC samples. Total RNA derived from 20 paired samples was isolated using TriZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (10 μg) was converted into cDNA by using Superscript II MMLV-reverse transcriptase (Gibco-BRL, Gaithersburg, MD). Expression of *EDNRB* (three isoforms, Δ1, Δ2, and Δ3) and *p16* in HCC samples was examined using RT-PCR as previously described (24,25). The mRNA expression of the ß2-microglobin gene was amplified as internal control.

Statistical analysis. The χ^2 test was applied to the association between promoter methylation status of *EDNRB* or *p16* and clinicopathological features, such as age, gender, and virus-infection status.

Results

Methylation status of EDNRB and p16 in HCC. MS-PCR was conducted using the 34 primary HCC samples. The promoter methylation status of CpG islands is summarized in Table I.

Promoter methylation of *EDNRB* was detected in 10 of 34 (29.4%) tumor tissue samples and in 5 of 34 (14.3%) adjacent non-malignant tissue samples. Aberrant *p16* methylation was also observed. These aberrations were significantly different in tumor tissue (11 of 34 samples; 32.3%) versus non-malignant tissue (3 of 34 samples; 9%) (p=0.02) (Fig. 1). Hyper-methylation of both genes was detected in six patients (6/34, 17.6%).

Expression of the EDNRB and p16 genes in HCC. Promoter hypermethylation is known to silence gene expression (26). To assess whether the promoter methylation status correlated with the mRNA expression of *EDNRB* and *p16*, RT-PCR was used to detect the mRNA levels in the HCC samples. As shown in Fig. 2, both mRNA transcripts were reduced in hypermethylated tumor tissue compared to correlated non-

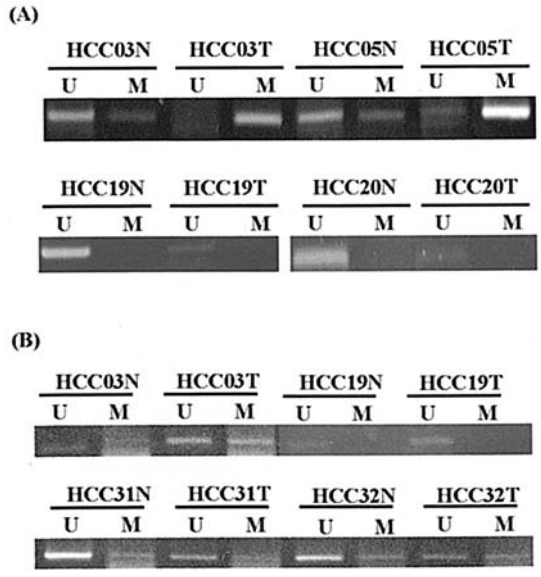

Figure 1. Methylation-specific PCR analysis of EDNRB and p16 of HCC. Paired genomic DNA derived from HCC samples underwent MS-PCR using primer specific for p16 (A) and EDNRB (B). PCR products were separated on 3% agarose gel, stained with ethidium bromide and visualized under UV illumination. U, amplification of unmethylated alleles; M, methylated alleles. The numbers shown are sample identification numbers.

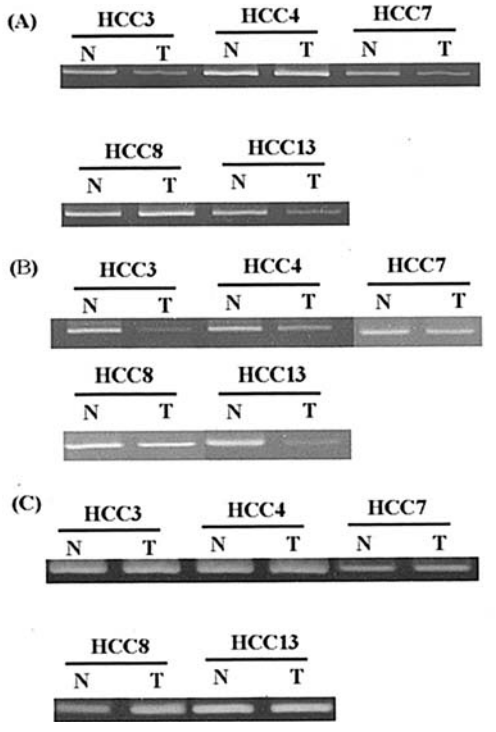

Figure 2. Reverse-transcription-PCR analysis of EDNRB and p16 gene expression levels in HCC samples. cDNAs derived from paired HCC patients were subjected to PCR analysis using specific primer sets for p16 (A) and EDNRB (B). A specific primer for microglobin was used as internal control (C).

tumor tissue, whereas no significant difference was observed in the non-hypermethylated paired samples.

Clinicopathological correlations with promoter hypermethylation. The association between the aberrant methylation status of *EDNRB* and *p16* and the clinicopathological characteristics of patients (including age, gender, tumor stage and virus-infection status) is summarized in Table I. The methylation status of *EDNRB* and *p16* was not related to gender, tumor stage or virus infection.

Discussion

Epigenetic alterations including promoter hypermethylation play an important role in hepatotumorigenesis (22). A growing number of tumor suppressor genes including *APC*, *E-cadherin*, *GSP*, and *p16* undergo CpG island hypermethylation during hepatocarcinogenesis (4,5).

In the present study, we observed that aberrant hypermethylation of *EDNRB* and *p16* is a frequent event in HCC samples obtained from patients of Taiwanese origin. To our knowledge, this is the first report of *EDNRB* methylation in HCC. No association was observed between the frequency of *EDNRB* and *p16* methylation and gender, tumor grade or virus-infection status.

Recently, the down-regulation of *EDNRB* has been suggested to be linked with tumorigenesis (9,11,27,28). The prevalence of *EDNRB* methylation (29.4%) in HCC (Table I) was substantially lower than that found in prostate cancers (29-83.3%) (27-29) and nasopharyngeal carcinoma (90.5%) (11). This difference may have resulted from the tissue-specific methylation status. Indeed, the discrepant methylation frequencies in different tumor types may reflect the different roles of distinct tumor suppressor genes in carcinogenesis and tissue specificity. For instance, *MLH1* is methylated in colorectal and gastric cancer but is infrequently methylated in HCC (30-32). Epigenetic epidemiological studies have revealed that the methylation frequency of *GSTP1* and *MGMT* display geographic variation. We cannot exclude a similar geographic variation from the present study.

Accumulating reports have focused on the correlation between *EDNRB* methylation and disease stages (27-29). Jeronimo *et al* evaluated the methylation status of *EDNRB* by performing MS-PCR using primers located -9 to -139 and found methylation of *EDNRB* in both normal and tumor tissues in 40 of 48 prostate tissue specimens (12). Moreover, *EDNRB* methylation in medulloblastoma reflects a normal level of tissue-specific methylation rather than a tumor-related event (33). On the other hand, Woodson *et al* found that *EDNRB* methylation correlates with the stage of prostate cancer but not with the tumor grade (29). It was recently demonstrated that aberrant methylation of *EDNRB* correlates with the pathological stage and Gleason score of primary prostate cancer (27).

The discrepancies in results may have risen from the use of different primer sets. Using the primer sets corresponding to the *EDNRB* promoter region (11), we presently revealed that the gene's methylation status is not correlated with tumor grade. Our results support the suggestion that methylation of *EDNRB* may not be a good candidate for distinguishing clinical stages in HCC patients.

Hypermethylation of *p16* has been well demonstrated with variable frequency in HCC and is suggested to occur in the early stage of HCC in HVB-infected patients (3,19,21). Aberrant methylation of *p16* has been detected in 47% of HCC tissue recovered from patients of Taiwanese origin and

correlates to aflatoxin B1-DNA adduct levels (34). We also presently observed a high prevalence of *p16* methylation. In contrast, Lin *et al* did not detect aberrant hypermethylation of CpG islands in *p16* and *p15* in 34 HCC samples from Taiwan (35).

Recent studies have implied that virus-associated epigenetic alternation and genetic alternation play roles in tumorigenesis (36,37). Silencing of several tumor suppressor genes through aberrant methylation has been found to relate to infection with HBV and/or HCV (21,38,39). Methylation of CpG islands in the promoter region of E-cadherin occurs predominantly in HBV-infected HCC (39). Using a methylation-specific PCR method, Zhong *et al* showed that GSTp1 CpG island hypermethylation is found in a majority of HBV-associated HCC patients (39). Kaneto *et al* also demonstrated promoter methylation of p16 in tumors positive for HBV or HCV and not in virus-negative tumors (40). However, in the present study, aberrant EDNRB methylation was slightly higher in virus-negative tumors than in HBV- or HCV-related tumors. We also observed promoter methylation of p16 in virus-negative tumors. Consistent with observations by Narimastu *et al* (41), we also found p16 hypermethylation in HCC with HBV or HCV and in virus-free HCC.

In conclusion, our data demonstrate that promoter hypermethylation of *EDNRB* and downregulation of *EDNRB* expression is highly prevalent in HCC patients. This prevalence is not associated with age, gender, clinical stage or virus infection. Aberrant methylation of *EDNRB* and *p16* appears to be a common event during hepatocarcinogenesis. The functional consequences of down-regulation of *EDNRB* in HCC await further investigation.

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