European Review for Medical and Pharmacological Sciences 2020; 24: 7972-7981

Reduced long noncoding RNA PGM5-AS1 facilitated proliferation and invasion of colorectal cancer through sponging miR-100-5p

B. ZHOU[1], F. YI[1], Y. CHEN[1], C.-H. LI[1], Y.-S. CHENG[1,2], K. YANG[1,2]

[1]Department of Diagnostic and Interventional Radiology, Shanghai Sixth People's Hospital East Affiliated to Shanghai University of Medicine and Health Sciences, Shanghai, China
[2]Shanghai University of Traditional Chinese Medicine, Shanghai, China

Zhou Bi and Yi Fei contributed equally to this work

Abstract. – OBJECTIVE: We aimed at investigating the expression of Long non-coding RNA (LncRNA) PGM5-AS1 and its facilitating effects on proliferation and invasion of colorectal cancer by sponging miR-100-5p.

PATIENTS AND METHODS: qRT-PCR was performed to detect the expressions of PGM5-AS1 and SMAD4 in human colorectal cancer tissues and cells. CCK-8 assay was performed to evaluate the SW403 cells proliferation and transwell assay was performed to evaluate the SW403 cells migration. The correlation between miR-100-5p and PGM5-AS1 was detected by statistical analysis. Bioinformatics prediction and Luciferase assay were performed to explore the interaction and binding site of PGM5-AS1 and miR-100-5p, miR-100-5p and SMAD4, respectively.

RESULTS: We found that both PGM5-AS1 and SMAD4 were downregulated in human colorectal cancer tissues and cells. qRT-PCR and CCK-8 assay showed that PGM5-AS1 expression is associated with the proliferation of colorectal cancer cells. Transwell assay showed that PGM5-AS1 regulated the migration ability of colorectal cancer cells. The bioinformatics prediction and Luciferase assay demonstrated that by sponging miR-100-5p, PGM5-AS1 can serve as a molecular sponge to further regulate the expression of SMAD4.

CONCLUSIONS: In this study, we found that lncRNA-PGM5-AS1 was low expressed in human colorectal cancer cells, which could promote tumor proliferation, migration and invasion by serving as a molecular sponge and by modulating the inhibitory effect of miR-100-5p on tumor suppressor gene SMAD4.

Key Words:
Colorectal cancer, MiR-100-5p, PGM5-AS1, Proliferation, Invasion.

Abbreviations

ARIMA: Autoregressive Moving Average; CCDC: Chinese Centre of Disease Control and Prevention; CoVID-19: Novel Coronavirus; GFS; Global Forecast System; IR; Short-Wave Irradiation; WD: Wind Direction; WHO; World Health Organization; WS: Wind Speed.

Introduction

Colorectal cancer is one of the most common malignancies in which cancer cells form in the colon tissues[1]. As the third most common carcinoma and the second leading cause of cancer-related deaths worldwide, the incidence and mortality rates of colorectal cancer are gradually increasing in China, with 376,300 new cases and 191,000 deaths in 2015[2,3]. Currently, the standard therapeutic methods for patients with colorectal cancer consist of surgery, radiation and chemotherapy alone or in combination[4]. Despite great advances in treatment technology, the 5-year survival rate of colorectal cancer is still <40% due to recurrence and metastasis[5]. Therefore, clarifying the intrinsic mechanisms and exploring potential new targets for colorectal cancer therapy are imperative.

As a class of non-coding RNAs, long non-coding RNAs (lncRNAs) are defined as >200 nucleotides in length and play an important role in diverse cellular processes, such as regulation of gene expression, posttranslational processing and tumorigenesis[6-8]. Alterations in lncRNA can result in abnormal expression of genes involved in biological function and disease[9]. Scholars[10] showed that lncRNAs were strongly related with proliferation and progression of colorectal cancer. Han et al[8]

Corresponding Author: Yang Kai, MD; e-mail: 98211yangkai@163.com

demonstrated that lncRNA-CRNDE contribute to cell proliferation and migration of colorectal cancer, and is associated with a poor prognosis. LncRNA PGM5 antisense RNA 1 (PGM5-AS1) is identified as a shared genetic susceptibility region related to several human diseases, including cancers, which had been showed that could predict a poor prognosis in patients with hepatocellular cancer[11]. However, the underlying mechanisms of lncRNA PGM5-AS1 involved in colorectal cancer remain to be elucidated at present.

MicroRNAs are short endogenous non-coding molecules (around 19-23 nucleotides in length) that regulate gene expression by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs, which play an important role in maintaining the homeostasis[12]. Currently, it is widely accepted that miRNAs may act as oncogenes or suppressor genes during tumor development[13]. Competing endogenous RNA (ceRNA) hypothesis was firstly stated by Esquela-Kerscher et al[14], Salmena et al[15] and Tay et al[16], suggesting that lncRNAs served as miRNA sponges to modulate the expression of miRNA target genes, thus participating in various biological processes including the progression of cancers. However, whether PGM5-AS1 could interact with miRNA to regulate the development of colorectal cancer remains to be elucidated.

Thus, the aim of the present study was to investigate the level of PGM5-AS1 expression in the progression of colorectal cancer and explore its underlying mechanism.

Patients and methods

Patients and Tumor Samples

In this study, we used NCCN clinical practice guidelines for patient diagnosis. 12 cases of colorectal cancer patients were diagnosis by colonoscopy. 16 pairs of colorectal cancer tissues and adjacent normal tissues were obtained from surgically treated and pathologically diagnosed colorectal cancer cases and then stored at -80°C. Patient information was included in Table I. No significant differences in the 12 pairs of samples in terms of diagnostic indicators and prognostic factors were reported. This study was approved by the Ethics Committee of our hospital. Patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients signed informed consent.

Table I. Demographic data.

Gender	Male	Female
Patients numbers	7	9
BMI (kg/m²) ± SD	22.1±3.8	24.1±5.2
Age (years)		
< 45	4	5
≥ 45	3	4
TNM stage		
I-II	5	6
III-IV	2	3
Lymph node metastasis		
Negative	4	4
Positive	3	5

Cell Culture

Human colorectal cancer cells SW403 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Rockville, MD, USA) supplied with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) and incubated at 37°C in an atmosphere of 5% CO_2.

Construction of Lentivirus and Cell Transfection

Lentiviral Lnc PGM5-AS1 and lnc PGM5-AS1 shRNA were synthesized and constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For miR analysis, the miR-100-5p mimic, miR-100-5p inhibitor and the negative control were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). To knockdown SMAD4, si-SMAD4 plasma and negative control plasma were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For transfection, 1×10^4 cells were seeded in 6-well plates and cultured with RANKL (100 ng/mL) and M-CSF (100 ng/mL). Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced serum medium were used for transfection. For analysis of Lnc-PGM5-AS1, cells were transfected with Lnc-PGM5-AS1 shRNA (referred as to sh) and negative control shRNA (referred as to nc), respectively. For analysis of miR-100-5p, the cells were transfected with miR-100-5p inhibitor, and control cells were transfected with empty vector, respectively. The cells without transfection were used as the control (referred as to control). After incubated for 30 min, the cultures were replaced with DMEM containing 10% FBS. Then, at indicated time point after transfection, the cells were harvested for further study.

Transwell Assay

To test the migration ability of SW403 cells, transwell plates with a pore size of 8 μm (Millipore Inc, Billerica, MA, USA) were used to conduct transwell assay. SW403 cells were treated differently and the lower chamber was added with DMEM supplemented with 20% FBS. The upper side of the membrane was wiped with a cotton swab to remove the cells that did not migrate, cell numbers in five random fields were counted in each sample.

RNA Extraction and qRT-PCR

After taking out the culture plates, the cells were washed with PBS. After treatment, total RNA of cells was extracted by using TRIzol reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. The samples were stored at room temperature for 30 min. The reverse transcription of cDNA was performed with a PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. For qRT-PCR, PCR primers were synthesized by GenePharma (ShangHai Gene Pharma, Shang-Hai, China) and sequences were listed in Table II. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

CCK-8 Assay

The CCK-8 kit (Dojindo, Kumamoto, Japan) was used to measure the cells proliferation according to the manufacturers' instructions. In brief, 5×10^3 cells were seeded in 96-well plates uniformly. After treated with regulated medium, the medium was removed, and cells were washed with PBS solution for 3 times. Then, CCK-8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 hours. After incubation, the plates were taken out, and cell proliferation was measured using multi-detection microplate reader. The absorbance (OD) value at 490 nm of each well was detected.

Luciferase Assay

After transfection for 48 h, the Luciferase activities were measured by using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla Luciferase activities were normalized to the firefly Luciferase activities and the data were expressed as the fold change relative to the corresponding control groups which were defined as 1.0.

Statistical Analysis

Unless otherwise indicated, all data are processed by Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Each assay was applied at least three independent experiments or replicates. All data were presented as mean ± SD. Student's *t*-test, one-way analysis of variance (ANOVA) and multiple comparison between the groups was performed by using SNK method, in which $*p<0.05$, $**p<0.01$ represented as the difference significance.

Results

PGM5-AS1 and SMAD4 Were Downregulated in Human Colorectal Cancer Tissues and Cells

To determine the roles of PGM5-AS1 and SMAD4 in colorectal cancer progression, we firstly performed qRT-PCR to examine the expressions of PGM5-AS1 and SMAD4 in colorectal cancer tissues and adjacent normal tissues. The results revealed that both the expressions of PGM5-AS1 and SMDA4 were significantly downregulated in colorectal cancer tissues compared with that of adjacent normal tissues (Figure 1A and 1B). To further interpret the biological functionality of PGM5-AS1 in colorectal cancer, we performed qRT-PCR to detect PGM5-AS1 expression in human colorectal cancer cell lines SW403. The results showed that both PGM5-

Table II. Primer sequences for qRT-PCR.

Genes	Forward	Reverse	Tm (°C)
PGM5-AS1	5'-AAAAGCTGTCGTAAACGTGGG-3'	5'-AAGCTCGTCGCATCTCTCGTCCA-3'	62
miR-100-5p	5'-ACGTCGTCATGGGGTACCCCA-3'	5'-GTACGATCGATGCGCTACGTCG-3'	61
SMAD4	5'-TAAGGGTGGCCAATGGTGAGA-3'	5'-GGGTTTTCCGACTCCTACAT-3'	62
U6	5'-ACTGATCGATGCCTGATCGATCG-3'	5'-AAAGCTGTCCCGGGGTACGTGCC-3'	61
GAPDH	5'-TGGATTTGGACGCATTGGTC-3'	5'-TTTGCACTGGTACGTGTTGAT-3'	62

AS1 and SAMD4 expressions were significantly downregulated in colorectal cancer cells compared with human epithelia cells HEK293 cells (p<0.05) (Figure 1C and 1D).

The Migration and Invasion of Colorectal Cancer Cells Were Significantly Inhibited After Upregulated the PGM5-AS1 Expression

To explore the functions of PGM5-AS1 in colorectal cancer progression, PGM5-AS1 overexpressing lentiviral (Lnc-PGM5-AS1) was constructed and transfected into SW403 cells. Besides, small interfering RNA for PGM5-AS1 (si-PGM5-AS1) was also synthesized and transfected into SW403 cells. The expression of PGM5-AS1 was subsequently detected by qRT-PCR. The results showed that the expression of PGM5-AS1 in the Lnc-PGM5-AS1 group was significantly enhanced compared with the vector1 group (p<0.05), while the expression levels of PGM5-AS1 were reduced in the si-PGM5-AS1 group compared with the negative control vector2 group (p < 0.05) (Figure 2A and 2B). To verify the role of PGM5-AS1 on cell proliferation, CCK-8 assay was performed on SW403 cells after regulation of PGM5-AS1 expression. The results revealed that the overexpression of PGM5-AS1 significantly reduced colorectal cancer cells proliferation compared with the control group, whereas the inhibition of PGM5-AS1 expression remarkably increased the cell proliferation number at 3 days (Figure 2C, 2D). These results suggested that alteration of PGM5-AS1 expression could influence the proliferation of

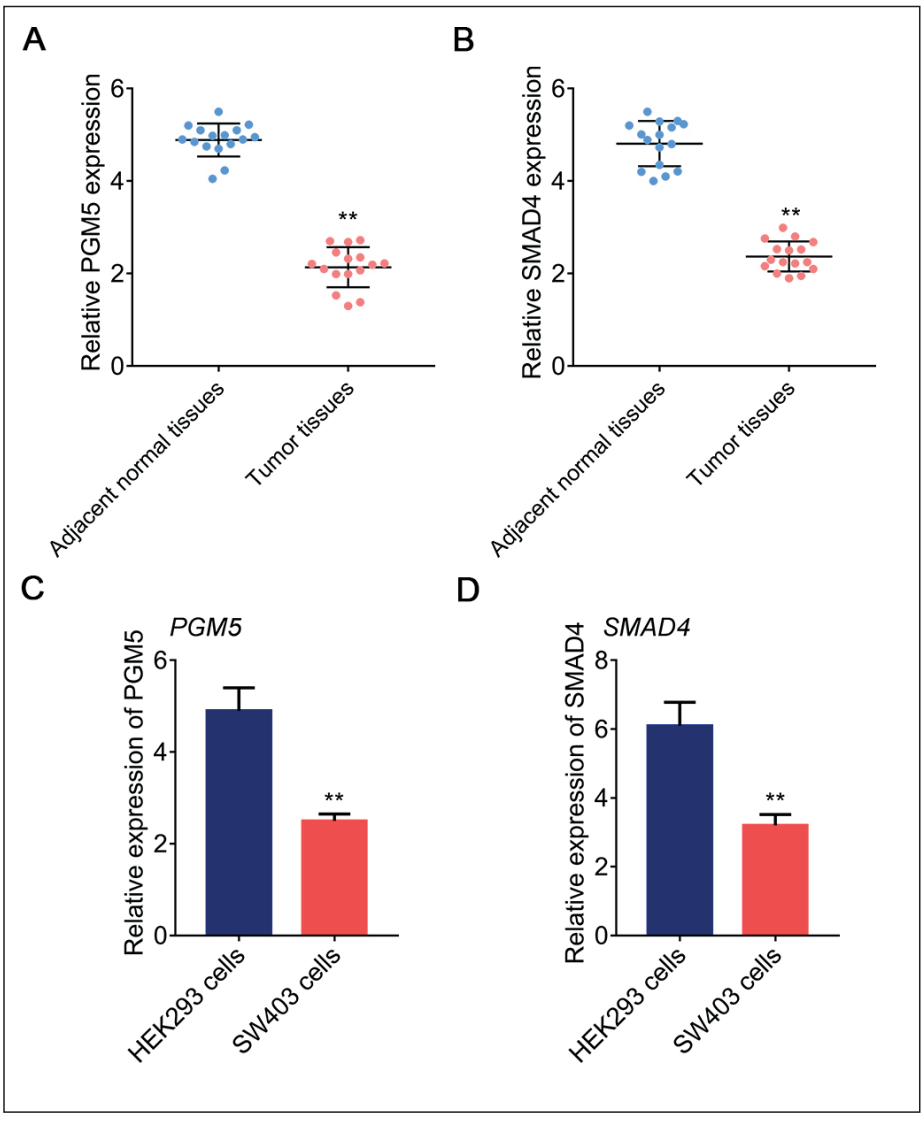

Figure 1. PGM5-AS1 and SMAD4 were downregulated in human colorectal cancer tissues and cells. Relative mRNA expression levels of (**A**) PGM5-AS1 and (**B**) SMAD4 in colorectal cancer tissues and adjacent normal tissues. Relative mRNA expression levels of (**C**) PGM5-AS1 and (**D**) SMAD4 in human colorectal cancer cell line SW403 and HEK293 cells. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as *(p<0.05) or **(p<0.01).

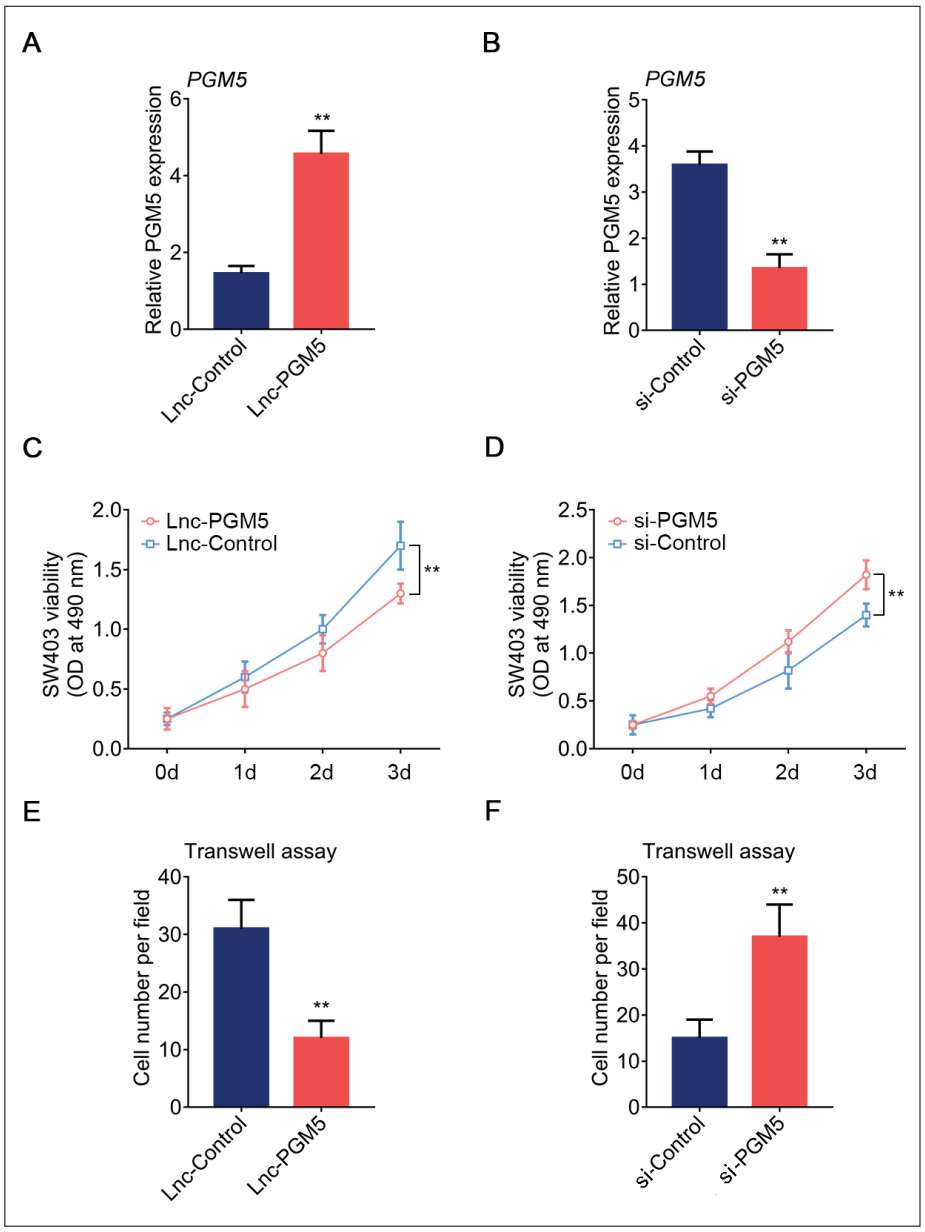

Figure 2. The migration and invasion of colorectal cancer cells were significantly inhibited after upregulated the PGM5-AS1 expression. **A**, Relative mRNA expression levels of PGM5-AS1 in SW403 cells transfected with PGM5-AS1 overexpressing lentiviral (Lnc-PGM5-AS1) and Lnc-Control. **B**, Relative mRNA expression levels of PGM5-AS1 in SW403 cells transfected with si-Control and si-PGM5-AS1. **C**, Absorption at 490 nm of SW403 cells treated with Lnc-PGM5-AS1 and Lnc-Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **D**, Absorption at 490 nm of SW403 cells treated with si-PGM5-AS1 and si-Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **E**, The number of migrated cells through transwell chambers was calculated after overexpression of PGM5-AS1. **F**, The number of migrated cells through transwell chambers was calculated after knockdown of PGM5-AS1. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as *(p<0.05) or ** (p<0.01).

colorectal cancer cells. To further determine that PGM5-AS1 influence the migration and invasion of colorectal cancer cells, we performed transwell assay to detect the migration ability of human colorectal cancer cells after the expression of PGM5-AS1 was regulated. The results revealed that after PGM5-AS1 upregulation, colorectal cancer cells migration through transwell chambers significantly decreased in response to fetal bovine serum compared with control group (Figure 2E). Then, when PGM5-AS1 was downregulated, the number of colorectal cancer cells migration through transwell chambers was significantly increased in response to fetal bovine serum compared with control group (Figure 2F). These data suggested that PGM5-AS1 alteration regulated the migration and invasion of human colorectal cancer cells, and upregulated PGM5-AS1 can effectively inhibit the migration and invasion of colorectal cancer cells.

MiR-100-5p Expression Was Upregulated in Colorectal Cancer Cells and Negatively Correlated With PGM5-AS1

In order to investigate whether PGM5-AS1 was correlated to miRNA, we used StarBase 2.0 to predict the target miRNA of PGM5-AS1 and found that miR-100-5p is one of the target miR-

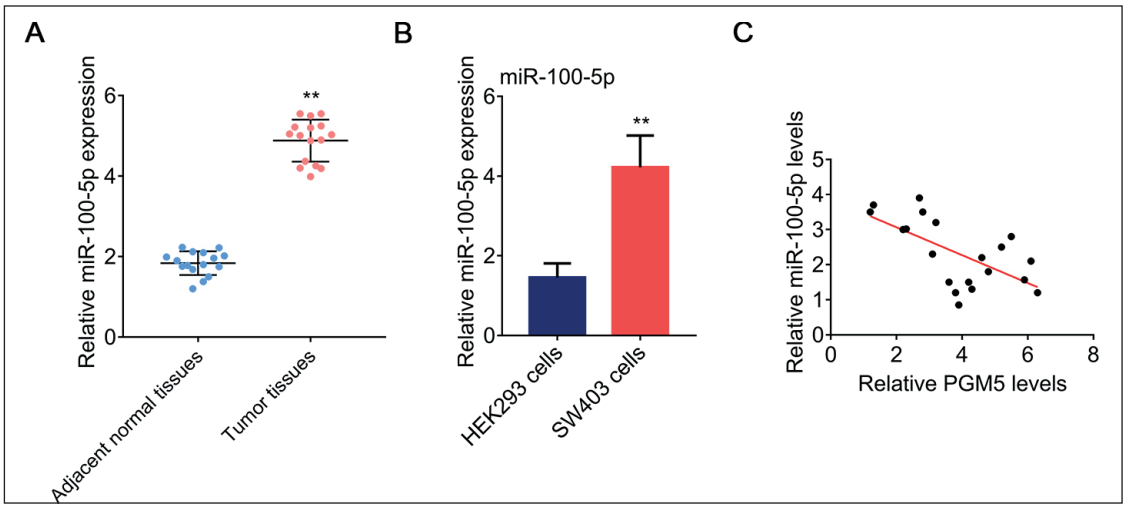

Figure 3. MiR-100-5p expression was upregulated in colorectal cancer cells and negatively correlated with PGM5-AS1. **A**, Relative miR-100-5p expression in colorectal cancer tissues and adjacent normal tissues detected by qRT-PCR. **B**, Relative miR-100-5p expression in SW403 cells and HEK293 cells detected by qRT-PCR. **C**, Correlation analysis was performed to evaluate the relationship between miR-100-5p and PGM5-AS1. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * (p<0.05) or ** (p<0.01).

NAs of PGM5-AS1. Therefore, we used qRT-PCR analysis to detect the miR-100-5p expressions of human colorectal cancer tissues and SW403 cells. Results showed that miR-100-5p was high-expressed in colorectal cancer tissues compared with that of adjacent normal tissues and was upregulated in SW403 cells compared with HEK293 cells (Figure 3A and 3B). We, then, used correlation analysis to further explore the relationship between PGM5-AS1 and miR-100-5p; the results showed that miR-100-5p was significantly negatively correlated with PGM5-AS1, indicating that miR-100-5p might be modulated by PGM5-AS1 (Figure 3C). These results suggested that miR-100-5p was high expressed in colorectal cancer tissues and SW403 cell line, which was negatively correlated with PGM5-AS1.

PGM5-AS1 Can Sponge With MiR-100-5p and Inhibit Its Expression in Colorectal Cancer Cells

It has been previously suggested that LncRNAs can act as a competing sponge in regulating the biological functions of miRNAs. As talked above, miR-100-5p was negatively correlated with PGM5-AS1, so we hypothesized that PGM5-AS1 can regulate the migration and invasion of colorectal cancer through interaction with miR-100-5p. To further confirm this, PGM5-AS1-wt Luciferase reporter vector and PGM5-AS1-mut 3'UTR Luciferase reporter vector were synthesized, and Luciferase reporter assay was performed (Figure 4A). Compared with the control, the Luciferase activity of SW403 cells that co-transfected with wild type PGM5-AS1 (PGM5-AS1-wt) and miR-100-5p mimic was significantly decreased (p<0.01), and it was reversely increased after mutation at the binding site of PGM5-AS1 (PGM5-AS1-mut) compared with PGM5-AS1-wt (p<0.01) (Figure 4B). These results demonstrated that PGM5-AS1 could directly bind to miR-100-5p. Besides, PGM5-AS1 overexpression suppressed miR-100-5p expression and PGM5-AS1 inhibition reversely facilitated miR-100-5p expression in SW403 cells (Figure 4C, 4D). Additionally, we also transfected miR-100-5p mimic and miR-100-5p inhibitor into SW403 cells; the results revealed that miR-100-5p mimic inhibited PGM5-AS1 expression and miR-100-5p inhibitor increased PGM5-AS1 expression (Figure 4E, 4F). All above, these results suggested that miR-100-5p directly bound to PGM5-AS1 at the recognition sites.

PGM5-AS1 Served as a CeRNA for MiR-100-5p to Further Modulate the Expression of SMAD4

SMAD4 is a tumor suppressor gene whose mutation or deletion is closely related to the occurrence of various cancers. To explore whether miR-100-5p interact with SMAD4, we performed qRT-PCR analysis for SMAD4 in the presence of miR-100-5p mimics or inhibitor. We observed

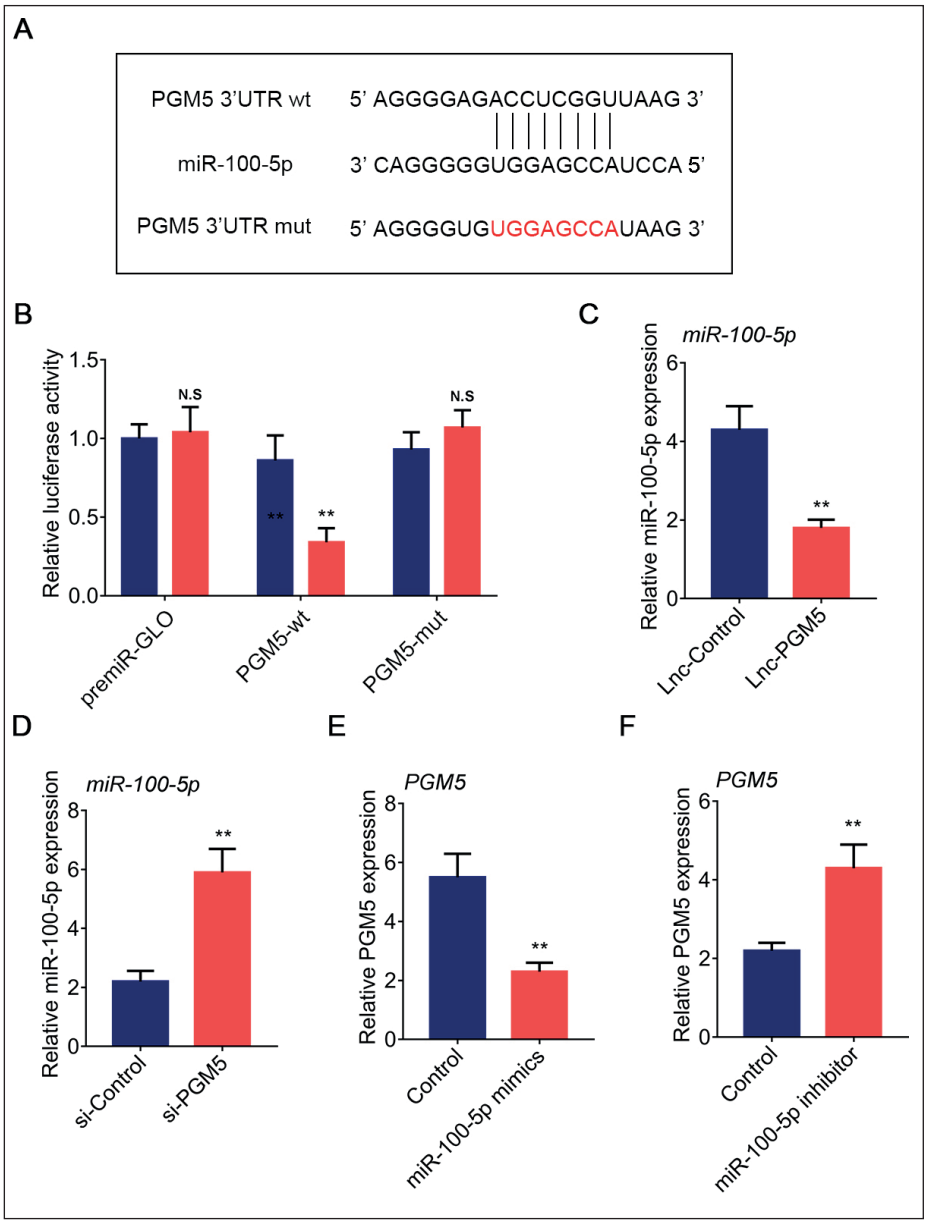

Figure 4. PGM5-AS1 can sponge with miR-100-5p and inhibit its expression in colorectal cancer cells. **A**, Sche¬matic illustration of the predicted miR-100-5p binding sites and mu¬tant sites in PGM5-AS1. **B**, Relative Luciferase activity of SW403 cells. **C-D**, qRT-PCR analysis of miR-100-5p expression level in SW403 cells transfected with lentiviral PGM5-AS1 and si-PGM5-AS1. **E-F**, Relative PGM5-AS1 expression was detected in SW403 cells after treated with miR-100-5p mimics and miR-100-5p inhibitor by RT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * (p<0.05) or ** (p<0.01).

decreased SMAD4 expression after SW620 cells were treated with the miR-100-5p mimics, which suggested that miR-100-5p could downregulate SMAD4 expression (Figure 5A). To validate this mechanism, we cloned the mice SMAD4 3'-UTR into the Luciferase reporter vector and constructed miR-100-5p binding mutants in which the putative miR-100-5p binding sites GGUCC in the NFTAc1 3'-UTR were mutated into CCAGG (Figure 5B). As expected, Dual-Luciferase report results showed that miR-100-5p mimics significantly downregulated the SMAD4 expression whereas point mutations in the SMAD4 3'-UTR abrogated the suppressed effect of miR-100-5p (Figure 5C). Then, we validated whether PGM5-AS1 can regulate SMAD4 expression *via* sponging with miR-100-5p. The results showed that PGM5-AS1 could significantly increase SMAD4 expression; however, mutation of the binding site with PGM5-AS1 of miR-100-5p eliminated the function effectively (Figure 5D). Conversely, inhibition of miR-100-5p overcame the suppression of SMAD4 by PGM5-AS1 knockdown (Figure 5E). Taken together, these data demonstrated that PGM5-AS1 could serve as a ceRNA for the miR-100-5p to further modulate SMAD4.

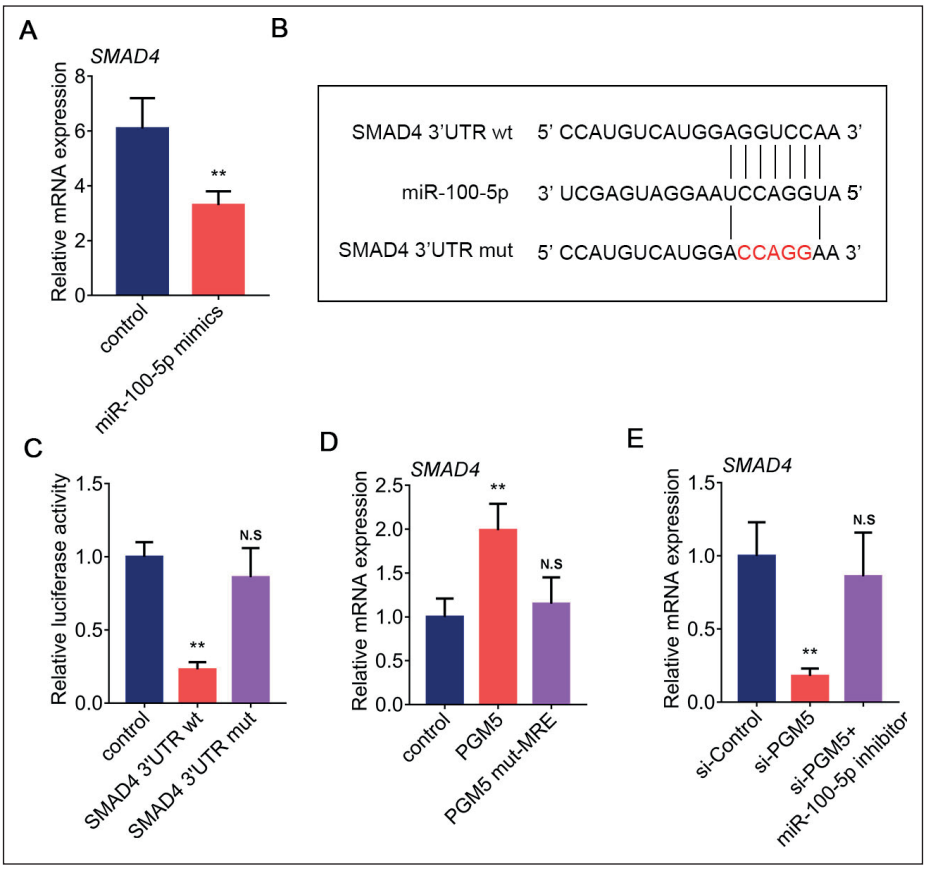

Figure 5. PGM5-AS1 served as a molecular sponge for miR-100-5p to further modulate the expression of SMAD4. **A**, qRT-PCR analysis of SMAD4 mRNA expression level in SW403 cells treated with the miR-100-5p mimics. **B**, Sche¬matic illustration of the predicted SMAD4 binding sites and mu¬tant sites in miR-100-5p. **C**, Relative Luciferase activity of SW403 cells. **D**, Relative mRNA expression levels of SMAD4 in SW403 cells transfected with PGM5-AS1 and PGM5-AS1 mut-MRE. **E**, Relative mRNA expression levels of SMAD4 in SW403 cells transfected with si-PGM5-AS1, si-PGM5-AS1 and miR-100-5p inhibitor by qRT-PCR analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * (p<0.05) or ** (p<0.01).

Discussion

Colon cancer is the most frequently diagnosed cancer in both Eastern and Western countries, affecting more than one million individuals each year[1]. Although advances in cancer diagnosis and therapy have improved the clinical outcome in recent years, the prognosis of patients with colon cancer remains frustrating.

As a "bridge" between DNA and protein, the complicated regulatory role of RNA has long been neglected. In eukaryotic cells, protein-coding RNA (mRNA) only accounts for about 2% of the genome, the rest large number of transcripts were categorized to non-protein coding RNAs (ncRNAs)[17]. Unlike ribosomal RNA (rRNA) and transfer RNA (tRNA), which have been well-acknowledged, other non-coding RNAs (ncRNAs) were thought to be transcriptional "noises" once upon a time. However, ncRNAs play a pivotal role in cellular process[18-20] and lots of noncoding RNAs (ncRNAs) are closely associated with colon cancer tumorigenesis[21,22]. As an important member of ncRNA, lncRNAs have been reported to play a pivotal role in colon cancer development and progression. However, the role and mechanism of PGM5-AS1 in colon cancer remained unknown.

PGM5-AS1 has been reported to play a crucial role in inhibiting the progression of esophageal squamous cell carcinoma[11]. Besides, downregulation of lncRNA PGM5-AS1 was closely correlated with poor prognosis of renal carcinoma[23]. However, the regulatory role of PGM5-AS1 in colorectal cancer development has not been reported. Our study revealed the regulatory role of PGM5-AS1 in colorectal cancer, which served as a ceRNA of miR-100-5p to upregulate suppressor gene SMAD4 expression and inhibit metastasis and invasion of colorectal tumor.

An increasing number of lncRNAs have been reported to serve as important mediators in human cancers[24]. Specific lncRNAs are significantly deregulated in colorectal cancer and have been demonstrated to promote tumor growth and metastasis. For instance, the growth arrest-specific 5 lncRNA is a prognostic marker in colorectal cancer that has been demonstrated to inhibit tumor cell proliferation, induce G0/G1 arrest and apoptosis[25]. In addition, the small nucleolar RNA host gene 12 lncRNA promotes the growth and inhibits apoptosis of col-

orectal cancer cells[26]. The present study observed that the expression levels of PGM5-AS1 were significantly decreased in colorectal cancer tissues and SW403 cell line when compared with adjacent normal tissues and non-tumor HEK293 cells, respectively. Besides, we first validate the important role of PGM5-AS1 in proliferation of human colorectal cancer cells SW403. Simultaneously, we found the expression of PGM5-AS1 was associated with the migration and invasion ability of SW403. Through bioinformatics prediction, we found miR-100-5p as a target miRNA of PGM5-AS1 and validate the combination relationship of PGM5-AS1 and miR-100-5p using Luciferase reporter assay. Furthermore, we found that miR-100-5p can interact with PGM5-AS1 co-expression gene SMAD4 and downregulate the expression of SMAD4.

Conclusions

All together these results revealed that overexpression of PGM5-AS1 can significantly promote SMAD4 gene expression. However, mutagenesis of the miR-100-5p recognition element in PGM5-AS1 alleviates the function effectively. Together, we suggested that PGM5-AS1 served as a ceRNA of miR-100-5p to upregulate SMAD4 expression.

Acknowledgments

Supported by the National Natural Science Foundation of China (No.81771943) and Shanghai Municipal Health and Family Planning Commission (No.201640191). Hospital-level Specialty Subject Construction (No.2019008).

Conflict of Interests

The authors declare no conflict of interest

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