Downregulation of lncRNA PMS2L2 in patients with gastric adenocarcinoma predicts poor prognosis

JUNPING BIAN, GUANGCHUN LI, ZHEN ZHANG and BIN LIU

Department of Gastroenterology, The Second Hospital of Shandong University, Jinan, Shandong 250033, P.R. China

Abstract. Long non-coding RNA PMS1 homolog 2 mismatch repair system component pseudogene 2 (PMS2L2) is a key player in lipopolysaccharide-induced inflammatory responses. Preliminary deep sequencing data revealed that PMS2L2 was downregulated in gastric adenocarcinoma (GA) tissues compared with healthy adjacent tissues and the aim of the present study was to investigate the role of PMS2L2 in GA. In the present study, reverse transcription-quantitative PCR assays were performed to analyze gene expression. Cell transfections were performed to analyze gene interactions and Transwell assays were performed to analyze cell invasion and migration. The results revealed that PMS2L2 expression was downregulated in cancer tissues obtained from patients with GA compared with healthy adjacent tissues and was not significantly affected by clinical stage. Furthermore, low levels of PMS2L2 in cancer tissues were closely associated with a low overall 5-year survival rate in patients. MicroRNA (miR)-25 was upregulated in GA tissues compared with healthy adjacent tissues and inversely associated with PMS2L2 levels. In GA cells *in vitro*, over-expression of PMS2L2 downregulated the expression of miR-25, while miR-25 overexpression did not significantly affect PMS2L2 expression. Furthermore, PMS2L2 overex-pression inhibited the migration and invasion of GA cells. miR-25 overexpression partially rescued the decreased migration and invasion of GA cells caused by PMS2L2 over-expression. Therefore, PMS2L2 may downregulate miR-25 expression to inhibit GA.

Introduction

Gastric cancer is a common malignancy worldwide that affects >1% of the worldwide population (1). A large number of patients with gastric cancer are diagnosed with inoperable tumors or experience high recurrence rates following curative resection (2). An unhealthy diet, cigarette smoking and alcohol consumption have been identified as risk factors for gastric cancer and reduced exposure to risk factors significantly reduces the incidence of the disease (3-5). It has been revealed genetic alterations as risk factors for gastric cancer (3-6); however, the limited number of oncogenes and tumor suppres-sors currently identified do not sufficiently explain the complex pathogenesis of gastric cancer (6).

Previous studies have shown that long non-coding RNAs (lncRNAs), transcripts >200 nucleotides in length, are not transcriptional 'noise', but serve important roles in growth and developmental processes (7,8). lncRNAs regulate gene expression at multiple levels, and altered expression of lncRNAs may result in dysregulated gene expression, thereby contributing to the occurrence of diseases, such as cancer (9,10). Therefore, characterization of the functions of lncRNAs may provide insights into disease prevention and treatment. However, the function of the majority of predicted or isolated lncRNAs remains unclear. PMS1 homolog 2 mismatch repair system component pseudogene 2 (PMS2L2) is a recently identified lncRNA that exhibits protective effects in chondrocytes during lipopolysaccharide-induced inflammation (11). Preliminary deep sequencing data revealed that PMS2L2 expression was downregulated in gastric adenocarcinoma (GA) tissues in comparison with adjacent non-tumor tissues and was inversely associated with microRNA (miR/miRNA)-25 expression levels (data not shown). A previous study revealed that miR-25 expres-sion was upregulated in GA tissues compared with adjacent non-tumor tissues and promoted the *in vitro* proliferation, invasion and migration of GA cells (12). Furthermore, high levels of miR-25 predicted poor prognosis of patients with GA. Therefore, the present study investigated the role of PMS2L2 in GA and its potential interactions with miR-25.

Materials and methods

Specimens and patients. Tissue specimens, including GA and healthy adjacent non-cancerous tissues (within 2 cm of the tumor margin), were obtained from 72 patients with GA at The Second Hospital of Shandong University between May 2010 and May 2015. Histopathological examination revealed that the cancer cell content in all adjacent non-cancerous tissues was

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Correspondence to: Dr Bin Liu, Department of Gastroenterology, The Second Hospital of Shandong University, 247 Bei Yuan Street, Jinan, Shandong 250033, P.R. China
E-mail: vx07412@163.com

<1%. The patients included 38 males and 34 females (age range, 33-68 years; mean age, 52.2±8.3 years). The inclusion criteria were as follows: i) New GA cases diagnosed by histopathological examination; ii) no previous history of malignancies; and iii) no familial history of malignancies. The exclusion criteria were as follows: i) Patients transferred from other hospitals; ii) patients who received treatment within 3 months prior to admission; and iii) patients with co-morbidities. Based on the criteria established by the American Joint Committee on Cancer (13), there were 14, 16, 28 and 14 patients with stage I-IV cancer, respectively. The present study was approved by the Ethics Committee of The Second Hospital of Shandong University and all patients provided written informed consent.

Follow-up. A 5-year follow-up study was performed to monitor the survival rate of the patients. Patients visited the outpatients department or were monitored remotely over the telephone every month. Patients who succumbed to other clinical disorders, including heart disease, or accidents, including traffic accidents, were excluded from the study. The aforementioned 72 patients completed the follow-up study.

Cell lines and cell culture. The human GA cell line AGS (Sigma-Adrich; Merck KGaA) was used to perform all *in vitro* experiments. The normal gastric cell line Hs 738.St/Int (cat. no. CRL-7869™; American Type Culture Collection) served as healthy control cells. Cells were cultured in F-12K medium (Sigma-Adrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Sigma-Adrich; Merck KGaA) and maintained at 37°C and 5% CO_2.

Transient transfections. The PMS2L2 expression vector was constructed by inserting full length PMS2L2 cDNA into a pcDNA3.1 vector (Sangon Biotech Co., Ltd.). Negative control (NC) miRNA (5'-CUAGUCGUGUAU ACAGUGUGA-3') and the miR-25 mimic (5'-CAUUGC ACUUGUCUCGGUCUGA-3') were purchased from Sigma-Aldrich, Merck KGaA. All cell transfections were performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) and 10 nM vector and 35 nM miRNA with all procedures performed following the manufacturer's instructions. Subsequent experiments were performed 24 h post-transfection. Negative controls included cells transfected with an empty vector or NC miRNA and controls included untransfected cells.

Reverse-transcription quantitiative polymerse chain reaction (RT-qPCR). Total RNA was extracted from GA and healthy adjacent non-cancerous tissues as well as the AGS and Hs 738.St/Int cell lines using RNAzol reagent (Sigma-Aldrich; Merck KGaA). Total RNA was reverse transcribed into cDNA using AMV Reverse Transcriptase XL (Clontech Laboratories, Inc.). The expression of PMS2L2 was analyzed by qPCR using the DyNAmo Flash SYBR Green qPCR kit (Thermo Fisher Scientific, Inc.) and 18S rRNA as an endogenous control.

The miRNA isolation kit (cat. no. RMI050; Geneaid Biotech Ltd.) was used to extract miRNA from AGS and Hs 738.St/Int cell lines. The TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to perform reverse transcription. The expression of miR-25 was
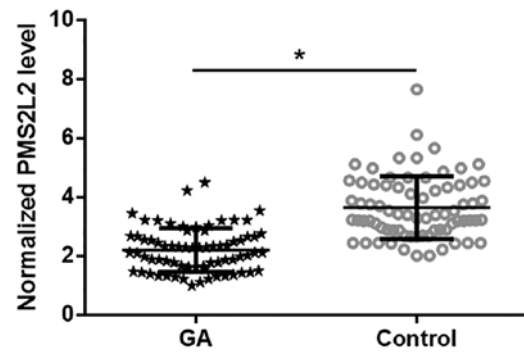

Figure 1. PMS2L2 was downregulated in GA. PMS2L2 expression data were analyzed by the paired t-test. PMS2L2 was significantly downregulated in GA tissues compared with healthy adjacent tissues. *P<0.05, as indicated. PMS2L2, PMS1 homolog 2 mismatch repair system component pseudogene 2; GA, gastric adenocarcinoma.

analyzed by qPCR using the TaqMan Real-Time PCR Master mix (Thermo Fisher Scientific, Inc.) and U6 as an endogenous control.

Primer sequences were: PMS2L2 forward, 5'-AGTCTA AGCACTGCGGTGAA-3' and reverse, 5'-GGTAGAAAT GGTGACATCAT-3'; 18S rRNA forward, 5'-CTACCACAT CCAAGGAAGCA-3' and reverse, 5'-TTTTTCGTCACT ACCTCCCCG-3'. The forward primer sequence of miR-25 was: 5'-CATTGCACTTGTCTCGGTC-3'. Universal reverse primers and U6 forward primer were from the kit. The thermocycling conditions were: 95°C for 1 min, then 40 cycles of 95°C for 10 sec and 60°C for 50 sec. All qPCR reactions were performed in triplicate and the $2^{-\Delta\Delta Cq}$ method (14) was used to quanitfy expression levels.

Cell migration and invasion. AGS and Hs 738.St/Int cells were harvested 24 h post-transfection. A single cell suspension was prepared using serum-free F-12K medium and the final cell density was adjusted to 3×10^4 cells/ml. A total of 0.1 ml cell suspension was plated in the upper chamber of Corning Transwell Cell Culture Plate Insert (8.0 μm pore; Corning, Inc.). The lower chamber was filled with F-12K medium (supplemented with 20% FBS). For invasion assays, the Transwell membranes were coated with Matrigel (EMD Millipore) for 6 h at 37°C. Following incubation at 37°C for 2.5 h, cells were fixed in ice-cold 70% ethanol for 20 min at 4°C. The migratory cells were then stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 16 min at room temperature. Stained cells were observed under an optical microscope and cells of five randomly selected visual fields were counted (magnification, x40). The control groups (untransfected cells) were set to 100% and all other groups were normalized to the control groups.

Statistical analysis. Data are presented as the mean ± standard deviation values calculated from experiments performed in triplicate. GraphPad prism 6 (GraphPad Software, Inc.) was used to perform all statistical analysis. Differences between GA tissues and healthy adjacent tissues were analyzed by the paired t-test. Differences among cell transfection groups or clinical stages were analyzed by the one-way ANOVA followed by Tukey's post hoc test. Associations between PMS2L2 and
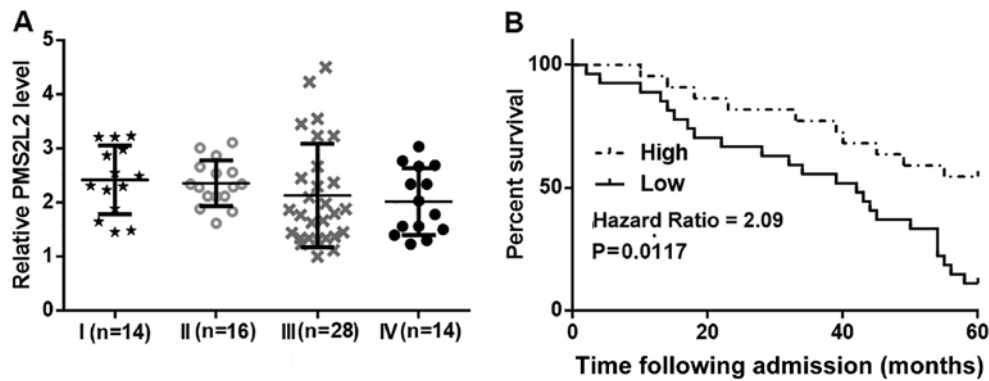
Figure 2. PMS2L2 was not affected by clinical stage but predicted the survival rate. (A) Analysis of PMS2L2 expression data by the one-way ANOVA followed by Tukey's post hoc test revealed no significant differences in PMS2L2 levels in patients with different clinical stages. (B) Survival curve analysis showed that low levels of PMS2L2 in gastric adenocarcinoma tissues were closely associated with a low overall 5-year survival rate. PMS2L2, PMS1 homolog 2 mismatch repair system component pseudogene 2.
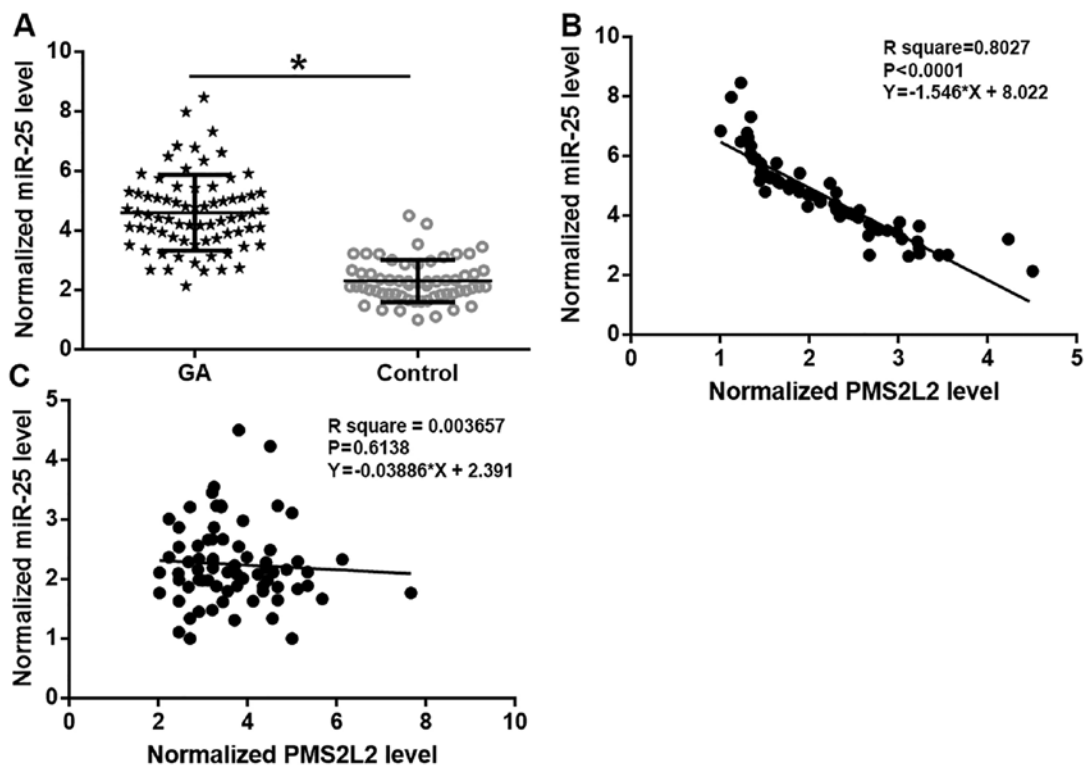

Figure 3. miR-25 was upregulated in GA and inversely associated with PMS2L2. miR-25 expression data were analyzed by the paired t-test. (A) miR-25 was significantly upregulated in GA tissues compared with healthy adjacent tissues. Associations between PMS2L2 and miR-25 were analyzed by linear regression and the results showed that miR-25 was inversely associated with PMS2L2 in (B) GA tissues but not in (C) healthy adjacent tissues. *P<0.05, as indicated. miR, microRNA; GA, gastric adenocarcinoma; PMS2L2, PMS1 homolog 2 mismatch repair system component pseudogene 2.

miR-25 were analyzed by linear regression. Patients were grouped into high (n=33) and low (n=39) PMS2L2 level groups based on Youden's index (cut-off value=2.14). Survival curves were plotted using the Kaplan-Meier method and compared by the log-rank test. $P<0.05$ was used to indicate a statistically significant difference.

Results

PMS2L2 is downregulated in GA. PMS2L2 expression in tissues was detected by RT-qPCR. The GA tissue with the lowest expression level was set to '1', and all other samples

were normalized to this sample. Expression data were analyzed by the paired t-test. PMS2L2 was significantly downregulated in GA tissues compared with healthy adjacent tissues (Fig. 1; $P<0.05$).

PMS2L2 is not affected by clinical stage but is associated with survival rate. PMS2L2 expression in GA tissues obtained from patients with different clinical stages was compared by the one-way ANOVA and Tukey's post hoc test. There were no significant differences in PMS2L2 levels among these groups of patients (Fig. 2A). Subsequently, patients were grouped into high (n=33) and low (n=39) PMS2L2 expression level groups
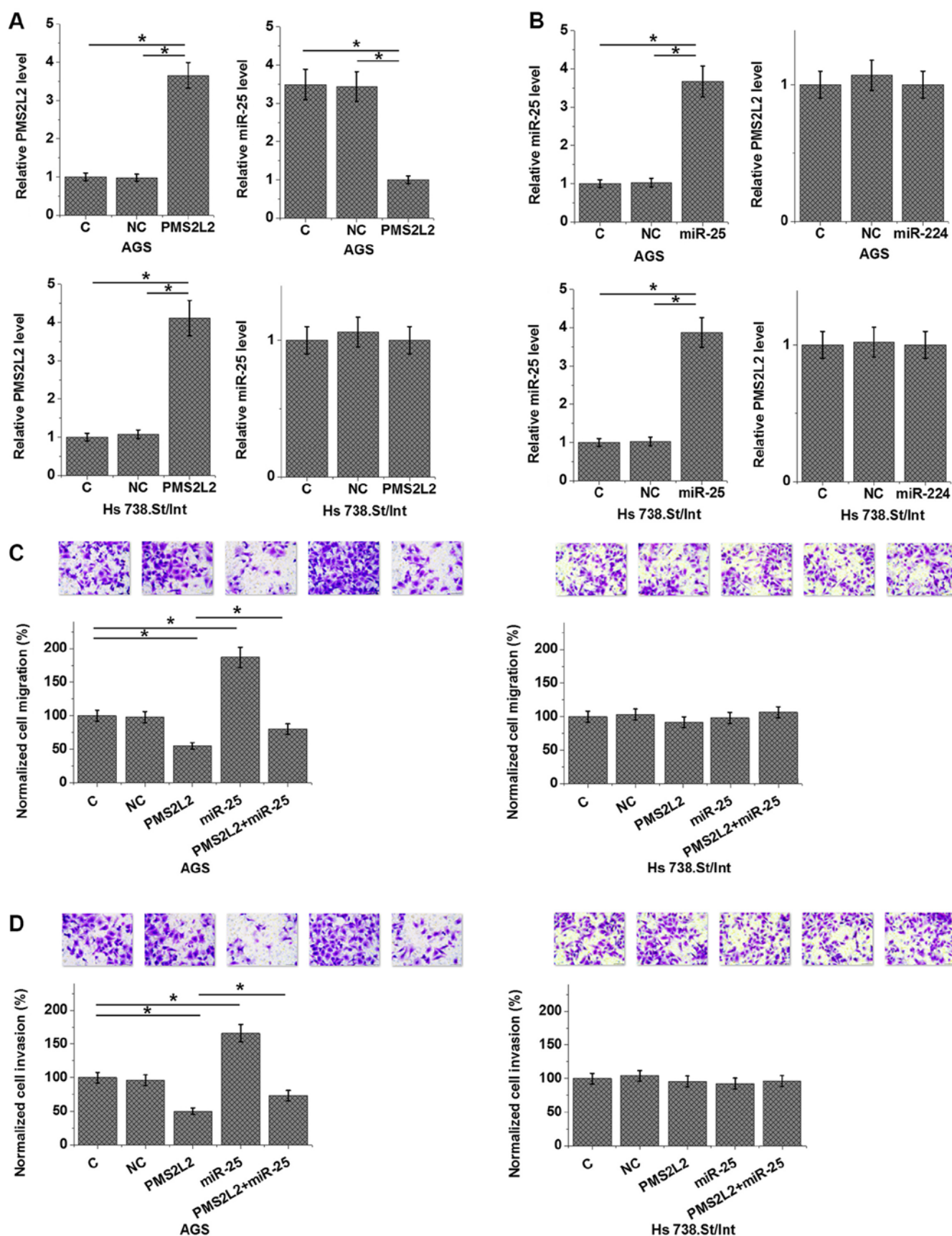
Figure 4. PMS2L2 downregulated miR-25 to regulate the migration and invasion of gastric adenocarcinoma cells, but not normal gastric tissue cells. Compared with the C and NC cells, cells transfected with a PMS2L2 overexpression vector and an miR-25 mimic exhibited significantly upregulated (A) PMS2L2 and (B) miR-25 levels 24 h post-transfection. Furthermore, overexpression of PMS2L2 downregulated the expression of miR-25 in AGS cells but not in Hs 738. St/Int, while miR-25 overexpression did not significantly affect PMS2L2 expression in both cell lines. Transwell assays revealed that PMS2L2 overexpression inhibited the (C) migration and (D) invasion of AGS cells but not Hs 738.St/Int cells. Moreover, miR-25 overexpression partially rescued the decreased migration and invasion of AGS cells caused by PMS2L2 overexpression. $^*P<0.05$, as indicated. PMS2L2, PMS1 homolog 2 mismatch repair system component pseudogene 2; miR, microRNA; C, control; NC, negative control.

based on Youden's index. As shown in Fig. 2B, patients with low levels of PMS2L2 exhibited a significantly shorter survival rate compared with patients with high expression (P=0.0117).

miR-25 is upregulated in GA and inversely associated with PMS2L2. miR-25 expression in tissues was detected by RT-qPCR. The GA tissue with the lowest expression level was set to '1', and all other samples were normalized to this sample. Expression data were analyzed by the paired t-test. miR-25 was significantly upregulated in GA tissues compared with healthy adjacent tissues (Fig. 3A; P<0.05). Associations between PMS2L2 and miR-25 were analyzed by linear regression. PMS2L2 and miR-25 expression levels were significantly inversely associated in GA tissues (Fig. 3B, R^2=0.8207, P<0.0001) but not in healthy adjacent tissues (Fig. 3C R^2=0.003657, P=0.6138).

PMS2L2 downregulates miR-25 to regulate the migration and invasion of GA cells but not normal gastric tissue cells. A PMS2L2 overexpression vector and miR-25 mimic were transfected into AGS and Hs 738.St/Int cells, and compared with the NC and control cells, PMS2L2 (Fig. 4A) and miR-25 (Fig. 4B) expression levels were significantly upregulated 24 h post-transfection (both P<0.05). In addition, overexpression of PMS2L2 downregulated the expression of miR-25 in AGS cells but not in Hs 738.St/Int (Fig. 4A; P<0.05), while miR-25 overexpression did not significantly affect PMS2L2 expression in both cell lines (Fig. 4B, both P>0.05). Transwell migration and invasion assays revealed that PMS2L2 overexpression inhibited migration (Fig. 4C) and invasion (Fig. 4D) of AGS cells (both P<0.05) but not Hs 738.St/Int cells. Moreover, miR-25 overexpression partially rescued the decreased migration and invasion of AGS cells caused by PMS2L2 overexpression.

Discussion

The present study investigated the role of PMS2L2 in GA. PMS2L2 was downregulated in GA tissues compared with healthy adjacent tissues and had prognostic value. Moreover, the results suggested that PMS2L2 may inhibit the migration and invasion of GA cells by downregulating miR-25, which is an oncogenic miRNA in this disease (12).

The prognosis and outlook of patients with GA is worse in China compared with developed countries (15). It has been revealed that lncRNAs serve important roles in GA (16). For example, lncRNA ncRuPAR inhibited protease-activated receptor-1 to suppress the development of GA (16). Therefore, the altered expression of lncRNAs may be associated with the survival time of patients with GA. The present study revealed that PMS2L2 expression was downregulated in GA tissues compared with healthy adjacent tissues. Interestingly, the level PMS2L2 expression in GA tissues was not significantly affected by the clinical stage of GA. Therefore, PMS2L2 is likely to be involved in the overall process of GA rather than specific stages. Furthermore, low expression of PMS2L2 in GA was closely associated with poor survival rate. Therefore, PMS2L2 expression may be used to predict the survival of patients with GA and aid the development of individualized treatment.

Preliminary deep sequencing data revealed that PMS2L2 and miR-25 were inversely associated in GA

tissues (data not shown). miR-25 is a well-characterized oncogenic microRNA and participates in several aspects of cancer development, including the regulation of cancer cell behavior and the responses of cancer cells to chemotherapy (17-19). The results of the present study suggested that PMS2L2 is likely to be an upstream inhibitor of miR-25 in GA cells, and the modulation of miR-25 by PMS2L2 is involved in the regulation of migration and invasion of GA cells but not normal gastric tissues cells. Therefore, PMS2L2 may serve as a potential therapeutic target for GA. It is known that lncRNAs can inhibit the function of miRNAs by serving as sponges (20,21). In effect, PMS2L2 sponges miR-203 and serves a protective role in chondrocytes protect against apoptosis (11). However, PMS2L2 does not sponge miR-25 as an appropriate binding site has not been identified (data not shown). In the present study, PMS2L2 and miR-25 were not significantly associated in healthy adjacent tissues. In addition, PMS2L2 overexpression did not affect miR-25 expression in normal gastric cancer cells. Therefore, the interaction between PMS2L2 and miR-25 is likely indirect or GA-specific. Future studies are required to identify the disease-related factors that mediate the interaction between PMS2L2 and miR-25.

In conclusion, the present study revealed that PMS2L2 was downregulated in GA and PMS2L2 may downregulate miR-25 to inhibit the migration and invasion of GA cells.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JB and BL designed the experiments. JB and GL performed experiments. ZZ collected and analyzed data. BL drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Second Hospital of Shandong University and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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