Promoter CpG Methylation of Caveolin-1 in Sporadic Colorectal Cancer

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Abstract. *Background: Caveolin-1 has been shown to be down-regulated in human colon cancer and involved in colon tumorigenesis. We investigated the mechanism. Materials and Methods: Cancerous and nearby non-cancerous tissues of 185 sporadic colorectal cancer samples were enrolled in this study. Methylation-specific PCR was performed to explore the mechanism of regulation of caveolin-1 gene expression. Results: Aberrant promoter methylation in the caveolin-1 gene was 3.8% and 5.9% for cancerous and nearby non-cancerous tissues, respectively. All the cancerous and non-cancerous tissue contained unmethylated promoters in the caveolin-1 gene. The methylation status of caveolin-1 had no clear relationship with age, cell grade, location of tumor or lymph node metastasis. However, female gender showed statistically significant difference (p=0.045). The immunohistochemistry study demonstrated that expression of caveolin-1 correlated with aberrant promoter methylation status in sporadic colorectal cancer tissues. Conclusion: Our findings suggested that aberrant promoter methylation of the caveolin-1 gene may occur at the precancerous stage, regulated by gender-related factors and is associated with gene silencing of caveolin-1 in the development of colorectal cancer.*

The caveolin proteins are the major integral protein components of membrane caveolae (1). Caveolin-1 has been shown to interact directly with and inhibit or sequester the inactive form of many key signaling molecules including H-Ras, EGF receptors, SRC family tyrosine kinases, protein kinase C and mitogen-activated protein kinases (2-5). Therefore, caveolin-1 may reduce cell tumorigenicity by inhibition of oncogenic signal molecules. Furthermore, down-regulation of caveolin-1 by antisense caveolin RNA is sufficient to transform NIH-3T3 (6) and re-expression of caveolin-1 abrogates the anchorage-independent growth in oncogene-transformed cells (7), suggesting that caveolin-1 may act as a candidate tumor suppressor gene.

DNA methylation is an enzyme-induced chemical modification of the DNA structure without alteration of the genomic sequences. In vertebrates, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide (8). DNA methylation in the CpG-rich areas known as CpG islands is associated with gene silencing and has been seen in X chromosome inactivation, genomic imprinting and embryogenesis (9-11). However, aberrant methylation of normally unmethylated CpG islands has been shown to occur frequently in immortalized and transformed cells (12) and has been associated with transcriptional inactivation of tumor suppressor genes (13,14). Therefore, analysis of the promoter CpG islands methylation pattern of the potential tumor suppressor genes has become an important issue in understanding the process of tumorigenesis.

Colorectal cancer is one of the leading causes of cancer-related morbidity and mortality in Western countries (15). In Taiwan, it is ranked the third most common cause of cancer mortality. Although intensive efforts have been made to study colorectal cancer, the molecular mechanism underlying its tumorigensis is still not clear. In accordance with caveolin-1 as a tumor suppressor gene, caveolin-1 levels are reduced in a variety of cancer cell lines or human cancers including colon cancer (16,17). However, no mutations were found in human cancer cells (18), suggesting

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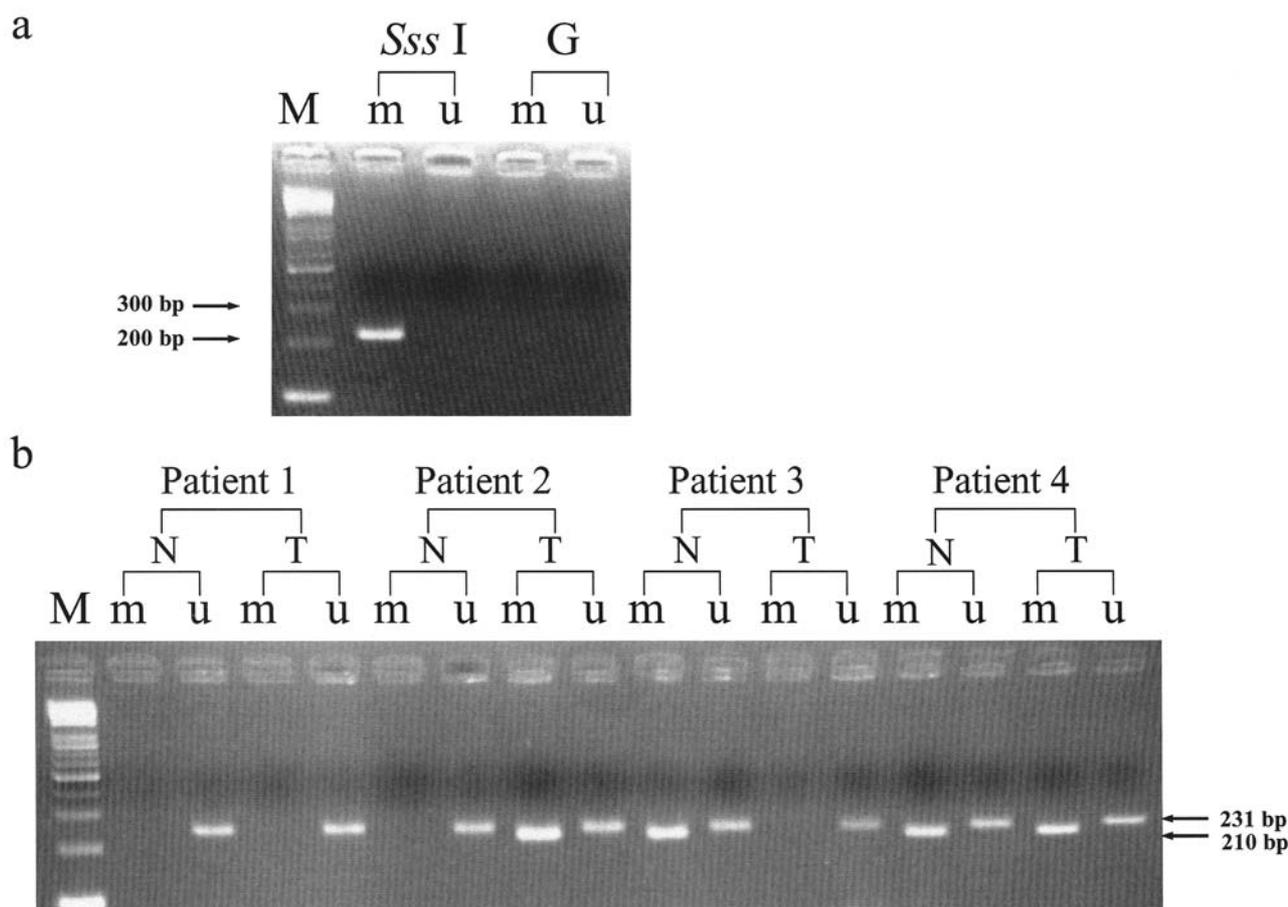
Figure 1. *Methylation-specific PCR analysis of caveolin-1 in normal colon epithelium and primary colorectal cancers. M denotes molecular markers. Sss I is Sss I-treated, bisulfite-modified DNA of a healthy individual which was used as a positive control for methylation-specific primers. G is unmodified genomic DNA of a healthy individual. m and u represent methylation-specific PCR using methylation and unmethylation-specific primer sets, respectively. N and T, normal colon epithelium and primary colorectal cancers, respectively. Sizes of the PCR products are indicated in bp. Four representative cases are shown.*

that epigenetic regulatory mechanisms may be involved in caveolin-1 gene down-regulation.

The purpose of this study was, by using methylation-specific PCR (MSP), to explore the mechanism of inactivation of the caveolin-1 gene in sporadic colorectal cancer and to search for possible clinical implications.

Materials and Methods

Specimens. One hundred and eighty-five colorectal cancerous tissues and their corresponding non-cancerous colon tissues obtained from patients who received surgical treatment at Changhua Christian Hospital (Changhua, Taiwan) were enrolled in this study. All the specimens were frozen immediately after surgical resection and stored in liquid nitrogen before experiments. Tumors were carefully dissected from adjacent normal tissues and all the malignant and non-malignant tissue specimens were confirmed by pathological examination. This study was approved by the Institute Review Board of the China Medical College Hospital and Changhua Christian Hospital, Taiwan.

DNA extraction, methylation PCR and sequencing. DNA extraction was performed as previously described (19). Sodium bisulfite modification of DNA and MSP were performed as previously described (20) and with some modifications. For detecting the methylated cytosines on the caveolin-1 gene promoter, 5'-GGTATTTTTGTAGGCGCGTC-3' (sense) and 5'-CTAACAACAAAAAACGAAAAACG-3' (antisense) were used as primers for the methylation-specific PCR reaction. These primers amplified a CpG-rich fragment of 210 base pairs on the promoter region. The primer sequences used for amplification of the unmethylated form were 5'-GTTTATATTGGGTATTTTTGTAG GTGTGT-3' (sense) and 5'-TCCCCAAAATTCTAACAACAAA AAACAAAAAAC A-3' (antisense). These primers amplified a 231-bp fragment containing CpG-island on the promoter. Generally, approximately 4 μg of genomic DNA in 40 μl H_2O was denatured by incubation with 10 μl of 1 M NaOH at 37°C for 10 min and then modified by 30 μl of 10 mM hydroquinone and 520 μl of 1.5 M sodium
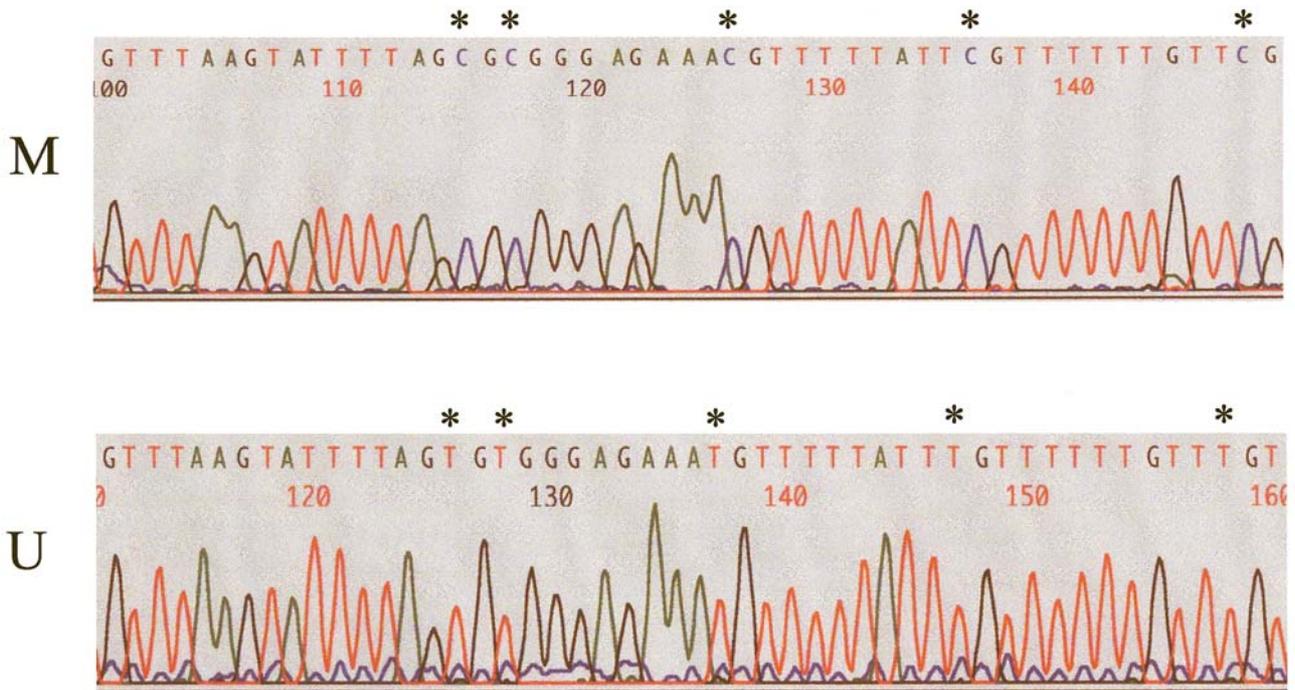
Figure 2. *CpG islands methylation analysis at promoter area of caveolin-1. Upper panel indicates modified DNA of a methylated sample (M) with all cytosines in CpG dinucleotides remaining as C while the lower panel indicates modified DNA of an unmethylated sample (U) with all the cytosines converted to thymidines. *, cytosines in the methylated CpG dinucleotides or thymidine converted from cytosines.*

bisulfite (pH 5.0) at 50˚C for 16 h. The DNA samples were eluted with 100 ml pre-warmed H_2O (65-70˚C) using the wizard DNA purification kit (Promega, Madison, WI, USA). Then 50 μl of 1 M NaOH was added to the elution and the mixture was incubated at room temperature for 5 min. After precipitation with 150 μl 100% isopropanol and washing with 70% ethanol, the DNA pellet was resuspended in 45 μl H_2O. Modified DNAs were amplified in a total volume of 20 μl solution containing 1X PCR buffer, 1 mM $MgCl_2$, 100 ng of each primer, 0.2 mM dNTPs and 2.5 units of Taq polymerase. PCR was performed in a thermal cycler for 35 cycles consisting of denaturation at 94˚C for 30 sec, annealing at 55˚C for methylation primers and 58˚C for unmethylation primers for 1 min and extension at 72˚C for 1 min, followed by a final extension at 72˚C for 5 min. PCR products were then loaded and electrophoresed on a 3.5% agarose gel, stained with ethidium bromide and visualized under UV illumination.

CpG methylase (*Sss* I)-treated genomic DNA was used as a positive control for methylation-specific primers because *Sss* I methylated all cytosines within the double-stranded dinucleotide CG. DNA samples from healthy individuals that were negative for the methylation-specific primer set and positive for the unmethylation-specific primer set were used as positive controls for unmethylation-specific primers. To ensure the specificity of the methylation or unmethylation primer sets for bisulfite-modified DNA, amplification using unmodified genomic DNA samples from healthy individuals was also performed.

For some representative cases, the PCR products were further analyzed using a direct sequencing method to confirm the results. Direct sequencing was performed on ABI Prism 310 Genetic Analyzer and BigDye or dRhodamine Terminator Cycle Sequencing Kits (Applied Biosystems, Rockville, MD, USA) and protocols followed for the reaction.

Immunohistochemistry study. As previously described (21), paraffin-embedded blocks were sectioned at 5-7 mm thickness, deparaffinized and rehydrated in PBS. After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, the slides were immersed in 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. After intensive washing with PBS, the slides were incubated overnight at 4˚C with the anti-caveolin-1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200. After a second incubation with a biotinylated anti-rabbit antibody, the slides were incubated with peroxidase-conjugated streptavidin (DAKO LSAB+ kit; Dako Corp., Carpinteria, CA, USA). Reaction products were visualized by immersing the slides in diaminobenzidine tetrachloride and finally counterstaining with hematoxylin.

Statistical analysis. Statistical analysis was performed using the SAS 8.e statistical software. The Z-test or Chi-square test was used to assess the association between the various parameters. Significance was accepted at $p < 0.05$.

Results

Caveolin-1 gene methylation in human colon tissues. We determined the methylation frequency of the caveolin-1
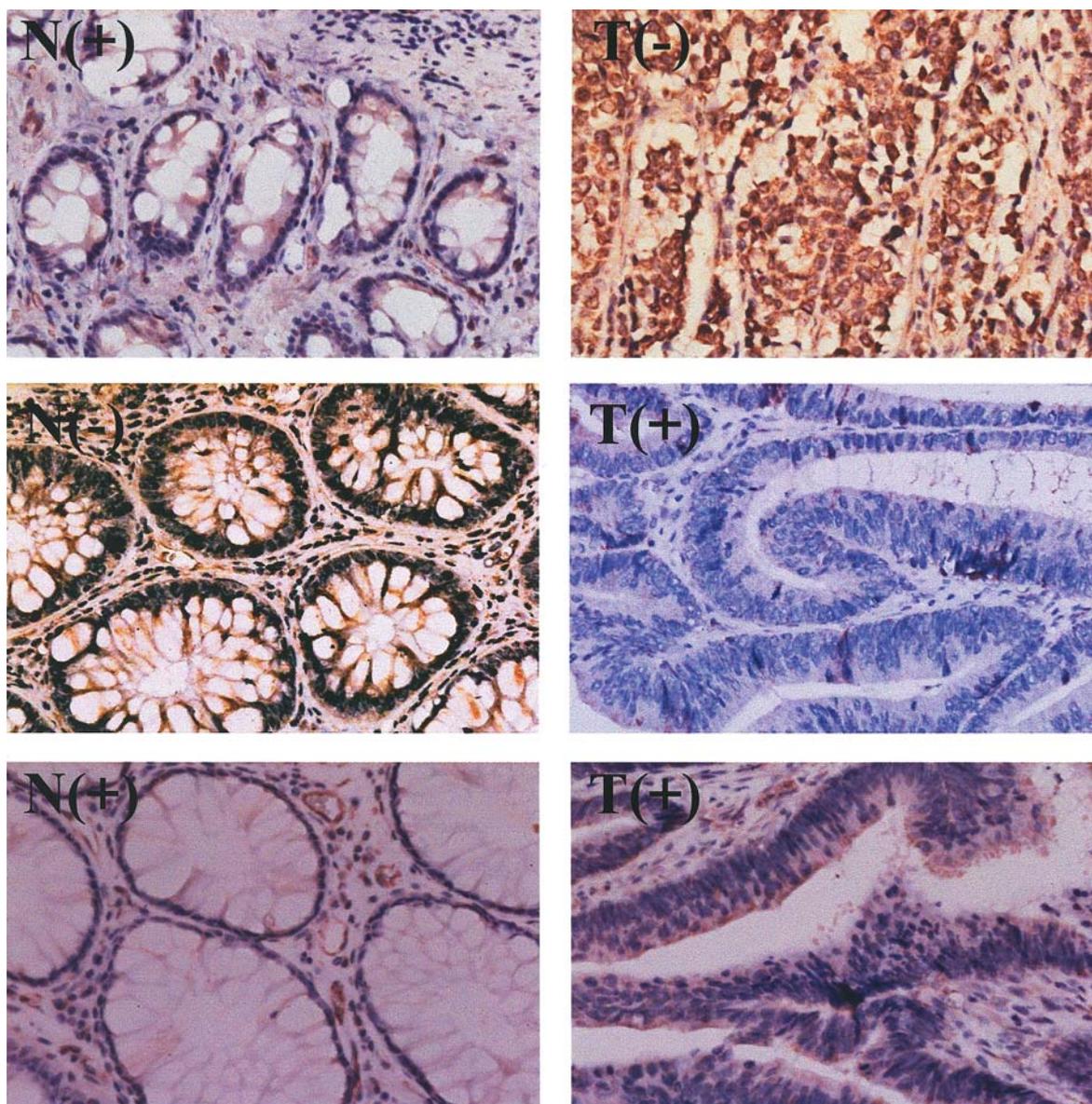
Figure 3. *The immunohistochemistry study of caveolin-1 in primary colorectal cancer tissues (T) and paired normal colon epithelium (N). The paraffin-embedded tissue blocks were cut into 5-7 mM thickness and stained with the specific polyclonal antibody against the human caveolin-1 protein. (-) or (+) denotes absence or presence of aberrant promoter methylation of the caveolin-1 gene, respectively. Immunoreactive cells are stained brown. (400X).*

gene in 185 paired cancerous and nearby non-cancerous tissues of colorectal cancer surgical specimens. The MSP analysis of representative examples of colorectal cancer primary tumors and normal colon epithelium are shown in Figure 1. There were four types of methylation pattern of the caveolin-1 gene in the colorectal tissues: unmethylation promoter in both cancerous and non-cancerous tissues; methylated and unmethylated promoter in cancerous tissues, with only unmethylated promoter in normal tissue; methylated and unmethylated promoter in normal tissues, with only unmethylated promoter in cancerous tissue; methylated and unmethylated promoter in both normal and cancerous tissues. We also performed direct sequencing to the PCR products to confirm the MSP results (Figure 2). The incidence of promoter hypermethylation in the caveolin-1 gene was 3.8% (7/185) for cancerous tissues whereas the frequency was 5.9% (11/185) for nearby non-cancerous tissues ($p > 0.05$, Z test). All the cancerous and non-cancerous tissue contained unmethylated promoters in the caveolin-1 gene.

Expression of tumor suppressor proteins was down-regulated in tissues with aberrant promoter methylation in CpG islands. To demonstrate whether DNA methylation in the CpG islands is associated with gene silencing, we performed an immunohistochemical study of the caveolin-1 gene in 13 colorectal cancer cases, which demonstrated aberrant promoter methylation in either normal or cancerous tissues or both. The results of representative cases for caveolin-1 are shown in Figure 3. Most of the colorectal epithelial cells in the unmethylated tissues were positively stained by the caveolin-1 antibody. In contrast, only a few colorectal epithelial cells were positively stained in the methylated tissues. These results suggested that aberrant promoter methylation in CpG islands of caveolin-1 gene is associated with its decreased protein expression.

Caveolin-1 gene promoter hypermethylation and clinical parameters. We determined the correlation between aberrant promoter methylation of the caveolin-1 gene and clinical parameters in the colorectal tissues. Clinical parameters included age ($\geq$60 *vs* <60), gender, cell grade (grade 1-2 *vs* grade 3), location of tumor: (right side *vs* left side) (descending, sigmoid and rectum) and lymph node metastasis. We found no statistically significant correlation between the methylation status of caveolin-1 with age, cell grade, location of tumor or lymph node metastasis. However, in colon tissue with aberrant promoter methylation of caveolin-1, female gender showed significant difference (p=0.045) (Table I).

Discussion

Our results demonstrated that inactivation of caveolin-1 through aberrant promoter methylation occurred in 3.8% of colorectal cancer tissue and 5.9% of normal colon tissues. These findings suggested that inactivation of the caveolin-1 gene could occur in the pre-cancerous lesion during colorectal tumorigenesis. In addition, in 13 colorectal tissues of caveolin-1 methylation, female gender showed a statistically significant difference suggesting that gender-related factors may be associated with methylation inactivation of caveolin-1. This observation is supported by a recent report indicating that ectopic expression of estrogen receptor alpha in human neuroblastoma cells leads to a ligand-independent transcriptional suppression of the caveolin-1 gene which is mediated through promoter hypermethylation of caveolin-1 (22).

It has been reported that methylation silencing of some tumor suppressor genes such as E-cadherin was associated with poorly-differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum (23). In addition, caveolin-1 haploinsufficient led to partial transformation of human breast epithelial cells (24) while caveolin-1 over-expression inhibited anchorage-independent growth and

Table I. *Caveolin-1 methylation status and clinical parameters in primary colorectal cancer patients.*

Clinical parameters	Promoter methylation		p*
	Positive	Negative	
Sex			
Female	9	70	0.045
Male	4	102	
Age			
$\geq$60	8	124	0.622
<60	5	48	
Tumor location			
right	5	30	0.134
left†	8	142	
Histological grade**			
1 or 2	11	164	0.311
3	2	8	
Lymph node metastasis			
Positive	6	117	0.192
Negative	7	55	

*Chi-square test;† Descending, sigmoid colon and rectum; **Grade 1,2 and 3, well, moderately and poorly-differentiated, respectively.

invasiveness of human breast cancer cells (25). In our 17 cases of mucinous or poorly-differentiated adenocarcinoma, none showed aberrant promoter methylation of caveolin-1 in the colorectal tissues. These findings suggested that aberrant promoter methylation of caveolin-1 is not associated with histological malignant potential in primary colorectal cancer.

According to previous reports, about 70 % of human colon cancer tissues showed decreased expression of the caveolin-1 gene compared to paired normal colon tissues (16,17). However, no mutation of caveolin-1 has been reported suggesting that epigenetic regulatory mechanisms were involved in the down-regulation of caveolin-1 (18). In our study, aberrant promoter methylation was observed in 3.8% of colorectal cancer tissue and 5.9% of normal colon tissues in 185 cases of primary colorectal cancer tissues. This result indicated that aberrant promoter methylation of caveolin-1 was not a frequent mechanism of epigenetic silencing of the caveolin-1 gene in colorectal cancers. Other mechanisms, such as histone deacetylation, histone methylation and transcriptional regulation may be involved in the process (26).

Acknowledgements

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