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Promoter hypermethylation of membrane type 3 matrix metalloproteinase is associated with cell migration in colorectal adenocarcinoma

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The gene *MT3-MMP* (also known as *MMP16*) encodes the membrane type 3 matrix metalloproteinase, which is a member of the matrix metalloproteinase (*MMP*) gene family. Several MMPs are associated with migration in colorectal cancer (CRC). However, the methylation status of the *MT3-MMP* promoter in CRC has not been reported. The methylation status and expression levels of *MT3-MMP* were investigated in primary tumor tissues and adjacent normal tissues in 105 patients with CRC, one normal colon cell line (CCD18Co), and three CRC cell lines (SW480, DLD-1, and LoVo) by quantitative methylation-specific PCR and real-time PCR. *MT3-MMP* was hypermethylated in 82 of 105 CRC tissues (78%), 30 of 105 adjacent normal tissues (29%), and two of 11 normal colon tissues (18%). *MT3-MMP* mRNA was significantly reduced in CRC compared with that in adjacent normal tissues ($P < 0.05$). The methylation-mediated downregulation of *MT3-MMP* was restored by treatment with 5-aza-2′-deoxycytidine in two CRC cell lines, and *MT3-MMP* promoter activity was significantly reduced by methylation. The knockdown of *MT3-MMP* induced cell migration, but overexpressed *MT3-MMP* reduced cell migration in CRC cells. These results demonstrate that the *MT3-MMP* promoter is frequently hypermethylated in CRC and that downregulation of *MT3-MMP* may be important for cell migration in CRC.

Keywords *MT3-MMP*, *MMP16*, colorectal cancer, hypermethylation, migration

Aberrant DNA methylation is an epigenetic event that inhibits the expression of tumor suppressor genes (1). In mammals, cytosine methylation at the CpG dinucleotide pairs in the genomic DNA is well-known in relation to the regulation of gene expression. Hypermethylation of CpG-rich or intermediate promoters inactivates downstream gene expression, and the promoters of tumor suppressor genes are frequently hypermethylated in many human malignancies (2–5). Epigenetic alterations that are commonly found in colorectal cancers (CRCs) include DNA methylation of tumor suppressor genes (6,7) and histone deacetylation (8).

MT3-MMP (also known as *MMP16*) is located on chromosome 8q21.3 and was cloned by Matsumoto and colleagues in 1997 (9). This locus encodes the membrane type 3 matrix metalloproteinase and matrix metalloproteinase (*MMP*) family genes. *MMP*s are zinc- and calcium-dependent endopeptidases that degrade extracellular matrix (ECM) proteins (10). *MMP*s, including *MT1-MMP,* regulate cell migration and metastasis in CRC (11). *MT1-*, *MT2-*, *MT3-*, and *MT5-MMP* have C-terminal transmembrane domains and short cytoplasmic tails, and they are localized at the cell surface (9,12,13). *MT1-MMP* is widely expressed in many tumors and is associated with poor prognosis in various cancers, including gastric cancer, breast cancer, and CRC (14–17). In addition, *MT1-MMP* is hypermethylated in a breast carcinoma cell line (18). The methylation status of *MT3-MMP* and its functional contribution to CRC is unclear.

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In this study, *MT3-MMP* hypermethylation was identified in CRC tissues, using quantitative methylation-specific PCR (QMSP). *MT3-MMP* expression in CRC tissues was compared with that in adjacent normal tissues using real-time PCR. We investigated the demethylating effects of *MT3-MMP* using 5-aza-2′-deoxycytidine (5-aza-dC), and *MT3-MMP* promoter activity was verified by luciferase assay. The effects of *MT3-MMP* knockdown and overexpression on the migration of CRC cell lines were also analyzed.

Materials and methods

Tissues

Fresh-frozen, paired primary tumor samples ($n = 105$) and adjacent normal tissue samples ($n = 105$) from CRC patients were collected at the time of surgery, and fresh-frozen normal colon tissue samples ($n = 11$) from colonoscopy patients were collected by biopsy at the Korea University Medical Center. The clinicopathologic features of CRC and colonoscopy patients are summarized in Table 1. The tissues were collected after obtaining informed consent from the patients, and the study was approved by the Institutional Review Board of Korea University (IRB no. KU-IRB-10-08-A-1, KU-IRB-13-84-A-1). The diagnosis of CRC tissues was acquired from pathology reports and histological evaluations.

Cell lines

One normal colon cell line (CCD18Co) and three CRC cell lines (SW480, Dukes' type B; DLD-1, Dukes' type C; LoVo, Dukes' type C and stage IV) were obtained from the American Type Culture Collection (Manassas, VA, USA). CCD18Co cells were cultured in Eagle's minimum essential medium, and the three CRC cell lines were cultured in RPMI 1640 medium. All of the culture media were supplemented with 10% FBS from HyClone (Logan, UT, USA) and 1% penicillin/streptomycin from Life Technologies (Carlsbad, CA, USA). The cells were maintained at 37°C and 5% carbon dioxide.

DNA extraction

Genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), according to the manufacturer's recommendations. Approximately 10–20 mg of tissue was homogenized and incubated at 65°C for 30 minutes before 600 μL of chilled nuclei lysis solution was added. RNase solution (3 μL) was added, and the mixture was incubated at 37°C for 15 minutes. The samples were mixed with 200 μL of protein-precipitation solution and centrifuged at 13,200 rpm for 5 minutes at room temperature. The supernatants were transferred to fresh tubes, and the genomic DNA was precipitated with isopropanol (Sigma-Aldrich, St. Louis, MO, USA), washed with 70% ethanol (Sigma-Aldrich), eluted in 100 μL of DNA rehydration solution, and quantified with a

Table 1 Clinicopathologic characteristics of CRC patients and methylation status of *MT3-MMP*

Characteristic	No. of cases	Frequency of *MT3-MMP* hypermethylation (%)	*P* value*	Methylation status of *MT3-MMP* (PMR)	
				Median (range)	*P* value*
Normal colon	11	1 (9.1%)	<0.001	4.44 (±1.06)	<0.001
Adjacent normal	105	30 (28.6%)		15.63 (±2.97)	
CRC	105	82 (78.1%)		175.60 (±19.02)	
Age, y			1.000		0.709
≤65	44	34 (77.3%)		184.01 (±30.08)	
>65	61	48 (78.7%)		169.51 (±24.70)	
Gender			0.329		0.672
Female	39	28 (71.8%)		165.04 (±30.14)	
Male	66	54 (81.8%)		181.83 (±24.60)	
Differentiation			0.602		0.893
Well	29	24 (93.1%)		176.76 (±36.75)	
Moderately, poorly	76	58 (76.3%)		174.00 (±22.37)	
Location			0.800		0.369
Colon	72	57 (79.2%)		187.23 (±23.75)	
Rectum	33	25 (75.8%)		150.20 (±31.30)	
TNM Stage			0.643		0.982
I, II	46	37 (80.4%)		175.09 (±27.59)	
III, IV	59	45 (76.3%)		175.98 (±26.34)	
Size, cm			0.817		0.328
≤20	53	42 (79.2%)		194.11 (±26.25)	
>20	52	40 (76.9%)		156.72 (±27.56)	

Abbreviation: TNM, tumor, lymph nodes, and metastasis.

PMR of 10 or higher was used as a cutoff to indicate a methylated state, whereas PMR of less than 10 was considered unmethylated. Statistical significance was evaluated by $\chi 2$ test, Fisher exact test, and ANOVA.

* *P* values less than 0.05 were considered statistically significant.