Original Article
Yonsei Med J 2021 Nov;62(11):1005-1015
https://doi.org/10.3349/ymj.2021.62.11.1005
Yonsei Medical Journal
YMJ
pISSN: 0513-5796 · eISSN: 1976-2437

Regulation of MUC6 Methylation Correlates with Progression of Gastric Cancer

Ding Shi and Xiao-xia Xi

Department of Gastroenterology, Hwamei Hospital, University of Chinese Academy of Sciences, Ningbo, China.

Purpose: This study aimed to investigate the mechanistic downregulation of mucin 6 (MUC6) and its influence on the progression of gastric cancer (GC).

Materials and Methods: The expression of MUC6 was examined in 40 GC patients. The methylation status of the MUC6 promoter region was investigated using GC cell lines and GC tissue specimens by immunohistochemistry and/or quantitative polymerase chain reaction (qPCR). MUC6 was knocked down in the gastric epithelial cells (GES-1) cell and overexpressed in the SGC7901 cell. The effects of MUC6 knockdown and overexpression on cell migration and invasion were examined using Transwell assays. The effects of demethylation and methylation on MUC6 expression were examined by western blot, qPCR, or double luciferase reporter assays.

Results: The expression of MUC6 in GC with lymph node metastasis and poor pathological stage was significantly lower than that in GC without lymph node metastasis and good pathological stage, respectively. While cell migration and invasion were significantly decreased after overexpression of MUC6, these abilities significantly increased after the knockdown of MUC6. The methylation levels of MUC6 in GC tissues and GC cell lines were significantly higher than those in para-cancerous tissues and normal GES. Promoter methylation could significantly reduce the binding of related transcription factors to the MUC6 promoter. The expression of MUC6 increased with the concentration and time of action of demethylation drugs.

Conclusion: Expression of MUC6 was regulated by promotor methylation. This methylation of the MUC6 promoter may lead to significant downregulation of MUC6 in GC and promote the progression of GC.

Key Words: MUC6, methylation, gastric cancer

INTRODUCTION

Mucin 6 (MUC6), one of the main components of the mucus barrier in the stomach, is secreted by the pyloric gland cells of the antrum and mucous neck cells located in the lower layer of the gastric mucosa.[1-4] MUC6 is known to be a marker of gastric foveolar and antral mucous glandular cells, reflecting gastric phenotypes and acting as a significant physiological barrier against various threats to the underlying epithelia.[5] It is also reported to be significantly correlated with epithelial growth factor receptor 2, which is related to the invasive behavior of gastric cancer (GC).[6] Nevertheless, MUC6 has not been thoroughly investigated. Therefore, not much is documented regarding changes to its expression during GC, the mechanism of such changes, and the effect of these changes on the progression of GC. Some studies have reported decreased expression of MUC6 in GC,[5,7-9] which may be associated with carcinogenesis, malignant potential, progression, clinical behavior, and poor prognosis of GC.[10-13] However, these were not independent studies and did not directly focus on MUC6. To date, the mechanism regulating the decreased expression of MUC6 and the direct effect of reduced MUC6 on the occurrence and progression of GC has not been fully explained.

Certain previous studies have suggested that mucins may be positioned at a hotspot for methylation in the genome,[14] and

Received: January 14, 2021 **Revised:** July 4, 2021
Accepted: July 23, 2021
Corresponding author: Xiao-xia Xi, MD, Department of Gastroenterology, Hwamei Hospital, University of Chinese Academy of Sciences, No.41 Northwest Street, Ningbo 315010, China.
Tel: 86-574-8387-1152, Fax: 86-574-8387-1267, E-mail: 17839974401m0@sina.cn

•The authors have no potential conflicts of interest to disclose.

methylation modifications play a significant role in regulating mucin gene expression in epithelial cancer cells.[15] An additional study has indicated that the repression of MUC2 expression in colonic carcinoma cells was linked to the methylation status of its promoter.[16] However, de novo expression of MUC2 was shown to be triggered by promoter demethylation or hypomethylation in pancreatic and gastric carcinoma cells.[17,18] Nevertheless, data regarding MUC6 are unavailable. The studies mentioned above motivated us to study the relationship between MUC6 and GC using data from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) and the Gene Expression Profiling Interactive Analysis software. The expression of MUC6 in GC tissue was significantly lower compared to normal tissue and was associated with tumor metastasis (Supplementary Fig. 1, only online). Retrieved data from the MethHC database indicated that the methylation level of the MUC6 promoter region in GC tissues was significantly higher compared to normal tissues (Supplementary Fig. 2, only online). Therefore, it can be hypothesized that MUC6 promotes the progression of GC through the methylation of its promoter region, resulting in the downregulation of its expression. The effect of MUC6 downregulation on the biological behavior of GC cells and the mechanism responsible for the downregulation of MUC6 expression were the focus of this study. The above hypothesis was investigated using clinical samples, MUC6 overexpression and knockdown in cell lines, and MUC6 promoter methylation and demethylation experiments.

MATERIALS AND METHODS

Patients and tissue samples

All tissue samples were supplied by the Department of Gastroenterology, Hwamei Hospital, University of Chinese Academy of Sciences, between January 2017 and October 2018. The study was approved by the Ethics Committee of Hwamei Hospital, University of Chinese Academy of Sciences (IRB NO: YJ-KYSB-NBEY-2019-105-01). All participating patients were informed about the procedure and written informed consent was obtained. The surgical sample from each patient was divided into two sections. One section was fixed in formalin and embedded in paraffin for histopathological investigation, and the other section was immediately refrigerated for detection of MUC6. None of the patients underwent chemotherapy or radiotherapy prior to tissue collection.

Cell lines and cell culture

SGC7901 cells (with the lowest expression of MUC6) were used for MUC6 plasmid transfection and overexpression experiments, whereas gastric epithelial cells (GES-1; with the highest expression of MUC6) were used for MUC6 knockdown experiments (Supplementary Fig. 3, only online). The SGC7901 gastric adenocarcinoma and GES-1 lines were obtained from the Shanghai Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1× penicillin-streptomycin at 37°C with 5% CO_2 in a saturated humidity incubator.

Transfection

The transfected plasmids included MUC6 overexpression and knockdown plasmids. For overexpression experiments, the cells were divided into the control group (pGL3) or MUC6 overexpression plasmid group (pGL3-MUC6-Promoter). For knockdown experiments, the cells were divided into MUC6 (NC), MUC6 (siRNA1), MUC6 (siRNA2), and MUC6 (siRNA3) groups. The vector used for the expression of MUC6 protein was the pGL3 plasmid obtained from Life Technologies (Thermo Fisher Scientific Inc.; Waltham, MA, USA). The MUC6-coding insert was established by total gene synthesis. For transfection, MUC6 plasmid (1 μg) and Lopti-MEM (25 μg) were gradually added to the liposome. The prepared 50 μL transfection mixture was added drop by drop to the pore containing SGC7901 cells and culture medium. It was then gently mixed and incubated at 37°C in 5% CO_2. Next, the transfection efficiency was detected. Thereafter, RNA and protein were extracted from the cells, and MUC6 expression was analyzed by quantitative reverse transcription polymerase chain reaction (qPCR) or western blotting (WB).

Immunohistochemistry (IHC)

Tumor tissues were fixed in 4% formalin and embedded in paraffin. Tissue sections (4 μm) were prepared and baked in a 65°C thermostat for 6–12 h, and IHC was performed. The sections were dewaxed using xylene and rehydrated using an alcohol gradient, and endogenous peroxidase activity was blocked with 3% H_2O_2. The sections were then boiled in citrate buffer (pH 6.0, 10 min) for antigen retrieval, allowed to slowly cool to room temperature, and rinsed three times with phosphate-buffered saline (PBS). The sections were then incubated with a primary antibody [MUC6, K167722B, 1:1000 dilution; GAPDH, Mab011-100, 1:5000 dilution; Beijing Zhongshan (Jingqiao) Biotech Co. Ltd., Hangzhou, China] at 4°C overnight. After rinsing three times with PBS, the sections were incubated with the second antibody [MUC6, K1566171, 1:1000 dilution; GAPDH, Mab011-040, 1:5000 dilution; Beijing Zhongshan (Jingqiao) Biotech Co. Ltd.] at 37°C for 30 min. After an additional wash, the sections were stained with 4′,6-diamidino-2-phenylindole (DAPI), then counterstained with hematoxylin. The IHC results were determined based on the staining intensity and the percentage of positive cells. Initially, the score was calculated by subtracting the background color from the intensity of the stain (no staining=0; mild staining=1; moderate staining=2; intense staining=3). Three different visual fields (×20) were selected from each section to observe the percent-

https://doi.org/10.3349/ymj.2021.62.11.1005

age of positive cells (0%–5%=0; 6%–25%=1; 26%–50%=2; 51%–75%=3; >75%=4). The final score was the sum of the percentage of positive cells and the staining intensity scores. Scoring criteria were as follows: 0=negative; 1–3=weakly positive; 4–5=moderately positive; and 6–7=strongly positive.

Hematoxylin-eosin (H&E) staining

Following dewaxing in xylene and rehydration in an alcohol gradient, the sections were immersed in a hematoxylin dyeing solution for 5 min at room temperature. The sections were then washed under running water for 1 min, immersed in a 1% hydrochloric acid alcohol solution for several seconds, rinsed under tap water, and returned to a blue stain. The sections were then immersed in the eosin dye for 3–5 min, and the excess dye on the slide was washed off with tap water. After dehydrating for 0.5 min with 80% ethanol, 95% ethanol I, 95% ethanol II, absolute ethanol I, and absolute ethanol II, the sections were immersed in xylene I and II for 3 min to make them clear and transparent. Finally, the sections were sealed with neutral gum.

Western blot protocol

Total protein extraction began by collecting samples from each group into Eppendorf (EP) tubes, and adding 200 mL of western and input pyrolysates into each tube (phenylmethylsulfonyl fluoride was also added at a final concentration of 1 mM). The samples were mixed and fully lysed at 4°C for 30 minutes, centrifuged at 4°C and 12000 rpm, and the supernatants were collected and stored separately. Polyacrylamide gels were used for electrophoresis. Firstly, 10% separating gels and 4% concentrating gels were prepared. The protein sample was then mixed with 5× sample buffer, heated at 100°C for 10 minutes, then rapidly cooled in an ice bath. Approximately 30 μg of protein was added to each lane. The electrophoretic buffer was added to the electrophoretic tank, and an 80-V power supply was provided. Constant voltage electrophoresis was performed until bromophenol blue ran out of the concentrated rubber layer. For the separating gel, 120-V constant voltage electrophoresis was used. When bromophenol blue migrated to the lower edge of the separation gel, the power supply was disconnected, and the electrophoresis was stopped. After being pretreated, a PVDF membrane was inserted into the electrophoresis cell, which was transferred at a constant current of 200 mA and was immersed in a sealed liquid containing 5% skimmed milk powder at room temperature for 1 h. The PVDF membrane was then incubated with a primary antibody [MUC6, K167722B, 1:1000 dilution; GAPDH, Mab011-100, 1:5000 dilution; Multisciences (Lianke) Biotech Co. Ltd., Hangzhou, China] overnight at 4°C. Membranes were washed three times in TBST, and then incubated with the second antibody [MUC6, GAM007, 1:1000 dilution; GAPDH, Mab011-040, 1:5000 dilution; Multisciences (Lianke) Biotech Co. Ltd.] for 1 h at room temperature. Membranes were washed three times with TBST, placed on fresh-keeping film, and exposed to a mixture of moderate amounts of solutions A and B from an enhanced chemiluminescence kit [Multisciences (Lianke) Biotech Co. Ltd.]. Blots were imaged using a gel imaging analyzer (SMA4000, Merinton, USA). The molecular weight of MUC6 was 252 kD. The supporting software was SMA4000 V4.2.3 (Chemidoc XRS+, BioRad, Hercules, CA, USA). The chemical photosensitive mode was used to capture the images, and photographs were exported in a TIFF format.

qPCR

Total RNA was prepared using a High Purity Total RNA Rapid Extraction kit (GK3016; Generay Corporation, Shanghai, China). A reverse transcription kit (HiScript-II Q RT SuperMix) for qPCR was obtained from Vazyme Biotech Company (R222-01; Vazyme Biotech Co. Ltd., Nanjing, China). Primers for qPCR were designed according to the MUC6 and β-actin sequences: forward primer of MUC6: 5'-TGGTGAACTCGTGGAAGGA-3'; reverse primer of MUC6: 5'-TGGCAGGTGGCAAAGGT-3'; amplification production of MUC6: 139 bp. Forward primer of β-actin: 5'-TGACGTGGACATCCGCAAAG-3'; reverse primer of β-actin: 5'- CTGGAAGGTGGACAGCGAGG-3'; amplification production of actin: 205 bp. After reverse transcription, qPCR was performed according to the manufacturer's instructions (ChamQ SYBR Color qPCR Master Mix, Vazyme, NanJing, Q411-02). The reaction system was as follows: SYRB Green mix (10 μL), forward primer (1 μL), reverse primer (1 μL), diluted cDNA (8 μL), for a total reaction volume of 20 μL. The reaction was mixed and run in a CFX Connect Real-Time PCR machine (Bio-Rad, Goodhere, Hangzhou, China). The amplification conditions were as follows: Pre-denaturation at 95°C for 30 seconds, denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and elongation at 70°C for 5 seconds, with a total of 45 cycles performed. β-actin was used as the internal control, and the relative expression level of MUC6 was determined using the $2^{-\Delta\Delta Ct}$ method.

Construction of MUC6 siRNA

Three different siRNAs against the human MUC6 gene were designed to specifically knockdown MUC6 expression in GES-1 cells. The siRNA sequences were as follows: siRNA-1: CGU CAAAUGUGGUAACAAAGGAG, siRNA-2: UGUAUUCAGUA GUCGUUCUUUGUU, and siRNA-3: GCACAUAAUAAGAAA CAGUAG. The control siRNA sequence was a small random fragment of the same length.

Methylation

The promoter region of MUC6 was inserted into a PGL-3 vector and treated with a methylation reagent. Plasmid DNA was methylated by M. SssI methyltransferase (CpG Methyltransferase, New England Biolabs, USA). The normal pGL3-MUC6-Promoter (or pGL3) and the methylated pGL3-MUC6-Promoter were transfected into GES-1 cells. The modified plasmid DNA was digested by BstUI restriction endonuclease to verify the methylation protection of M. SssI. Luciferase activity was stud-

ied 48 h later. Renilla luciferase was used as an internal reference.

Demethylation

SGC7901 cell lines in the logarithmic phase were digested using 0.25% trypsin, centrifuged at 1000 rpm for 5 minutes, and plated into three six-well plates with four holes for each six-well plate and 200000 cell holes in each plate, and counted under the counter plate. Plates were stored in the incubator overnight. Three gradients of the demethylation drug (5-aza-2'-deoxycytidine) concentrations were prepared: 1, 5, and 10 μM. Each six-hole plate consisted of four groups: untreated cells (CK), 1, 5, and 10 μM groups. Drugs were added to each group. RNA was extracted at 24, 48, and 72 h, respectively.

Double luciferase report experiment

The double fluorescein luminescence was detected according to the instructions in the Dual-Luciferase Reporter Assay System kit (E1910, Promega, Madison, WI, USA). Briefly, the procedure followed five steps. 1) The 24-well plate was removed, and the culture medium was discarded using an aspirator. The cells were then gently rinsed with PBS. 2) Passive lysis buffer (PLB; 100 μL) was added to each well, and the plate was oscillated at room temperature for 20 minutes. The lysis buffer was transferred to 1.5 mL EP tubes and centrifuged to isolate the supernatant. 3) Then, 20 μL of supernatant was transferred to a new 1.5 mL EP tube, and 100 μL of the luciferase substrate luciferase assay reagent II was added to each well to prevent light penetration. The plate was inserted into the instrument for the first luciferase reading. 4) Approximately 100 μL of Stop & Glo Reagent was added into the EP tube and inserted into the instrument for the second reading. 5) Data analysis: The final fluorescence value of each well was the first fluorescence reading of Firefly luciferase, and the second fluorescence reading was that of Renilla luciferase.

Cell migration

After 48 h, the treated cells were digested with 0.25% trypsin+0.02% EDTA and centrifuged, suspended in 2% serum medium, counted, transferred to a 24-well plate at a density of 2.0×10^5 cells/well, and incubated with 10% serum in the lower chamber in an incubator with 5% CO_2 at 37°C.

Cell invasion

Matrigel was removed from storage at -20°C and bathed in ice overnight in the fridge. In the ice bath, 10 mg/mL matrix glue was gently mixed with serum-free medium of equal volume and then added to the upper chamber of a Transwell and incubated at 37°C for 4–6 h. The serum-free medium was once gently rinsed in the Transwell chamber for later use. The cells in each group were digested with 0.25% trypsin+0.02% EDTA, centrifuged at 1500 rpm for 5 minutes, counted using a counter plate, suspended in 2% serum medium, diluted to 5.0×10^5 cells/well, and placed in the upper chamber of the Transwell at a density of 2.0×10^5 cells/well, and cultured with 500 μL of media containing 10% serum in the lower chamber. After 16 h of culture, the cells were rinsed three times with PBS, fixed at room temperature with 4% paraformaldehyde for 15 minutes, and then washed three times with PBS. The upper chambers were wiped with cotton swabs to remove cells, and crystal violet stain was used for 15 min to stain cells that had migrated to the bottom of the Transwell. Membranes were washed three times with PBS, air-dried at room temperature, and photographed under a microscope.

Statistical analysis

Bands from WB or qPCR were quantified using the Quantity software (CFX Connect Real-Time System, Bio-Rad, Goodhere). Numerical data were presented as the mean±standard deviation. The difference between means was assessed using the Student's t-test. The multigroup average was analyzed with the help of CFX Manager 3.1 (Bio-Rad, Goodhere) statistical software. The homogeneity test of variance was first performed for the data. If the variance was homogeneous, a single factor analysis of variance was used to compare the differences among all groups. The mean comparison was made between multiple-dose groups and one control group using the one-to-one comparison method. The rank-sum test was used to analyze the data of non-normal or heterogeneous variance. All statistical analyses were performed using the SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). p-values <0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 40 specimens were collected directly from GC sites or gastric mucus membranes near the cancer sites. Forty patients were confirmed by histopathology of surgical samples to have adenocarcinoma, and the tissue surrounding the cancer site was confirmed as normal gastric mucosal membrane by H&E staining. The age range of the patients was 51–83 years (median age=67.6), and the patient group included 24 males and 16 females. Demographic data are provided in Table 1.

MUC6 expression levels in GC and para-cancerous tissues of different histological types, pathological stages, and lymph node metastasis

IHC and qPCR were performed in 40 cases of GC and the corresponding normal adjacent tissues. The results of either IHC ($p<0.05$) or qPCR ($p<0.01$) indicated that the expression of MUC6 in GC tissues was significantly lower than that in normal para-cancerous tissues (Figs. 1 and 2).

The qPCR results revealed that the expression of MUC6 in mucinous adenocarcinoma, signet ring cell carcinoma, and undifferentiated cell carcinoma was significantly lower than that

https://doi.org/10.3349/ymj.2021.62.11.1005

Table 1. Clinicopathological Features

Features	Number
Sex	
Male	24
Female	16
Age (yr)	
<60	8
60–80	28
>80	4
Histological classification	
Tubular adenocarcinoma	16
Mucinous adenocarcinoma	14
Signet ring cell carcinoma	6
Undifferentiated carcinoma	4
Lymph node metastasis	24
Differentiated degree	
Well	2
Moderately	6
Poorly	32
Tumor stage	
I	4
II	8
III	28
IV	0
Location	
Antrum	26
Angle	12
Gastric body	2

in tubular adenocarcinoma (p<0.01). Furthermore, the MUC6 expression in GC with lymph node metastasis was significantly lower than that in GC without lymph node metastasis (p<0.01). The expression of MUC6 in GC with stage III+IV was significantly lower than that of stage I+II (p<0.01) (Figs. 3, 4, and 5).

Effects of overexpression and knockdown of MUC6 on cell invasion and migration

SGC7901 cells with the lowest expression of MUC6 for transfection were selected for cell invasion and migration (Supplementary Fig. 3, only online). After the transfection of MUC6 plasmid, the expression of MUC6 in SGC7901 cells was found to significantly increase compared to the control group, which was confirmed by qPCR (p<0.001) (Fig. 3A) and WB (p<0.001) (Fig. 3B). The migration (p<0.01) and invasion (p<0.05) abilities of SGC7901 cells were significantly decreased after overexpression of MUC6 (Fig. 4).

GES-1 cells with the highest MUC6 expression were chosen (Supplementary Fig. 3, only online) for the knockdown. After knocking down MUC6, the expression of MUC6 mRNA and protein in GES-1 cells transfected with siMUC6 decreased compared to NC (p<0.01), and the MUC6 expression in siMUC6-3-transfected cells was the lowest (p<0.001) (Fig. 5). The migration and invasion abilities of the siMUC6-transfected cells increased significantly compared to siNC cells (p<0.001) (Fig. 6).

The methylation level of MUC6 promoter region

The methylation of the MUC6 promoter region was detected by qPCR in five GC patient samples and adjacent tissues. The methylation level of MUC6 in para-cancerous tissues was sig-

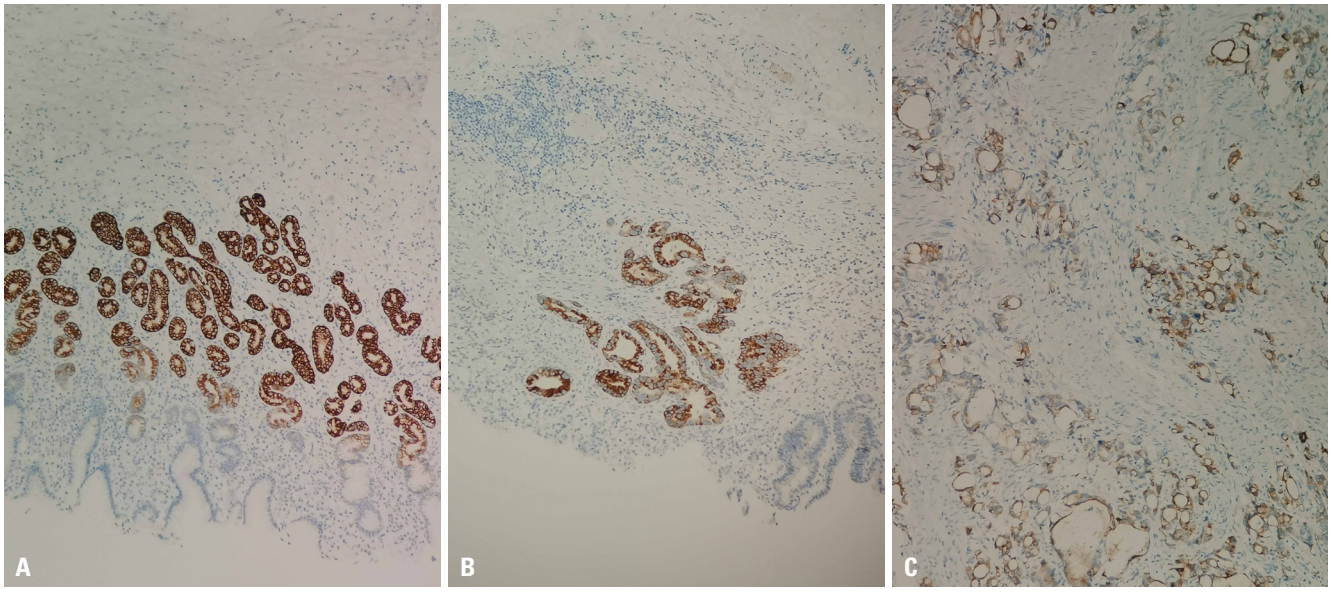

Fig. 1. IHC findings of gastric samples. Immunoreactivity to MUC6 distributed in the cytoplasm. (A) IHC staining of MUC6 in normal para-cancerous tissues. (B) IHC staining of MUC6 in intestinal-type GC tissues. (C) IHC staining of MUC6 in diffuse-type GC tissues. Compared to normal para-cancerous tissues, the expression of MUC6 was lower in intestinal-type GC, and the lowest in diffuse-type GC. ×40 magnification. IHC, immunohistochemistry; MUC6, mucin 6; GC, gastric cancer.

nificantly lower than that in GC tissues (Fig. 7).

The methylation levels of the MUC6 promoter regions in several GC and epithelial cell lines were detected by qPCR. The methylation levels of MGC803, MKN45, AGS, SGC7901, and BGC823 were recorded to be significantly higher than those of normal GES-1 cells (Fig. 8).

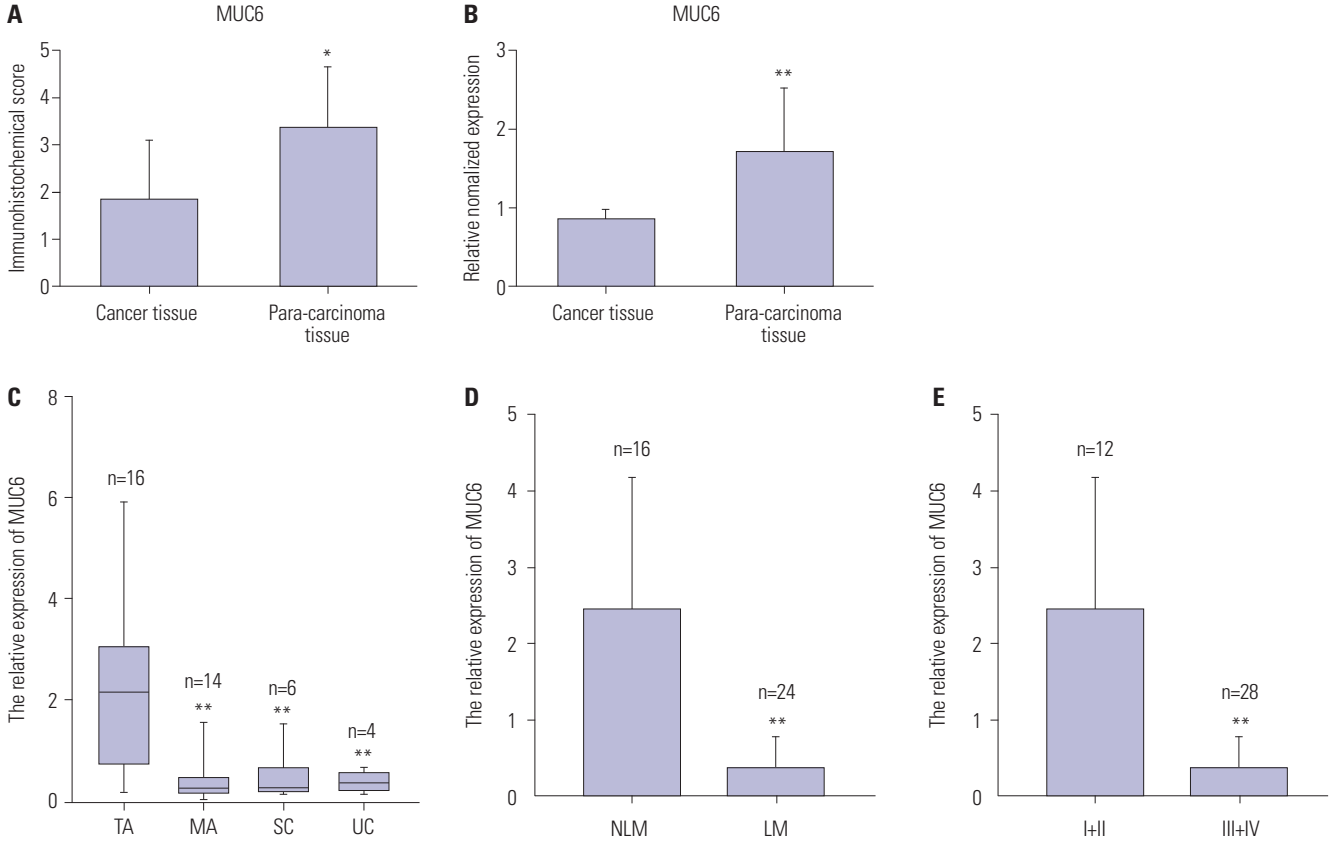

Fig. 2. Detection of MUC6 in GC and adjacent tissues by IHC and qPCR. (A) IHC showed that MUC6 in GC tissue was significantly downregulated compared to that in adjacent tissues (*p<0.05). (B) qPCR showed that MUC6 in GC tissues was significantly downregulated compared to that in adjacent tissues (**p<0.01). (C) qPCR showed that MUC6 had low expression in poorly differentiated GC (**p<0.01). (D) The expression of MUC6 was low in GC patients with lymph node metastasis (**p<0.01). (E) The higher the pathological stage of GC, the lower the expression of MUC6 (**p<0.01). MUC6, mucin 6; GC, gastric cancer; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; TA, tubular adenocarcinoma; MA, mucinous adenocarcinoma; SC, signet ring cell carcinoma; UC, undifferentiated carcinoma; NLM, non-lymph node metastasis; LM, lymph node metastasis; I, II, III, IV, pathological staging of GC.

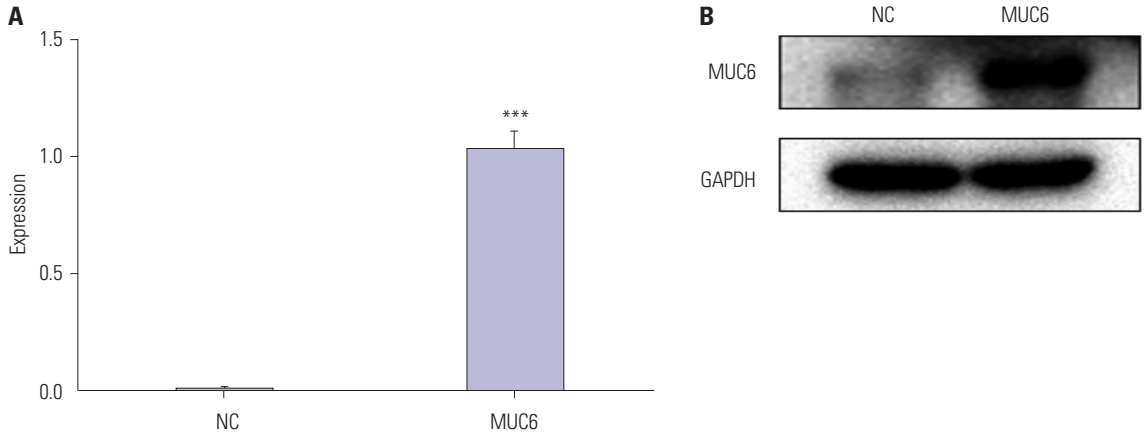

Fig. 3. Detection of MUC6 expression by qPCR and WB 48 h after transfection. (A) qPCR showed that the expression of MUC6 mRNA increased significantly after transfection with overexpressed plasmid (n=3, ***p<0.001). (B) WB showed that the expression of MUC6 protein increased significantly after transfection with overexpression plasmid. MUC6, mucin 6; qPCR, quantitative polymerase chain reaction; WB, western blot; NC, control group.

https://doi.org/10.3349/ymj.2021.62.11.1005

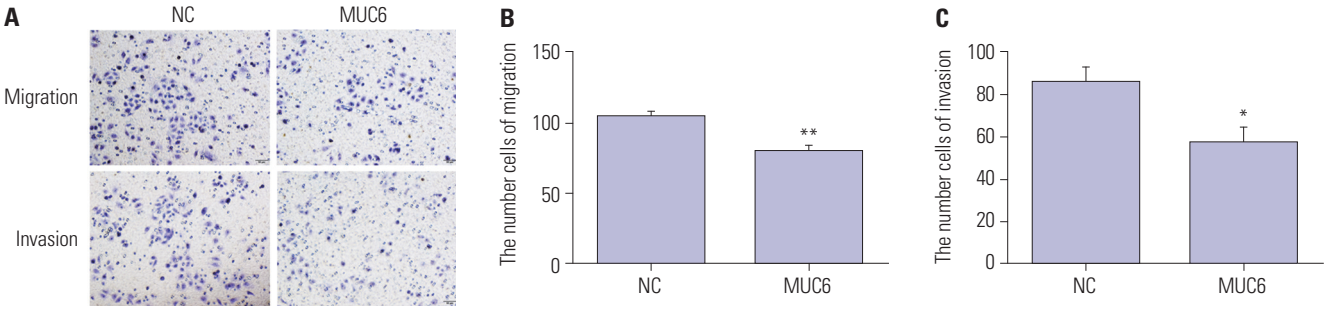

Fig. 4. Detection of migration and invasion of SGC7901 cells by Transwell assays after overexpression of MUC6. Compared to NC, cell migration and invasion ability decreased significantly after MUC6 overexpression (n=3, *p<0.05, **p<0.01). (A) Migration and invasion results. (B) Migration statistical analysis. (C) Invasion statistical analysis. NC, no-load plasmid group; MUC6, MUC6 overexpression group; MUC6, mucin 6.

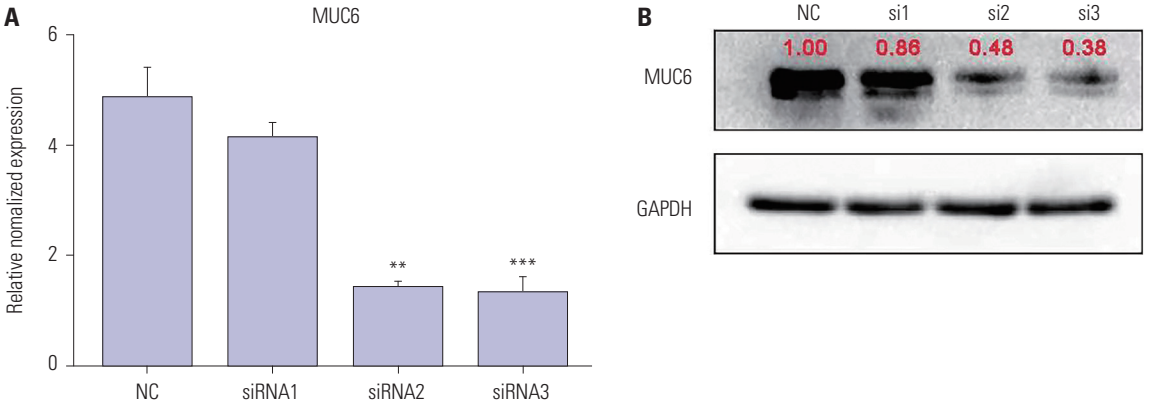

Fig. 5. Detection of MUC6 expression after knockdown by qPCR and WB. Compared to NC, the expression of MUC6 (A, mRNA; B, protein) in GES-1 cells transfected with siRNA decreased, and the MUC6 expression of cells transfected with siRNA3 was the lowest (n=3, **p<0.01, ***p<0.001). NC, siRNA irrelevant sequence; si1, siRNA; si2, siRNA2; si3, siRNA3; MUC6, mucin 6; qPCR, quantitative polymerase chain reaction; WB, western blot; GES-1, gastric epithelial cells.

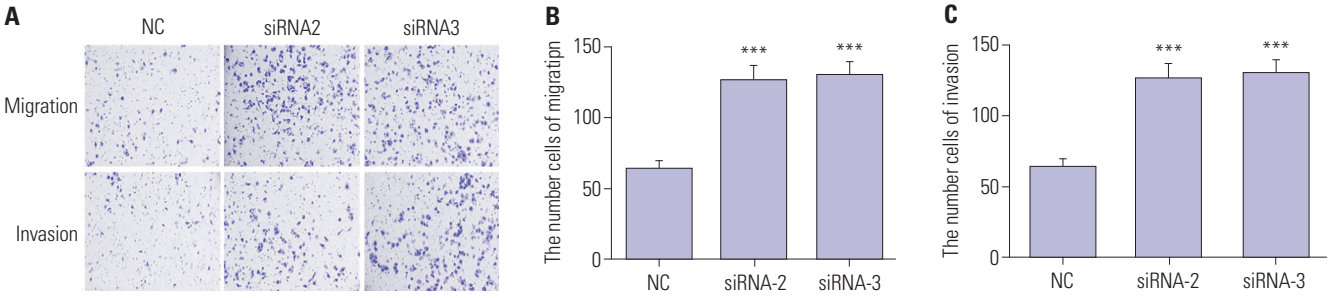

Fig. 6. Cell migration and invasion assay. Transwell assay revealed that the capacity of cells to migrate and invade increased significantly after knocking down MUC6 by siRNA (n=3, ***p<0.001). (A) Migration and invasion results. (B) Migration statistical analysis. (C) Invasion statistical analysis. NC, siRNA irrelevant sequence; siRNA, knockdown of MUC6; MUC6, mucin 6.

Effect of promoter methylation and demethylation on MUC6 expression

The pGL3-MUC6-Promoter plasmid was methylated by M.SssI methyltransferase, and the protective degree of M.SssI methylation was verified by restriction endonuclease digestion with BstUI. Compared to pGL3, the fluorescence activity of the MUC6 promoter region after methylation was significantly decreased (p<0.001), suggesting that the BstUI enzyme could cut plasmid DNA without M. SssI modification, but not when modified by M. SssI (Fig. 9). This indicated that promoter methylation could significantly reduce the binding of related transcription factors to the MUC6 promoter region.

Different concentrations of the demethylation drug 5-aza-2'-deoxycytidine were added to SGC7901 cells, and the expression of MUC6 in SGC7901 cells was detected using qPCR and WB. The expression of MUC6 increased with the concentration of demethylation drugs and the time of action (Fig. 10), suggesting that the expression of MUC6 was regulated by the methylation status of its promoter.

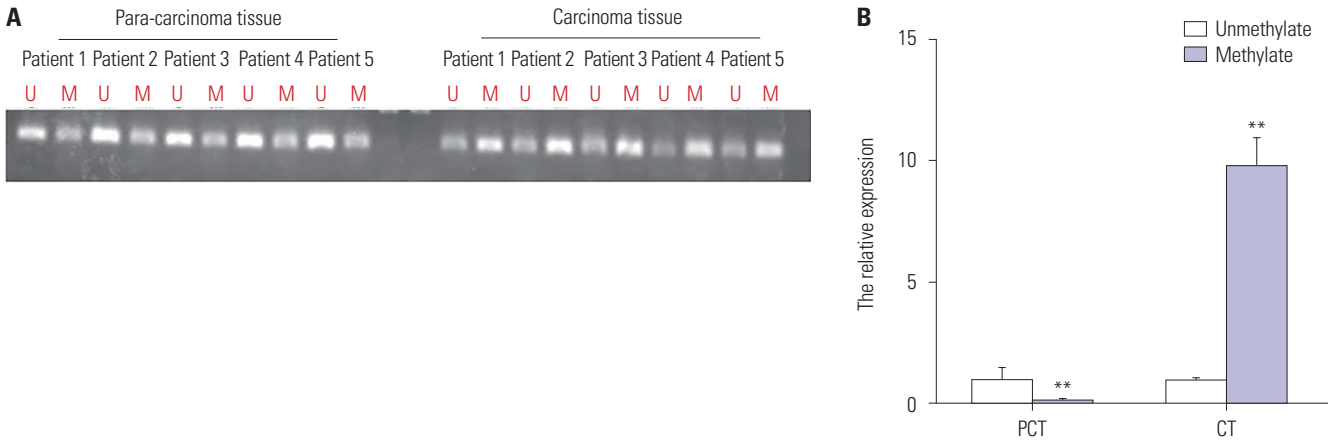

Fig. 7. Detection of MUC6 methylation in gastric cancer and adjacent tissues by qPCR. The results showed that the methylation level of the MUC6 promoter region in para-cancerous tissues was significantly lower than that in cancer tissues (**p<0.01). (A) qPCR results. (B) Statistical analysis. MUC6, mucin 6; qPCR, quantitative polymerase chain reaction; U, non-methylation; M, methylation; PCT, para-carcinoma tissue; CT, carcinoma tissue.

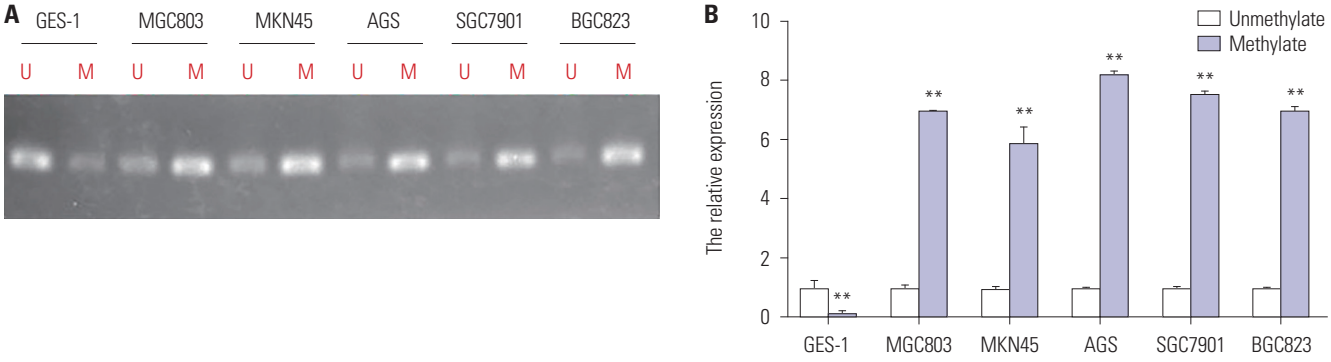

Fig. 8. Detection of the methylation level of the MUC6 promoter region in gastric cell lines by PCR. The results showed that the methylation levels of MGC803, MKN45, AGS, SGC7901, and BGC823 were significantly higher than those of normal GES-1 cells (**p<0.01). (A) PCR results. (B) Statistical analysis. MUC6, mucin 6; PCR, polymerase chain reaction; U, non-methylation; M, methylation.

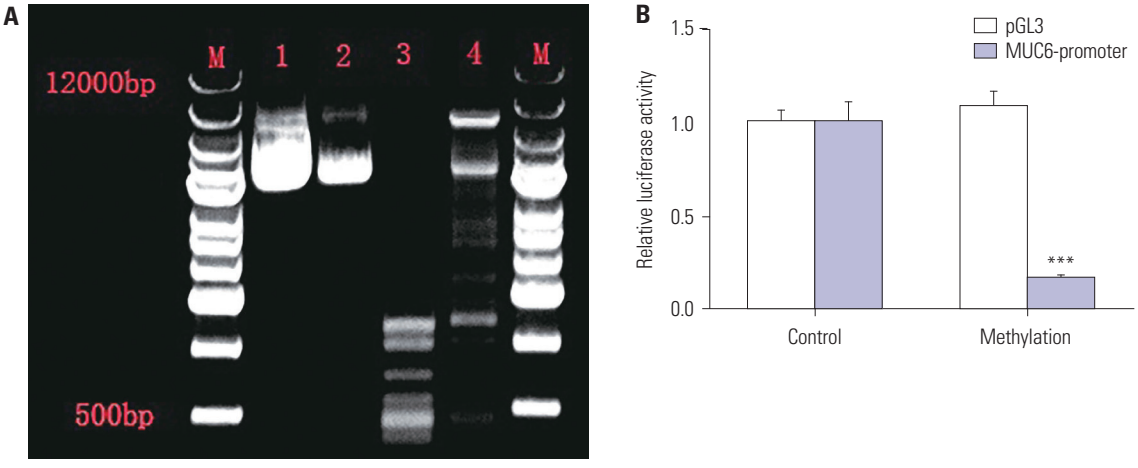

Fig. 9. Fluorescence activity analysis of methylation in the MUC6 promoter region. (A) Validation of methylation modification in MUC6 promoter region. The figure showed that BstUI enzyme could cut the plasmid DNA without M. SssI modification, but not the plasmid with DNA modified by M. SssI. (B) Compared to pGL3, the fluorescence activity of the MUC6 promoter region after methylation decreased significantly (n=3, ***p<0.001). M, marker; 1, plasmid DNA; 2, plasmid DNA treated by M. SssI methylation modifying enzyme; 3, plasmid DNA digested by BstUI enzyme; 4, plasmid DNA digested by M. SssI methylation modifying enzyme; Control, the unmethylated plasmids were transfected into the cells. For methylation, the methylated plasmid was transfected into the cells. MUC6, mucin 6.

https://doi.org/10.3349/ymj.2021.62.11.1005

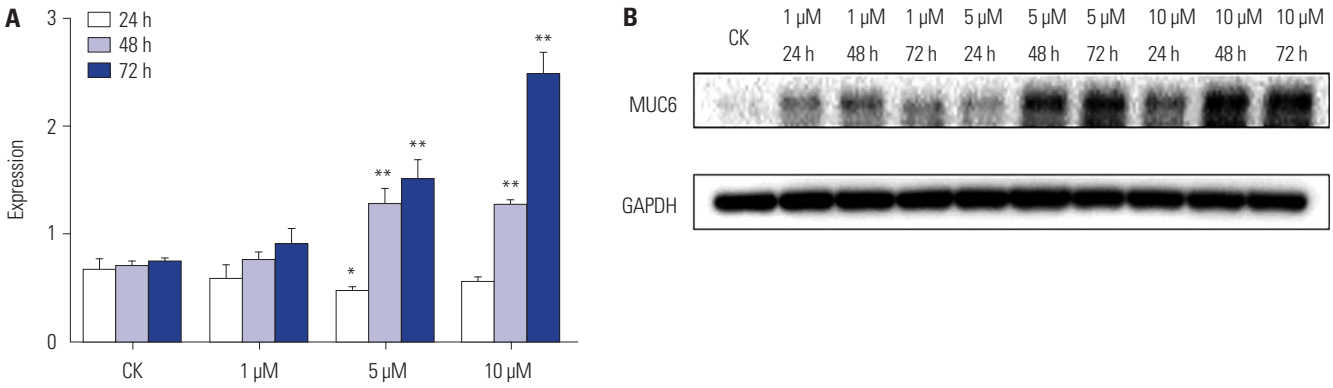

Fig. 10. Detection of the MUC6 expression in cells treated with different concentrations of demethylation drugs by qPCR (A) and WB (B). The results showed that the expression of MUC6 increased with the concentration of demethylated drugs and the time of action (n=3, *p<0.05, **p<0.01). CK, Untreated cells; 1 μM, 5 μM, and 10 μM, Cells were treated with 5-aza-2'-deoxycytidine at concentrations of 1 μM, 5 μM, and 10 μM. MUC6, mucin 6; qPCR, quantitative polymerase chain reaction; WB, western blot.

DISCUSSION

For the first time, to the best of our knowledge, the methylation status of the MUC6 promoter region was shown to cause a significant downregulation of MUC6 expression in GC. The downregulation of MUC6 may promote the metastasis of GC. Earlier studies have focused on detecting the expression of MUC6 in GC and normal tissues.[6] A decrease in MUC6 expression in GC has been established,[9,19] and MUC6 gene polymorphism appears to be associated with an increased tendency to develop GC.[20] Varied expression of the MUC6 gene in GC has been described in the literature.[11] The expression of MUC6 was highly correlated with the progression of GC[13] and was found to be repressed by methylation in KATO-III cells.[21,22] However, these conclusions were only drawn from studies based on clinical specimen detection, and there has not been any direct evidence from basic mechanistic studies. Therefore, the regulation of MUC6 gene expression in gastric cells and the effect of decreased MUC6 expression on the progression of GC have yet to be explored.

The expression of MUC6 in GC and adjacent tissues was investigated, and MUC6 expression was significantly lower in GC than in the adjacent tissues, which was consistent with the findings of previous studies,[9,10] indicating that the downregulation of MUC6 might be related to the progression of GC. To further explore the effect of decreased MUC6 on the progression of GC, MUC6 in GC cells was knocked down by siRNA. After knocking down MUC6, the migration and invasion abilities of GC cells increased significantly. Overexpression of MUC6 in GC cells significantly reduced cell invasion and migration, which suggested that the upregulation of MUC6 might reduce the progression or metastasis of GC. Therefore, at the cellular level, the hypothesis that MUC6 downregulation promotes the metastasis of GC was confirmed. We investigated the expression of MUC6 in 40 GC specimens according to the histological type, lymph node metastasis, and pathological stage. The results showed that the lower the differentiation degree of GC, the lower the expression of MUC6; the worse the stage of GC, the lower the expression of MUC6. The expression of MUC6 in GC patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis. These results suggest that the low expression of MUC6 may be an important factor in GC progression and can be considered a biomarker of GC progression. Since the downregulation of MUC6 leads to decreased adhesion of GC cells,[23] GC cells lacking MUC6 showed more aggressive behavior[10] and were significantly correlated with factors such as the depth of invasion, venous invasion, stage, and poorer patient prognosis,[13] which also correlated with our results.

The methylation of MUC6 in GC and adjacent tissues was investigated based on large data retrieval results of the MUC6 promoter region. The results suggested that the methylation of MUC6 in GC tissues was significantly higher than that in adjacent tissues. To verify the downregulation of MUC6 expression caused by the high methylation status in GC, the promoter region of MUC6 was treated with M. SssI methylation modifying enzyme and verified by BstUI cleavage. The fluorescence of the MUC6 promoter region was observed to be decreased after methylation, indicating that the expression of MUC6 was downregulated. It was also confirmed that methylation interferes with the binding of transcription factors to the MUC6 promoter region. The promoter region of MUC6 was demethylated and transfected into SGC7901 cells with the control plasmid. As a result, the expression of MUC6 in SGC7901 cells was found to be significantly upregulated after demethylation. Previous studies have concluded that regional hypermethylation occurring preferentially at promoter CpG islands plays a significant role in carcinogenesis[24-26] and results in the inactivation of tumor suppressor genes.[27,28] Many CpG sites have been identified throughout the MUC6 promoter,[15] which became the material basis of methylation affecting MUC6.

It can be concluded that our results are in complete agreement with earlier findings;[22] that is, methylation of the promoter region leads to downregulation of MUC6 expression. There-

fore, the hypothesis that promoter methylation causes MUC6 downregulation and promotes GC progression was verified at the molecular level in this study.

The limitation of the present study was that only in vitro experiments revealed that the downregulation of MUC6 promoted the metastasis of GC, and no evidence was reported from animal experiments. In addition, although we observed that methylation in the MUC6 promoter region was related to the progression of GC, we did not provide any information regarding the sites of methylation. These will be investigated in our future research.

In conclusion, the expression of MUC6 was regulated by methylation of its promoter, and the methylation status of the MUC6 promoter might lead to significant downregulation of MUC6 in GC and promote the progression of GC.

ACKNOWLEDGEMENTS

We would like to thank the Hangzhou Hebei company for their technical assistance.

The work was supported by the Medical Scientific Research Foundation of Zhejiang Province, China (Grant No. 2020KY836). Regarding the experiments, the funders provided some experimental techniques.

AUTHOR CONTRIBUTIONS

Conceptualization: Ding Shi. Data curation: Xiao-xia Xi. Formal analysis: Xiao-xia Xi. Funding acquisition: Ding Shi. Investigation: Ding Shi. Methodology: Xiao-xia Xi. Project administration: Xiao-xia Xi. Resources: Ding Shi. Software: Xiao-xia Xi. Supervision: Xiao-xia Xi. Validation: Ding Shi. Visualization: Xiao-xia Xi. Writing—original draft: Ding Shi. Writing—review & editing: Xiao-xia Xi. Approval of final manuscript: all authors.

ORCID iDs

Ding Shi https://orcid.org/0000-0002-3310-7854
Xiao-xia Xi https://orcid.org/0000-0002-8733-741X

REFERENCES

1. Pinto-de-Sousa J, Reis CA, David L, Pimenta A, Cardoso-de-Oliveira M. MUC5B expression in gastric carcinoma: relationship with clinico-pathological parameters and with expression of mucins MUC1, MUC2, MUC5AC and MUC6. Virchows Arch 2004;444:224-30.
2. Senapati S, Sharma P, Bafna S, Roy HK, Batra SK. The MUC gene family: their role in the diagnosis and prognosis of gastric cancer. Histol Histopathol 2008;23:1541-52.
3. Jia Y, Persson C, Hou L, Zheng Z, Yeager M, Lissowska J, et al. A comprehensive analysis of common genetic variation in MUC1, MUC5AC, MUC6 genes and risk of stomach cancer. Cancer Causes Control 2010;21:313-21.
4. Nakayama J. Dual roles of gastric gland mucin-specific O-glycans in prevention of gastric cancer. Acta Histochem Cytochem 2014;47:1-9.
5. Babu SD, Jayanthi V, Devaraj N, Reis CA, Devaraj H. Expression profile of mucins (MUC2, MUC5AC and MUC6) in Helicobacter pylori infected pre-neoplastic and neoplastic human gastric epithelium. Mol Cancer 2006;5:10.
6. Park KK, Yang SI, Seo KW, Yoon KY, Lee SH, Jang HK, et al. Correlations of human epithelial growth factor receptor 2 overexpression with MUC2, MUC5AC, MUC6, p53, and clinicopathological characteristics in gastric cancer patients with curative resection. Gastroenterol Res Pract 2015;2015:946359.
7. Reis CA, David L, Correa P, Carneiro F, de Bolós C, Garcia E, et al Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, and MUC6) expression. Cancer Res 1999;59:1003-7.
8. Reis CA, David L, Carvalho F, Mandel U, de Bolós C, Mirgorodskaya E, et al. Immunohistochemical study of the expression of MUC6 mucin and co-expression of other secreted mucins (MUC5AC and MUC2) in human gastric carcinomas. J Histochem Cytochem 2000;48:377-88.
9. Ho SB, Shekels LL, Toribara NW, Kim YS, Lyftogt C, Cherwitz DL, et al. Mucin gene expression in normal, preneoplastic, and neoplastic human gastric epithelium. Cancer Res 1995;55:2681-90.
10. Zheng H, Takahashi H, Nakajima T, Murai Y, Cui Z, Nomoto K, et al. MUC6 down-regulation correlates with gastric carcinoma progression and a poor prognosis: an immunohistochemical study with tissue microarrays. J Cancer Res Clin Oncol 2006;132:817-23.
11. Kwon JA, Lee SY, Ahn EK, Seol SY, Kim MC, Kim SJ, et al. Short rare MUC6 minisatellites-5 alleles influence susceptibility to gastric carcinoma by regulating gene. Hum Mutat 2010;31:942-9.
12. Terada T. An immunohistochemical study of primary signet-ring cell carcinoma of the stomach and colorectum: II. Expression of MUC1, MUC2, MUC5AC, and MUC6 in normal mucosa and in 42 cases. Int J Clin Exp Pathol 2013;6:613-21.
13. Shiratsu K, Higuchi K, Nakayama J. Loss of gastric gland mucin-specific O-glycan is associated with progression of differentiated-type adenocarcinoma of the stomach. Cancer Sci 2014;105:126-33.
14. Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS, Galiegue-Zouitina S, d'Hooge MC, et al. Human mucin genes assigned to 11p15.5: identification and organization of a cluster of genes. Genomics 1996;38:340-52.
15. Vincent A, Perrais M, Desseyn JL, Aubert JP, Pigny P, Van Seuningen I. Epigenetic regulation (DNA methylation, histone modifications) of the 11p15 mucin genes (MUC2, MUC5AC, MUC5B, MUC6) in epithelial cancer cells. Oncogene 2007;26:6566-76.
16. Gratchev A, Böhm C, Riede E, Foss HD, Hummel M, Mann B, et al. Regulation of mucin MUC2 gene expression during colon carcinogenesis. Ann N Y Acad Sci 1998;859:180-3.
17. Siedow A, Szyf M, Gratchev A, Kobalz U, Hanski ML, Bumke-Vogt C, et al. De novo expression of the Muc2 gene in pancreas carcinoma cells is triggered by promoter demethylation. Tumour Biol 2002;23:54-60.
18. Mesquita P, Peixoto AJ, Seruca R, Hanski C, Almeida R, Silva F, et al. Role of site-specific promoter hypomethylation in aberrant MUC2 mucin expression in mucinous gastric carcinomas. Cancer Lett 2003;189:129-36.
19. Bartman AE, Buisine MP, Aubert JP, Niehans GA, Toribara NW, Kim YS, et al. The MUC6 secretory mucin gene is expressed in a wide variety of epithelial tissues. J Pathol 1998;186:398-405.
20. Garcia E, Carvalho F, Amorim A, David L. MUC6 gene polymorphism in healthy individuals and in gastric cancer patients from northern Portugal. Cancer Epidemiol Biomarkers Prev 1997;6:1071-4.
21. Williams SJ, McGuckin MA, Gotley DC, Eyre HJ, Sutherland GR, An-

talis TM. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. Cancer Res 1999;59:4083-9.
22. Perrais M, Pigny P, Buisine MP, Porchet N, Aubert JP, Van Seuningen-Lempire I. Aberrant expression of human mucin gene MUC5B in gastric carcinoma and cancer cells. Identification and regulation of a distal promoter. J Biol Chem 2001;276:15386-96.
23. Shi D, Qiu XM, Yan XJ. The changes in MUC5AC expression in gastric cancer before and after Helicobacter pylori eradication. Clin Res Hepatol Gastroenterol 2014;38:235-40.
24. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683-92.
25. Toyota M, Issa JP. Epigenetic changes in solid and hematopoietic tumors. Semin Oncol 2005;32:521-30.
26. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223-31.
27. Bird AP, Wolffe AP. Methylation-induced repression--belts, braces, and chromatin. Cell 1999;99:451-4.
28. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21:163-7.