

Methylation regulation of MUC6 correlates with metastasis of gastric cancer

Ding Shi

Huamei Hospital, University of Chinese Academy of Sciences

Xiaoxia Xi (✉ 17839974401m0@sina.cn)

Henan University of Chinese Medicine <https://orcid.org/0000-0002-8733-741X>

Research article

Keywords: MUC6, methylation, gastric cancer, metastasis

Posted Date: February 13th, 2020

DOI: <https://doi.org/10.21203/rs.2.23506/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: The aim of this study was to investigate the mechanism of the downregulation of MUC6 and its influence on GC metastasis.

Methods: The expression of MUC6 was examined in cancer tissues and their corresponding adjacent normal tissues in 40 gastric adenocarcinoma patients. The investigation of methylation level of MUC6 promoter region in gastric cell lines and gastric specimen tissues was performed through immunohistochemistry and/or quantitative polymerase chain reaction (qPCR)s. MUC6 was knocked down in GES-1 cell lines and overexpressed in SGC7901 cell lines; the effects of MUC6 knockdown and overexpression on cell migration and invasion were examined using Transwell migration assay. The effects of demethylation and methylation on MUC6 expression were examined using Western blot, qPCR, or double luciferase report experiment.

Results: The expression of MUC6 in GC tissues was significantly lower than that in normal paracancerous tissues. While the cells migration and invasion abilities were decreased significantly after overexpression of MUC6, these abilities increased significantly after the knocking down of MUC6. The methylation levels of MUC6 in GC tissues and GC cell lines (MGC803, MKN45, AGS, SGC7901, and BGC823) were significantly higher than those in paracancerous tissues and gastric epithelial cells. The promoter methylation could significantly reduce the binding of MUC6 promoter region to the related transcription factors. The expression of MUC6 increased with the concentration of demethylated drugs and the time of action.

Conclusion: The expression of MUC6 was regulated by methylation of its promoter, and this methylation of MUC6 promoter may lead to significant downregulation of MUC6 in GC and promote the metastasis of GC.

Background

MUC6 is one of the main components of the mucus barrier secreted by pyloric gland cells of the antrum and mucous neck cells located in the lower layer of the gastric mucosa of the stomach [1-4]. MUC6 is known to be a marker of gastric foveolar and antral mucous glandular cells, reflecting gastric phenotypes and acts as a significant physiological barrier against various aggressions of 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 is a relatively less-investigated molecule; thus, not much is documented about its expressional changes in GC, the mechanism of such changes, and the effect of these changes on the metastasis of GC. Few studies have reported the downregulation of MUC6 expression in GC [5,7-9], which may be associated with carcinogenesis, malignant potential, progression, clinical behaviors, and poor prognosis of GC[10-13]. These were not independent studies and did not directly focus on MUC6. Thus, till date, the mechanism of the

downregulation of MUC6 and the effect of MUC6 change on the occurrence and progression of GC have not been fully explained.

Some of the earlier studies have recorded that mucins may be positioned at a hotspot for methylation in the genome^[14] and that methylation modifications played a significant role in regulating mucin genes in epithelial cancer cells^[15]. A few studies indicated that the repression of MUC2 expression in colon carcinoma cells was linked to methylation of its promoter^[16]. However, de novo expression of MUC2 was exhibited to be triggered by promoter demethylation or hypomethylation in pancreatic and gastric carcinoma cells^[17,18]. Nevertheless, data regarding MUC6 are unavailable. The above-mentioned studies motivated us to study the relationship between MUC6 and GC using the TCGA database and the software Gene Expression Profiling Interactive Analysis. The expression of MUC6 in GC tissue was found to be significantly lower than that in normal tissues and to be closely associated with the metastasis of tumors (Supplementary Figure 1). Retrieved MethHC database indicated that the methylation level of MUC6 promoter region in GC tissues was higher than that in the normal tissues, and a significant difference between the two levels was found (Supplementary Figure 2). Thus, MUC6 is assumed to promote the metastasis of GC through methylation of its promoter region, resulting in the downregulation of its expression. The effect of downregulation of MUC6 on the biological behavior of GC cells and the mechanism of downregulation of MUC6 expression are the focus of this study. The above hypothesis was investigated through clinical samples, MUC6 overexpression and knockdown, and MUC6 methylation, and demethylation.

Methods

Patients and tissue samples

All tissue samples were supplied by the Department of Gastroenterology, Huamei Hospital, University of Chinese Academy of Sciences between January, 2017 and October, 2018. The study was approved by the Ethics Committee of Huamei Hospital, University of Chinese Academy of Sciences. All participating patients were informed regarding the procedure, and written informed consent was obtained from them. Each patient's surgical sample was divided into two sections. One section was fixed in formalin and embedded in paraffin and then its histopathology was investigated. The other section was immediately refrigerated to be used for MUC6 detection. None of the patients underwent chemotherapy and radiotherapy prior to the tissue harvest.

Cell lines and cell culture

SGC7901 cells with the lowest expression of MUC6 for MUC6 plasmid transfection and gastric epithelial cells (GES-1) with the highest expression of MUC6 for MUC6 knockdown were selected (Supplementary Figure 3). The SGC7901 gastric adenocarcinoma and GES-1 lines were procured from the Shanghai Cell Bank (Shanghai, China). The cells were cultured in a DMEM medium containing 10% FBS and 1× penicillin–streptomycin at 37°C, 5% CO₂, and saturated humidity incubator.

Transfection

The transfected plasmids included MUC6 overexpression and knockdown plasmids. The former was divided into control group (pGL3) and MUC6 overexpression plasmid group (pGL3-MUC6-Promoter). The latter was 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 the total gene synthesis. For transfection, 1 µg MUC6 plasmid and 25 µg Lopti-MEM were gradually added to liposome. Thus prepared 50 µL transfection complex was added drop by drop to the pore containing SGC7901 cells and the culture medium and was gently mixed and incubated at 37°C incubator containing 5% CO₂. Next, the transfection efficiency was detected. Thereafter, RNA and protein were extracted from the cells, and MUC6 expression was analyzed using reverse transcription qPCR and Western blot (WB) protocol.

Protein detection

Immunohistochemistry (IHC)

Tumor tissues were fixed in 4% formalin and embedded in paraffin. Slices of thickness of 4µm were prepared and baked in a 65°C thermostat for 6–12 hours), and IHC was performed. Sections were dewaxed using xylene and rehydrated using gradient alcohol, were blocked using endogenous peroxidase, and inactivated with 3% H₂O₂. The sections were then boiled in citrate buffer (pH 6.0) for 10 minutes to retrieve antigenicity, naturally cooled to room temperature and rinsed thrice with phosphate-buffered saline (PBS), followed by incubation with the first antibody at 4°C overnight. After rinsing thrice with PBS, the sections were incubated with the second antibody at 37°C for 30 min. The sections were stained with DAPI reaction after another wash and then restained with hematoxylin. The staining intensity and positive cell score were observed and photographed to analyze the staining results under light microscopy. Scoring criteria: 0 was negative, 1–3 was weakly positive, 4–5 was moderately positive, and 6–7 was strongly positive.

Hematoxylin–eosin(H&E) staining

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

Western blot (WB) protocol

The total cell protein was extracted as per the following steps: Samples from each group were collected into Eppendorf tubes, and 200 µl Western and Input pyrolysates were added into each tube (PMSF was added before using, and the final concentration was 1 mM). The samples were mixed and fully decomposed at 4°C for 30 minutes, centrifuged at 4°C and 12,000 rpm, and the supernatant were collected and stored separately. Polyacrylamide gel was used for electrophoresis. First, 10% separating gum and 4% concentrating gum were prepared. The sample was then mixed with 5×sample buffer, stored at 100°C for 10 minutes, and rapidly cooled in an ice bath. The sample size was about 30µg per swimming lane. The electrophoretic buffer was added to the electrophoretic tank, and 80V power supply was provided. Constant voltage electrophoresis was performed until bromophenol blue runs out of the concentrated rubber layer. For separating the gel, 120V 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 electrophoresis cell, which was transferred at a constant current of 200 mA and was immersed in a sealed liquid containing 5% skimmed milk powder and sealed at room temperature for 1 hour. The closed PVDF membrane was added with the first antibody and incubated overnight at 4°C (the dilution ratio of MUC6 was 1:1000 and that of GAPDH was 1:5000) (MULTISCIENCES LIANKE) BIOTECH,CO.,LTD, Hangzhou, China). PVDF membranes were washed thrice with 1× TBST buffer, and the second antibody was added to incubate for 1 hour at room temperature and re-washed thrice with 1× TBST buffer. Then, the PVDF membrane was placed on the fresh-keeping film, which was added with the mixture of a moderate amount of solutions A and B from the ECL kit (MULTISCIENCES LIANKE) BIOTECH,CO.,LTD, Hangzhou, China) and moved into the gel imaging analyzer (SMA4000, Merinton, USA). The supporting software was SMA4000 V4.2.3, Merinton, USA. The chemical photosensitive mode was exposed and developed. Photographs were exported in a TIFF format.

Molecular Detection

Quantitative polymerase chain reaction (qPCR) protocol

The total RNA was prepared with the help of a High Purity Total RNA Rapid Extraction kit (No.,GK3016; Generay Corporation, Shanghai, China). Reverse transcription kit (HiScript-II Q RT SuperMix) for qPCR was supplied by Vazyme Biotech Company (No.,R222-01; Vazyme Biotech Co. Ltd., Nanjing). Primers for real-time PCR were designed as per the MUC6 and b-actin sequence: forward primer of MUC6:5'-TGGTGAACCTCGTGGAAGGA-3'; reverse primer of MUC6: 5'-TGGCAGGTGGCAAAGGT-3'; amplification production of MUC6: 139bp. Forward primer of Actin: 5'-TGACGTGGACATCCGCAAAG-3'; reverse primer of actin reverse: 5'-CTGGAAGGTGGACAGCGAGG-3'; amplification production of actin: 205 bp. After reverse transcription, quantitative PCR was performed according to the manufacturer's instructions (ChamQ SYBR Color qPCR Master Mix, Vazyme, NanJing, Q411-02). The reaction system was as follows: SYBR Green mix 10µl, upstream primer 1µl, downstream primer 1µl, diluted cDNA 8µl, and the overall system was of the quantity 20µl. The system was mixed and reacted with a CFX Connect Real-Time PCR (Bio-Rad, Goodhere, Hangzhou, China). instrument. 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, totaling to 45 cycles was performed. Actin was taken as the internal parameter, and the relative expression level of MUC6 was expressed by $2^{-\Delta\Delta Ct}$ value.

Construction of MUC6 siRNA

Three different siRNAs against the human MUC6 gene were designed to specifically knock down MUC6 expression in GES-1 cells. The siRNA sequences were as follows: siRNA-1: CGUCAAAUGUGGUAACAAAGGAG, siRNA-2: UGUUAUCAGUAGUCGUUCUUUGUU, and siRNA-3: GCACAUAUAAGAAACAGUAG. The control siRNA sequence was a random small fragment with the same length.

Methylation

The promoter region of MUC6 was constructed on PGL-3 vector and treated with the methylation reagent. Plasmid DNA was methylated by M. SssI methyltransferase (CpG Methyltransferase, New England Biolabs, USA). Normal pGL3-MUC6-Promoter (or pGL3) and 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 studied 48 hours later. Renilla 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 laid into three six-well plates, four holes for each six-well plate, 200,000 cell holes in each plate, and counted under the counter plate. They were stored in the incubator overnight. Three gradients of the demethylation drug (5-aza-2'-deoxycytidine) concentrations were prepared: 1, 5, and 10. Each 6-hole plate consisted of four groups: CK, 1 μ M, 5 μ M, and 10 μ M groups. Drugs were added into each group. RNA was extracted at 24 hour, 48 hour, and 72 hour, respectively.

Double luciferase report experiment

The double fluorescein luminescence was detected as per the instructions in the Dual Luciferase Reporter Assay System kit (E1910, Promega, Madison, Wisconsin, USA). The concrete procedure is as follows: (1) The 24-hole plate was taken out and the culture medium discarded using a gun head, it was then gently cleaned with 1 \times PBS until it was exhausted. (2) Each hole was added with 100 μ L 1 \times PLB cracking fluid, which was oscillated at room temperature for 20 minutes. The cracking solution was transferred to 1.5 ml EP tube and centrifuged for supernatant. (3) Then, 20 μ L cracking solution was transferred into 1.5 mL EP tube, and 100 μ L luciferase substrate LAR II was added into each hole to prevent light penetration and inserted into the instrument for the first luciferase reading. (4) About 100 μ L 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 hole was the first fluorescence reading of a firefly. The second fluorescence reading was that of sea kidney.

Phenotype Detection

Cell migration

After 48 hours, the treated cells were digested and centrifuged with 0.25% trypsin + 0.02% EDTA, suspended with 2% serum medium, counted, transferred to a 24-well plate with 2.0×10^5 /well/density, and incubated with 10% serum medium in the basement, 5% CO₂, and 37°C incubator temperature.

Cell invasion

Matrix gel was removed from the refrigerator at -20°C and was ice bathed overnight at 4°C. Under the ice bath, 10mg/mL matrix glue was gently mixed with a serum-free medium of equal volume and then added to the upper layer of the Transwell chamber, incubated at 37°C for 4–6 hours. The serum-free medium was gently rinsed once in the Transwell chamber for later use. Cells in each group were digested with 0.25% trypsin + 0.02% EDTA, centrifuged at 1500rpm for 5 minutes, counted on a counter plate, suspended in a 2% serum medium, diluted to 5.0×10^5 /well, and placed in the upper chamber of the Transwell chamber, 2.0×10^5 /well, and cultured in 10% serum of 500µL in the lower chamber. After 16 hours of culture, the above-mentioned cells were cleaned thrice with 1 × PBS, fixed at room temperature with 4% paraformaldehyde for 15 minutes, then cleaned thrice with 1 × PBS again, wiped the upper chamber cells with cotton swabs, the cells with crystal violet stain for 15 minutes, cleaned thrice with 1 × PBS, air-dried at room temperature, and photographed under a microscope.

Statistical analysis

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

Results

Patient characteristics

A total of 40 specimens were collected from GC and gastric mucus membranes from the near cancer sites. Forty patients were confirmed by histopathology of surgical samples for adenocarcinoma, and the tissues surrounding the cancer site was confirmed as normal gastric mucus membrane through H&E

staining. The patient's age range was 51–83 years (median age =67.6), including 24 males and 16 females. The patients' demographic data are given in Table 1.

MUC6 expression levels in GC and paracancerous tissues

IHC and qPCR were performed in 40 cases of GC and corresponding normal adjacent tissues. By way of either IHC ($p<0.05$) or qPCR ($p<0.01$), the results indicated that the expression of MUC6 in GC tissues was significantly lower than that in normal paracancerous tissues (Figures 1 and 2).

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

SGC7901 cells with the lowest expression of MUC6 for transfection were selected (Supplementary Figure 3). After the transfection of MUC6 plasmid, the expression of MUC6 in SGC7901 cells was found to increase significantly to a value higher than that in the control group, which was confirmed by qPCR ($p<0.001$; Figure 3A) and WB detection ($p<0.001$; Figure 3B). The migration ($p<0.01$) and invasion ($p<0.05$) abilities of SGC7901 cells were observed to be decreased significantly after overexpression of MUC6 (Figure 4).

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

Methylation level of MUC6 promoter region

The methylation of MUC6 promoter region was detected by way of qPCR in 5 GC patients in the GC and adjacent tissues. The methylation level of MUC6 in paracancerous tissues was found to be significantly lower than that in GC tissues (Figure 7).

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

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 with pGL3, the fluorescence activity of MUC6 promoter region after methylation was observed to decrease significantly ($p<0.001$), suggesting that BstUI enzyme could cut plasmid DNA without M. SssI modification, but not that modified by M. SssI (Figure 9). This indicated that promoter methylation could significantly reduce the binding of MUC6 promoter region to related transcription factors.

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

Discussion

For the first time, as per our knowledge, the methylation of MUC6 promoter region was shown to cause a significant downregulation of MUC6 in GC and that the downregulation of MUC6 may promote the metastasis of GC. Earlier studies focused on detecting the expression of MUC6 in GC and normal tissues^[6]. A decrease in MUC6 expression in GC has been elucidated^[9,19], and MUC6 gene polymorphism appears to be associated with the tendency of GC^[20]. Varied expression of 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 studies only draw conclusions based on clinical specimen detection, and there was no direct evidence of basic tests, lack of systematic and complete demonstration. Therefore, regulation of MUC6 gene expression in gastric cells and the effect of downregulation of MUC6 expression on metastasis of GC are yet to be further explored.

The expression of MUC6 in GC and adjacent tissues was investigated, and it was indicated to be significantly lower in GC than in the adjacent tissues, which was consistent with the findings of earlier studies^[9,10], indicating that downregulation of MUC6 might be related to the progression of GC. To further explore the effect of downregulation of MUC6 on metastasis of GC, MUC6 in GC cells was knocked down by siRNA. After knocking down MUC6 gene in GC cells, the migration and invasion abilities of GC cells increased significantly. MUC6 plasmid was also transfected into SGC7901 cells and performed cell migration and invasion experiments. Overexpression of MUC6 in GC cells could significantly reduce cell invasion and migration as per our results, which proved that upregulation of MUC6 may reduce the metastasis of GC. Earlier studies concluded that GC lacking MUC6 expression showed aggressive behavior^[10] and was significantly correlated with the factor such as the depth of invasion, venous invasion, stage, and poorer patient prognosis^[23], which mutually correlated with our results. Therefore, at the cellular level, the hypothesis that MUC6 downregulation promotes the metastasis of GC was confirmed.

The methylation of MUC6 in GC and adjacent tissues was investigated on the basis of large data retrieval results of MUC6 promoter region. The results suggested that the methylation of MUC6 in GC tissues was found to be significantly higher than that in adjacent tissues. To verify the downregulation of MUC6 expression caused by high methylation level in GC, the promoter region of MUC6 was treated with M. SssI methylation modifying enzyme and verified by BstUI cleavage. The fluorescence of MUC6 promoter region was observed to be decreased after methylation, indicating that the expression of MUC6 was downregulated. It was confirmed that methylation interferes with the binding of MUC6 promoter region to

transcription factors. 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 upregulated significantly after demethylation. Earlier studies have concluded that regional hypermethylation occurring preferentially at promoter CpG islands has a significant role in carcinogenesis^[24-26] and results in the inactivation of tumor suppressor genes^[27,28]. A high number of CpG sites throughout MUC6 promoter had been identified^[15], which became the material basis of methylation affecting MUC6.

It may be concluded that our results completely correlate with those of earlier findings^[22], that is, methylation of promoter region leads to downregulation of MUC6 expression. Thus, the hypothesis that promoter methylation caused MUC6 downregulation and promoted GC metastasis was verified at the molecular level.

The limitation of this 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 metastasis of GC, we did not provide any information on the related sites of methylation. These will be investigated in our next study.

Conclusion

To conclude, the expression of MUC6 was evidently regulated by methylation of its promoter, and the methylation of MUC6 promoter might lead to significant downregulation of MUC6 in GC and promote the metastasis of GC.

Abbreviations

GC: gastric cancer

GES-1: gastric epithelial cells

PBS: phosphate-buffered saline

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Huamei Hospital, University of Chinese Academy of Sciences. All participating patients were informed regarding the procedure, and written informed consent was obtained from them.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

Funding

The work was supported by Supported by Medical Scientific Research Foundation of Zhejiang Province, China (Grant No. 2020KY836);In our experiment, the funders provided some experimental techniques.

Authors' contributions

DS. Contributed to the study design, acquisition and analysis of data, paper revision; X X. Wrote the paper; All authors read and approved the final manuscript.

Acknowledgments

We'd like to thank Hangzhou Hebei company for our technical assistance.

Authors' information

1. Ding Shi Department of Gastroenterology, Huamei Hospital, University of Chinese Academy of Sciences, Ningbo 315010, China.
2. Xiao-xia Xi Department of Gastroenterology, Henan University of Chinese Medicine, Zhengzhou 450000, Henan Province, China.

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(3): 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(12): 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(2): 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): 1-9.
- [5] Babu SD1, 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; 19; 5: 10.
- [6] Park KK1, Yang SI1, Seo KW1, Yoon KY1, Lee SH, 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. doi: 10.1155/2015/946359.
- [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(5): 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(3): 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(12): 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 ClinOncol*. 2006;132(12): 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(8): 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(4): 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(1): 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(3): 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(45): 6566-76.

- [16] Gratchev A, Bohm C, Riede E, Foss HD, Hummel M, Mann B, et al. Regulation of mucin MUC2 gene expression during colon carcinogenesis. *Ann NY Acad Sci.* 1998; 859: 180-183.
- [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(1): 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; 89: 129-136.
- [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(4): 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(12): 1071-4.
- [21] Williams SJ, McGuckin MA, Gotley DC, Eyre HJ, Sutherland GR, Antalis TM. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. *Cancer Res.* 1999; 59(16): 4083-9.
- [22] Perrais M, Pigny P, Buisine MP, Porchet N, Aubert JP, Van Seuningem-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(18): 15386-96.
- [23] 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(1): 126-33.
- [24] Jones PA, Baylin SB. The epigenomics of cancer. *Cell.* 2007; 128(4): 683-92.
- [25] Toyota M1, Issa JP. Epigenetic changes in solid and hematopoietic tumors. *Semin Oncol.* 2005; 32(5): 521-30.
- [26] Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer.* 2005; 5(3): 223-31.
- [27] Bird AP1, Wolffe AP. Methylation-induced repression—belts, braces, and chromatin. *Cell.* 1999; 99(5): 451-4.
- [28] Jones PA1, Laird PW. Cancer epigenetics comes of age. *Nat Genet.* 1999; 21(2): 163-7.

Table

Table 1. Clinicopathological Features

Features	Number
Sex	
Male	24
Female	16
Age	
<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

Figures

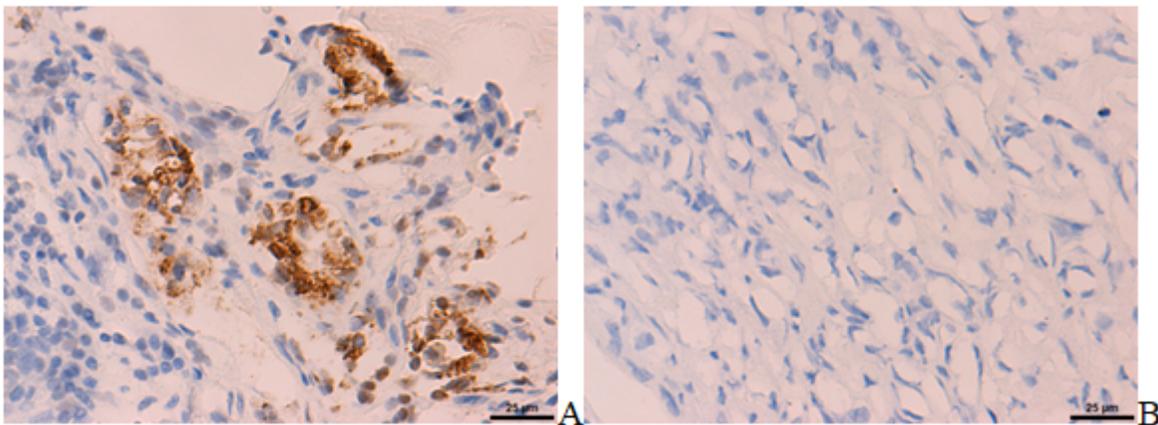


Figure 1

IHC findings of gastric samples. Immunoreactivities to MUC6 distributed to the cytoplasm. A, Immunohistochemical staining of MUC6 in normal paracancerous tissues; B, Immunohistochemical staining of MUC6 in GC tissues. There was a strong MUC6 expression in paracancerous tissues, but not in gastric carcinoma tissues. Immunostaining $\times 40$.

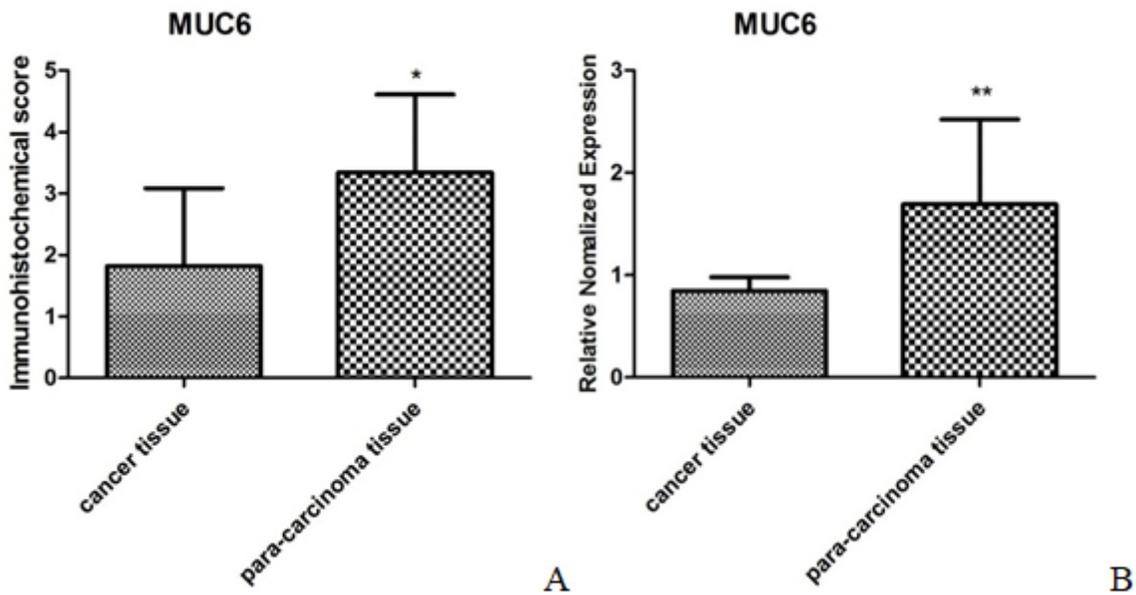


Figure 2

Detection of MUC6 in GC and adjacent tissues by IHC and qPCR. A. IHC showed that MUC6 in GC tissue was significantly downregulated compared with that in adjacent tissues (* $p < 0.05$); B. qPCR showed that MUC6 in GC tissues was significantly downregulated compared with that in adjacent tissues (** $p < 0.01$).

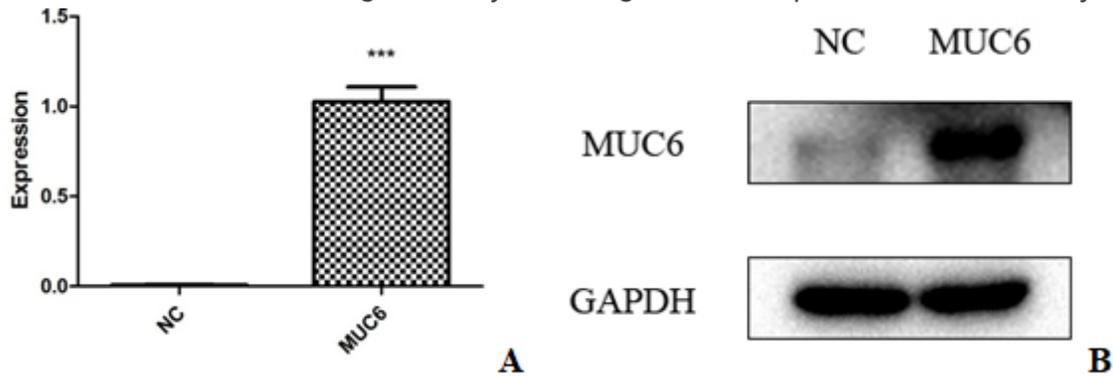


Figure 3

Detection of MUC6 expression by qPCR and WB 48 hours after transfection. A. qPCR showed that the expression of MUC6 mRNA increased significantly after transfection of overexpressed plasmid ($n = 3$, *** $p < 0.001$); WB showed that the expression of MUC6 protein increased significantly after the transfection of overexpressed plasmid ($n = 3$, *** $p < 0.001$). NC, control group.

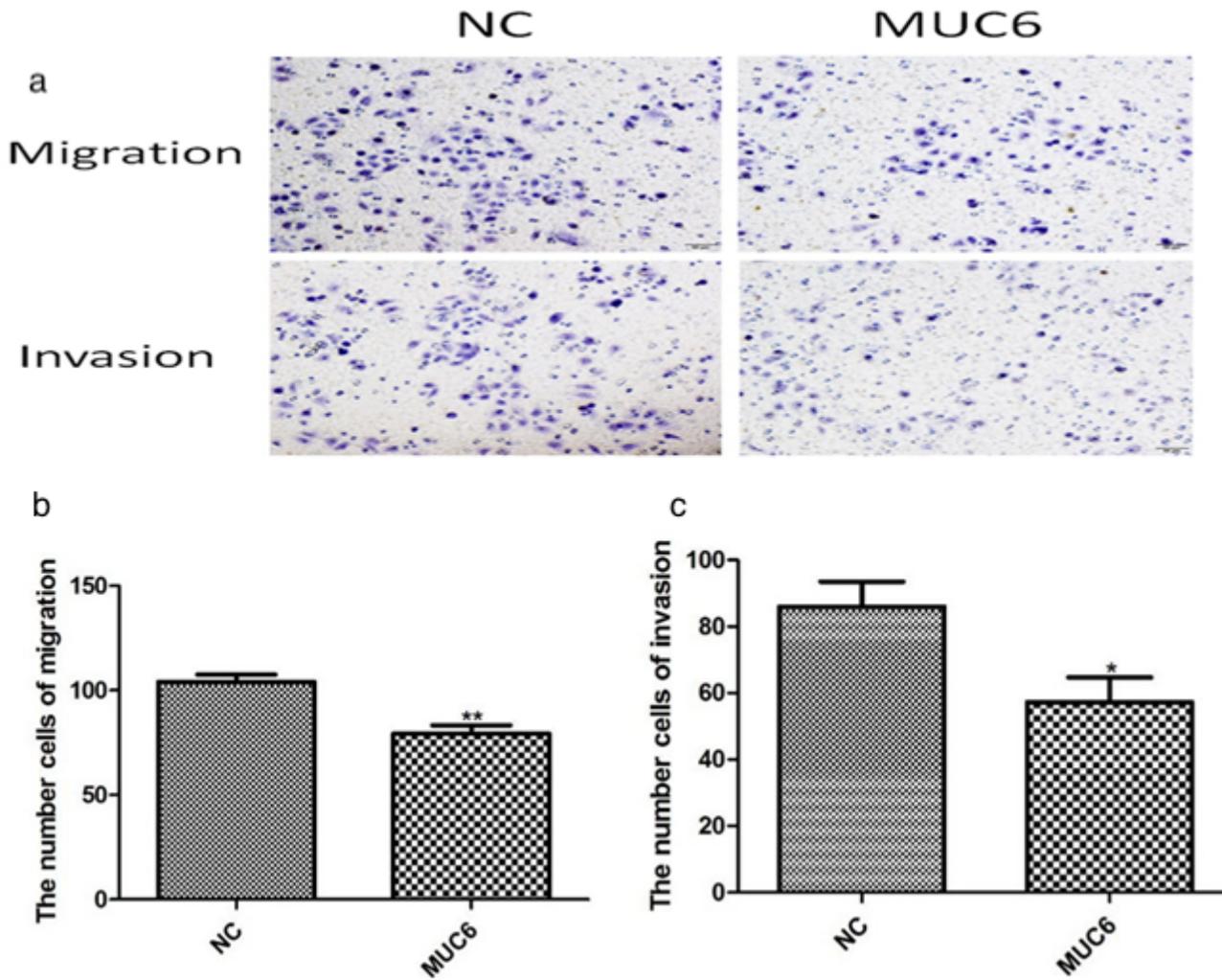


Figure 4

Detection of migration and invasion of SGC7901 cells by Transwell after overexpression of MUC6. Compared with NC, the ability of cell migration and invasion decreased significantly after MUC6 overexpression (n=3, **p<0.01, *p<0.05). NC, No-load plasmid group. MUC6, MUC6 overexpression group.

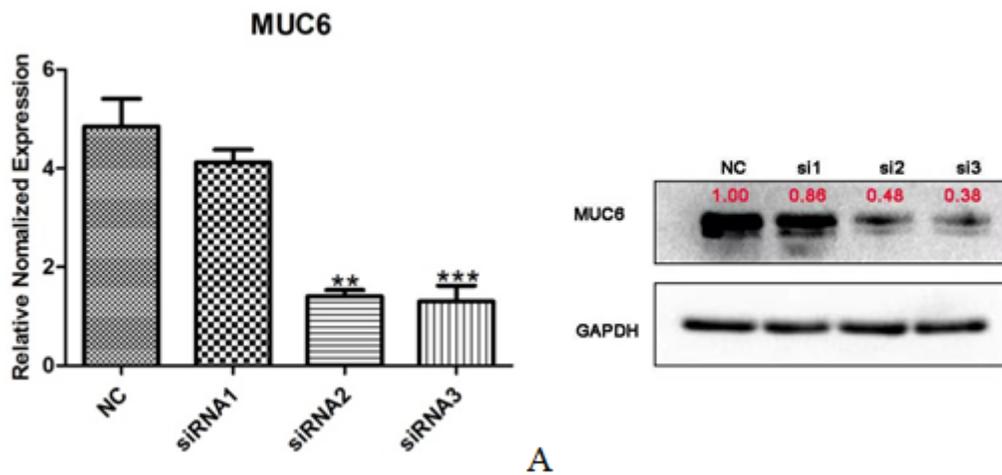


Figure 5

Detection of MUC6 expression after knockdown by qPCR and WB. Compared with 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.

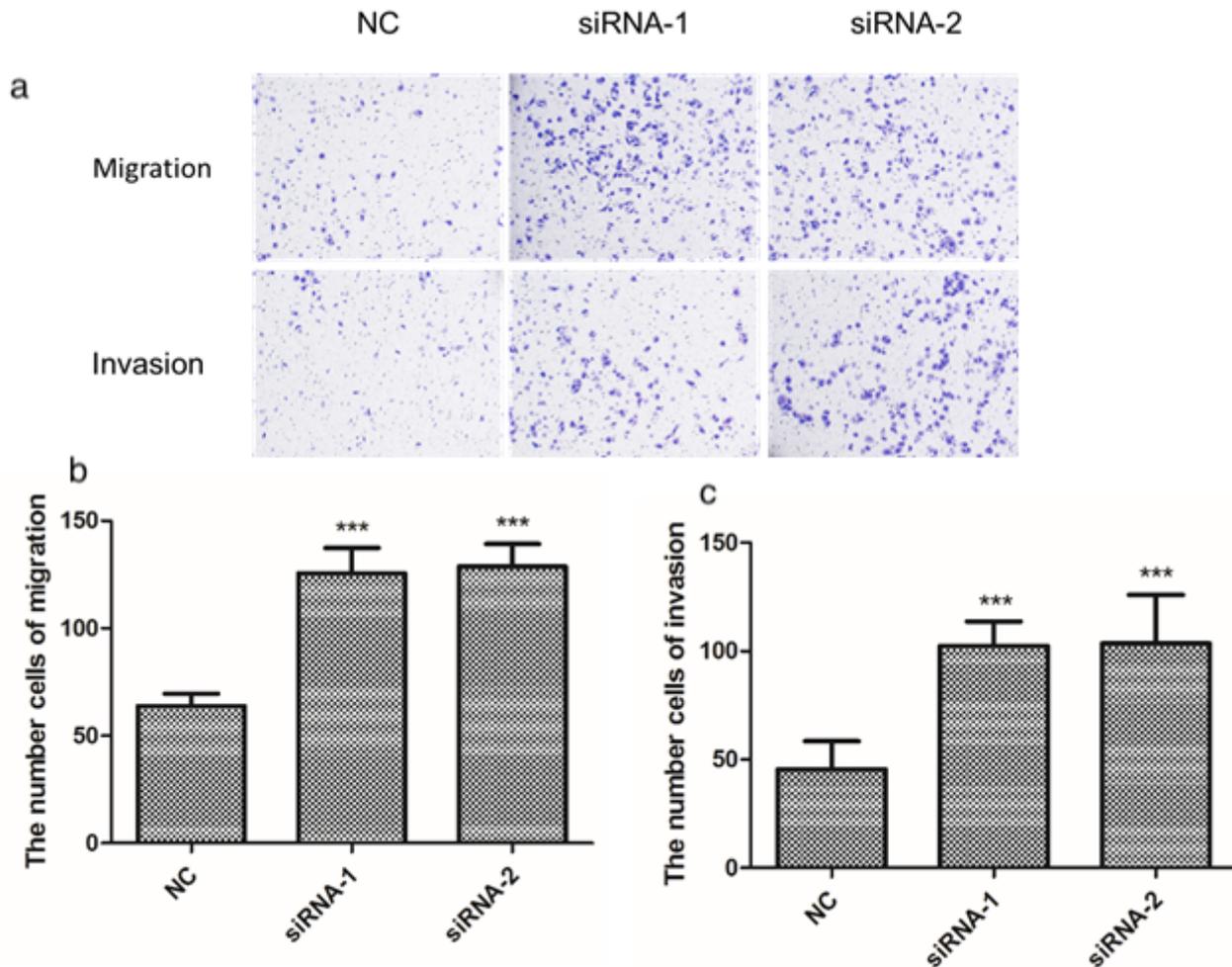


Figure 6

Cell migration and invasion assay. Transwell assay showed that the migration and invasiveness of cells increased significantly after knocking down MUC6 by siRNA (n=3, ***p<0.001). NC, siRNA irrelevant sequence; siRNA, knockdown of MUC6.

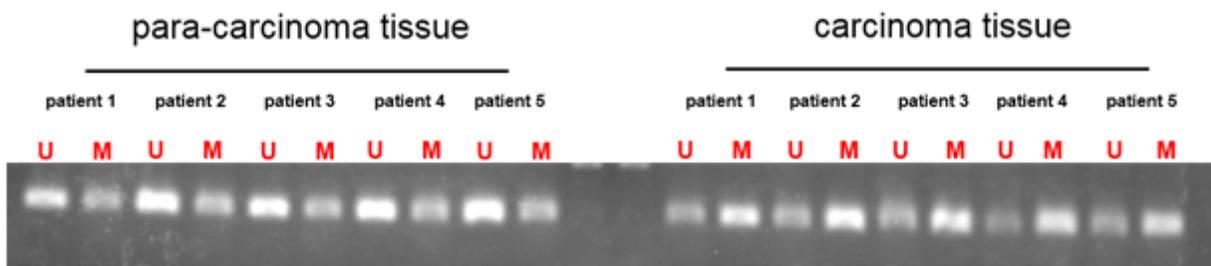


Figure 7

Detection of MUC6 methylation in GC and adjacent tissues by qPCR. The results showed that the methylation level of MUC6 promoter region in paracancerous tissues was significantly lower than that in cancer tissues. U, non-methylation; M, methylation.

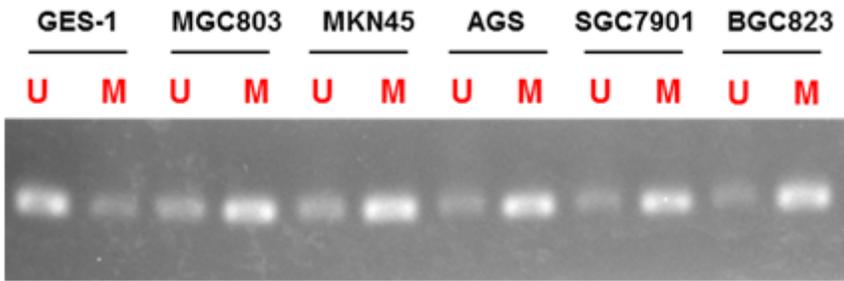


Figure 8

Detection of methylation level of 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. U, non-methylation; M, methylation.

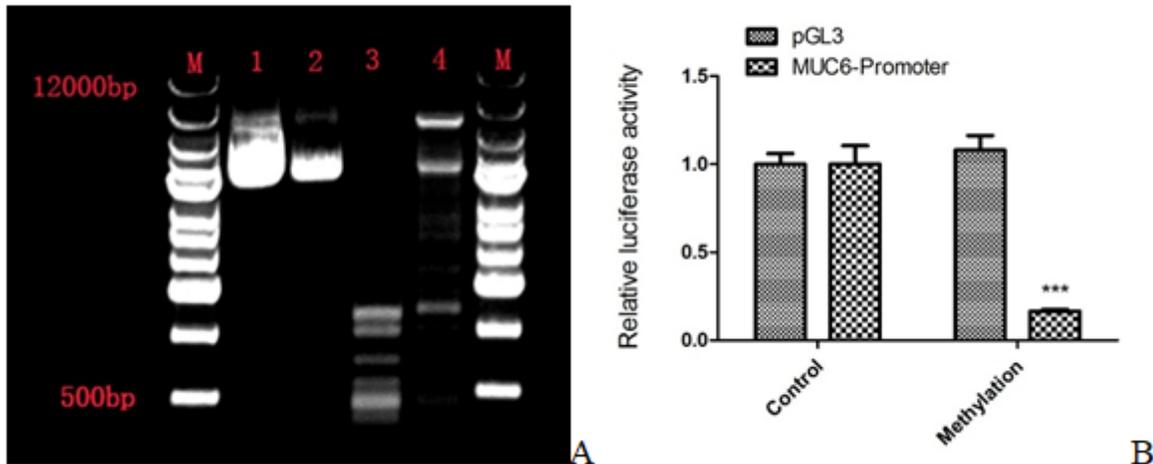
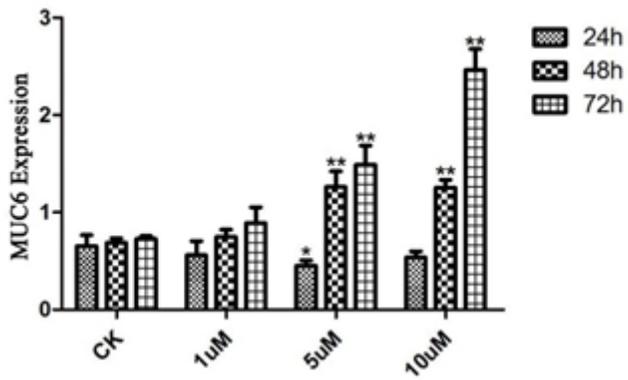
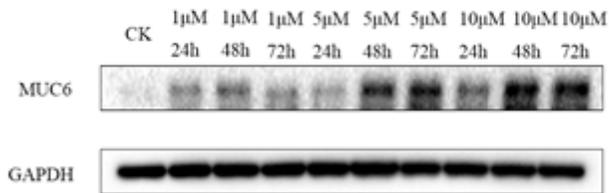


Figure 9

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



A



B

Figure 10

Detection of 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 ($n=3$, $**p<0.01$, $*p<0.05$). CK, Untreated cells; 1µM, 5µM, and 10µM, Cells were treated with 5-aza-2'-deoxycytidine at concentrations of 1, 5, and 10µM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure1.pdf](#)
- [SupplementaryFigure3.pdf](#)
- [SupplementaryFigure2.pdf](#)