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Original Article

MicroRNA-212 functions as an epigenetic-silenced tumor suppressor involving in tumor metastasis and invasion of gastric cancer through down-regulating PXN expression

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Abstract: Altered expression of paxillin (PXN) is closely linked to the pathogenesis progression, metastasis and prognosis of different malignancies including gastric cancer (GC). Epigenetic silencing of tumor-suppressive microRNAs (miRNAs) is a crucial component of the mechanism underlying activation of oncogenes in tumor. To screen for epigenetically silenced miRNAs which target PXN in GC, we performed bioinformatics algorithms and real-time PCR analysis, and identified miR-212 as the optimum candidate gene. A luciferase reporter gene assay validated that miR-212 directly targets the 3'UTR region of PXN. Importantly, miR-212 levels were inversely correlated with PXN expression in GC cell lines and clinical tumor tissues. The use of miR-212 minics decrease PXN mRNA and protein level in GC cell lines. Moreover, low expression of miR-212 and its promoter hypermethylation were causally related and were associated with aggressive tumor phenotype and adverse prognosis in GC. Restoring mir-212 expression by exogenous mirprecursor molecules transfection or reexpression of endogenous miR-212 treated by 5-aza-2'-deoxycytidine (5-aza) can exert similar effect that reduce GC cells invasion and metastasis abilities in vitro by interacting PXN gene. In addition, 5-aza-induced PXN reduction could be partically blocked by miR-212 inhibitor, resulting in a reversal of weankening cell migration and invasion ability of 5-aza. A rescue experiment and a loss-of-function experiment in vitro and vivo showed that PXN restoration rescues migration and invasion phenotype in miR-212 overexpressed GC cell lines and PXN knockdown blocks GC cells migration and invasion in the presence miR-212 inhibitors. Taken together, our results clearly show that overexpression of PXN induced by methylation-suppressed miR-212 promotes tumor metastasis and invasion, and regulation of miR-212 expression may be a novel therapeutic strategy for gastric cancer.

Keywords: MiR-212, methylation, gastric cancer, cancer metastasis, cancer invasion, PXN, 5-aza-2'-deoxycytidine

Introduction

Gastric cancer is one of the most frequent causes of death from cancer in both sexes around word [1]. Metastasis is strongly implicated in GC aggressiveness and outcome. Statistics point out that it is responsible for more than 90% of mortality [2]. So a better understanding of the signaling networks regulating this phenomenon become more and more important. Recent evidence has indicated that the significant role of paxillin (PXN) involved in the progression and metastasis of different malignancies including gastric carcinoma [3-6]. The PXN gene encodes for a focal adhesion molecule of 68 kDa, which is a multidomain adaptor protein connecting extracellular matrices to the cytoskeleton [5, 7]. By protein-protein interactions and phosphorylation events, the PXN signaling hub controls the dynamics of focal adhesion assembly and disassembly, and in turn involved in variety of

physiological process, such as gene expression, matrix organization, tissue remolding, cell proliferation and survival, cell motility and metastasis [8-10]. Previous studies have shown that PXN was overexpressed and acted as a pro-oncogene in various malignancies, such as oral cavity squamous cell carcinoma [4], lung carcinoma [5], colorectal cancer [11], breast cancer and prostate cancer [3]. In GC, it has been found that PXN was up-regulated in GC tissues and cell lines as compared with adjacent normal tissues and normal gastric epithelial cell lines. PXN expression was negatively correlated with tumorsize, depth of invasion and lymph node metastasis; Multivariate analysis indicated that PXN expression was an independent prognostic factor [6, 12]. Moreover, ectopic expression of PXN stimulates cell proliferation and migration in AGS cells whereas knockout of PXN suppress these capacities [6]. Therefore, PXN may be employed as a promising therapeutic target and an indicator of pathobiological behaviors and prognosis in GC. Despite that the clinicopathological implication of PXN in GC has been identified, the underlying molecular mechanism, especially the upstream regulatory mechanism remains elusive.

MicroRNAs are endogenous small non-coding RNAs that bind to specific target mRNAs, leading to direct mRNA degredation or translational repression [13]. miRNAs regulated multiple target and pathways simultaneously and involved in various biological process including cell growth, differentiation, proliferation and apotosis [14]. Importantly, recent studies have shown that the miRNAs play a pivotal role in modulating the metastatic process in solid tumors [14-16]. Tumor-specific downregulation of subsets of miRNAs have generally been observed in various types of human cancer, suggesting that some of these miRNAs act as tumor suppressor gene in specific tumor [15, 16]. In addition, evidence has emerged that the silencing of several miRNAs is tightly linked to epigenetic mechanism, especially the promoter hypermethylation of these tumor suppressor miRNAs playing a critical component of the mechanism underlying tumorigenesis [17, 18]. To evaluate the possible role of this epigenetic regulation in metastatic progress, previous studies have assessed miRNA expression level in metastatic cell lines treated by DNA demethylating agent 5-aza-2'-dexoxycytidine, which lead to the discovery of several miRNAs with cancer-specific methylation, just as miR-137 [19], miR-34b/c [20], miR-148a [21] have identified in GC.

In consideration of the crucial prognostic effect of PXN and the emerging role of miRNAs in GC, we postulate that the PXN can be inhibited by certain tumor-suppressive miRNAs which silenced through aberrant DNA methylation in GC. To explore such miRNAs, we searched for miRNAs that potentially target mRNA of PXN and down-regulated in GC cell lines. Then screened human genome database for the existence of CpG island around these miRNAs and identified that miR-212 was the optimum candidate gene. A luciferase assay further confirmed this result. Downregulation miR-212 and its promoter hypermethylation is closely linked to the progress and prognosis of gastric carcinomas, importantly, abnormal express of paxillin, miR-212 and methylation levels of miR-212 are causally related in GC. Transfection of exogenous miR-212 mimics of reexpression of endogenous miR-212 treated by 5-aza can exert similar effects that inhibited the metastasis and invasion abilities of GC cells by targeting PXN.

Materials and methods

Clinical samples

Paired fresh gastric cancer tissues and adjacent non-tumorous tissues (71) were collected from GC patients who underwent surgery in the General surgery department of the first affiliated hospital of Nanchang University (Nanchang China). The median age of patients (31 women and 40 men) was 63 years (range 25-80). All specimens were immediately frozen in liquid nitrogen until further use. All samples were obtained with informed consent and the study was approved by the institutional review committee. The histological grade of cancers was assessed according to criteria set by the World Health Organization. The tumor-node-metastasis stage was performed using standard criteria of the seventh tumor-node-metastasis staging system. All the patients were followed-up regularly every three months after surgery, the median follow-up time was 43 months (range from 3-120 months). Relevant clinicopathological information including gender, age, tumor size, and the differentiation status, depth of invasion, lymph node invasion, UICC staging, luaren's grade, live metastasis, venous inva-

sion and peritoneal metastasis were obtained from patients' medical files.

Cell lines and cell culture

GC cell lines SGC-7901, MKN-45, AGS, MKN-28, BGC-823, MGC-803 and an immortalized normal gastric epithelial cell line GES-1 were obtained from Cell Bank of Type Culture Collection (Shanghai China). All cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS) (TransGen Biotech, Beijing, China), 100 U/ml penicillin, and 100 ug/ml streptomycin and maintained at 37 and, and 100 hinaatmosphere of 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells and frozen fresh tissues using TRIZOL (Invitrogen, Carlsbad, CA, USA) and cDNA was obtained using an Expand Reverse Transcriptase Kit (Takara, Danian, China) according to the manufacturer's instructions. Quantitative PCR was performed using the Applied Biosystems 7500 Fast real-time PCR System with the SYBR Green PCR Master Mix (Applied Biosystems, USA) to detect PXN mRNAs. β-actin was amplified in parallel as an internal control. For microRNAs, 100 ng of total RNA was reverse transcribed by their respective stem-loop RT primers, followed by RT-qPCR on the ABI 7900HT Fast real-time PCR system using the SYBR Green PCR Master Mix and U6 snRNA was served as an endogenous control, Expression of each gene was quantified by measuring cycle threshold (Ct) values and normalized by means of the 2^{-easu}. All the primers which designed by software Primer Premier 6.0 and Oligo7 were list in Supplementary Table 1.

DNA extraction, methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP)

Cell line and tissue sample genomic DNA was isolated using Easypure Genomic DNA kit (TransGen Biotech, Beijing, China). Aliquots of the genomic DNA were treated with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Then, Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) were performed and analyzed as previously described [22, 23]. All the primer involved in BSP and MSP in this study were list in Supplementary Table 1.

5-Aza-2'-deoxyazacytidine demethylation treatment

To block DNA methylation, SGC-7901 and MGC-803 cells were treated with 2.5 μ treat, 5 μ reated with μmol/l 5-Aza-2'-deoxyazacytidine (Sigma-Aldrich, St Louis, MO) for 72 h, respectively. Cells were harvested for RT-qPCR, bisulfite sequencing and West blot.

Synthetic miRNA transfection

GC cell lines SGC7901 and MGC-803 were plated for approximately 24 h before transfection, miR-212 mimics, inhibitor and their respective miR-negative controls (Genepharma, Shanghai, China) were transfected using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's procedures. The sequences of the primer of Hsa-miR-212 mimics, inhibitor and negative control (NC mimic) were used in this article list in Supplementary Table 1. For overexpression of endogenous PXN and knockdown endogenous PXN expression (si-PXN) in cells, the method was adopted in this article as described previously [6]. Total RNA and protein were extracted at 48 h post-transfection and subjected to RT-qPCR, western blotting. For cotreated with 5-aza and synthetic miRNAs study, GC cell lines were transfected with miR-212 mimic, miR-212 inhibitor, and their respective NCs for 48 h, 5-aza was added to the cell culture medium at 7 h after each transfection. Then cells were harvested for further experiments.

Dual luciferase reporter assay

The wild-type human PXN 3'UTR fragment containing predicted miR-212 target sites were amplified by by polymerase chain reaction (PCR). Mutant 3'UTR were generated by overlap-extension PCR method. MGC-803 and SGC-7901 cells were cultured in 24-well plates and performed co-transfections of PXN-3'UTR or mut-PXN-3'UTR plasmid with miR-212 mimics or negative control precursor by using Lipofectamine 2000 (Invitrogen). PXN-TK (Promega) was also transfected as a normalization control. Luciferase activities were measured with a dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions.

Experiments were performed in triplicate in three independent experiments.

Protein extraction and western blot analysis

Protein of GC cell lines was extracted using a Total Protein Extraction Kit (KeyGen, Nanjing, China), and separated by SDE-PAGE gels. Western blot analysis was carried out as descripted previously [23], the primary antibodies used was rabbit PXN antibody (1:1000, Cell Signaling Technology, USA), and an anti-β-actin (Santa Cruz Biotechnology) was used to normalized the amount of the analyzed samples.

Cell migration and invasion analysis

Cell migration and invasion of parental and treanted MGC-803, SGC-7901 cell lines were assayed using a Transwell chamber of diameter 6.5 mm with an 8um membrane (Corning, NY, USA), which coated with Matrigel (BD Bioscience) in invasion assays. 5×10^4 cells were harvested and placed in the upper chamber without serum. The lower chamber contained 10% fetal bovine serum for use as a chemoattractant. After several hours of incubation at 37°C with 5% CO_2, the cells that did not migrate or invade through the pores were carefully wiped out with cotton wool. Cell morphology was observed by staining with 20% methanol and 0.2% crystal violet, photo, and counted with an inverted microscope (Olympus, Japan).

Metastasis assays in vivo

Four week-old BALB/c nude mice were obtained from the Center of Experimental Animal Nanchang University (Nanchang, China). All of the in vivo experimental protocols were approved by the Animal Care Committee of Nanchang University. Peritoneal metastatic model by intraperitoneal inoculation in vivo was performed as described previously [24]. On day 28, all mice were sacrificed and the intestine and liver metastatic lesions were analyzed.

Bioinformatics analyses

The bioinformatics analyses about miRNA target gene prediction, the gene promoter and CpG islands searching in the human genome database, metlylation status analysis by QUMA, CpGviewer and Biq-analyzer have been descripted in our previous study [22, 23].

Statistical analysis

All data were analyzed using the SPSS 19.0 computer software (SPSS Inc., Chicago, IL, USA) and software program GraphPad Prism (Version 6.02 for Windows, Graphpad Software, La Jolla, CA, USA). Quantitative variables were analyzed using Student's test, Wilcoxon test (non-parametric paired analysis) and Mann-Whitney U test (non paired analysis). Qualitative variables were analyzed using either the Chi Square Test or the Fisher's test. Methylation status of each DNA clone sequence was analyzed comprehensively and comparatively by QUMA, CpGviewer and Biq-analyzer. Correlations between miR-212 expression and promoter CpG methylation were determined by calculating r^2 as a measure of the goodness of fit for the linear regression, Spearman's correlation coefficient (r) was used to determine the correlation between miR-212 and PXN mRNA levels. Survival analysis was performed using the Kaplan-Meier method. Unless otherwise noted, $P<0.05$ was accepted as significant.

Results

Screening for epigenetically silenced miRNAs which target PXN in GC cells

The present study, strategy and partial results of which are shown in **Figure 1A**, To screen for epigenetically silenced miRNAs, we initially performed computational searches of miRNA target databases (microRNA.org, Target Scan Human 6.2, Cometa, PicTar) and choosed conserved and overlapping miRNAs targeting PXN. As shown in **Figure 1B**, 5 miRNAs (miR-216b, miR-137, miR-132, miR-212, miR-145) were confirmed. Then, we performed Real-time quantitative RT-PCR analysis of 5 miRNAs expression in 3 GC cell lines (namely SGC-7901, AGS and BGC-823) relative to the levels in normal gastric epithelial cell line GES-1. Among 5 miRNAs, miR-132 was abundantly expressed in 3 GC cell and excluded from further analysis (**Figure 1C**). Interesting, although miR-132 and miR-212 belong to the human miR-212/132 cluster, they exhibited different expression in our study, and these results are same to the previous study [25, 26]. Subsequently, we searched the human genome database (UCSC Genome Bioinformatics, CpG Island Searcher) for the existence of CpG islands around these 4 miRNAS and confirmed that miR-137 and miR-

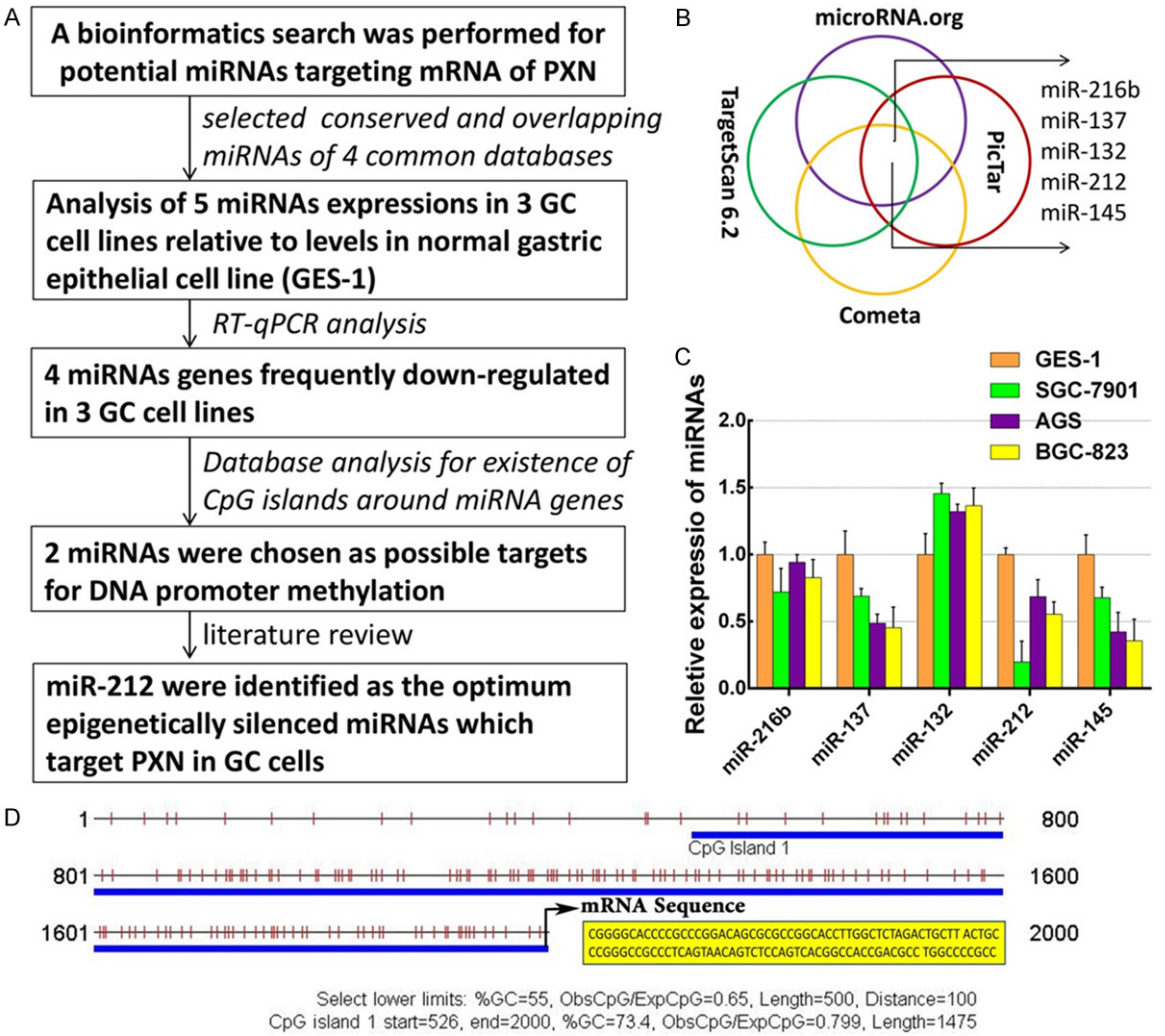

Figure 1. Strategy for the identification of epigenetically silenced miRNA genes targeting PXN in GC. A. Schematic strategy for the identification of epigenetically silenced tumor suppressor miRNAs which target PXN in GC. B. Bioinformatic prediction of potential miRNAs targeting PXN by 4 common databases. C. Relative expression of five miRNAs was detected by real-time PCR in 3 GC cell lines and a normall gastric epithelial cell lines GES-1. The expression levels of GC cell lines were independently calculated relative to those of GES-1, which are normalized to 1. D. Scanning for CpG islands with the submitted sequence of miR-212 in CpG Island Searcher, under this figure is the tlimited parameters for scanning and the detail information of CpG island. The CpG islands were shown in the Figure with blue line. The yellow rectangle reprent the mRNA sequence of miR-212.

212 were located on CpG islands (with 1000 bp) (**Figure 1D**). On consider of previous studies that miR-137 is not silenced its "promoter" methylation [27]. We, therefore, further analyzed miR-212.

miR-212 directly target and down regulated PXN expression in GC cell lines

By using bioinformatic algorithms we have identified that PXN is the potential target site of miR-212, the representative result of miRNA and TargetScan was shown in **Figure 2A**. To explore whether the expression of PXN is inversely correlated with miR-212, firstly, we detected their levels by RT-qPCR in six GC cell lines and a normal gastric epithelial cell lines. As shown in **Figure 2B**, the expression level of PXN mRNA in all the cell lines was negatively associated with miR-212 expression. To validate a direct binding and repression effect, we performed dual luciferase reporter assays, the wt-PXN 3'UTR and its mutant type mut-PXN 3'UTR were amplified and su-cloned downstream of the firefly luciferase reporter. Co-transfection of MGC-803 and SGC-7901 GC cell lines with miR-212 precursor and wild-type

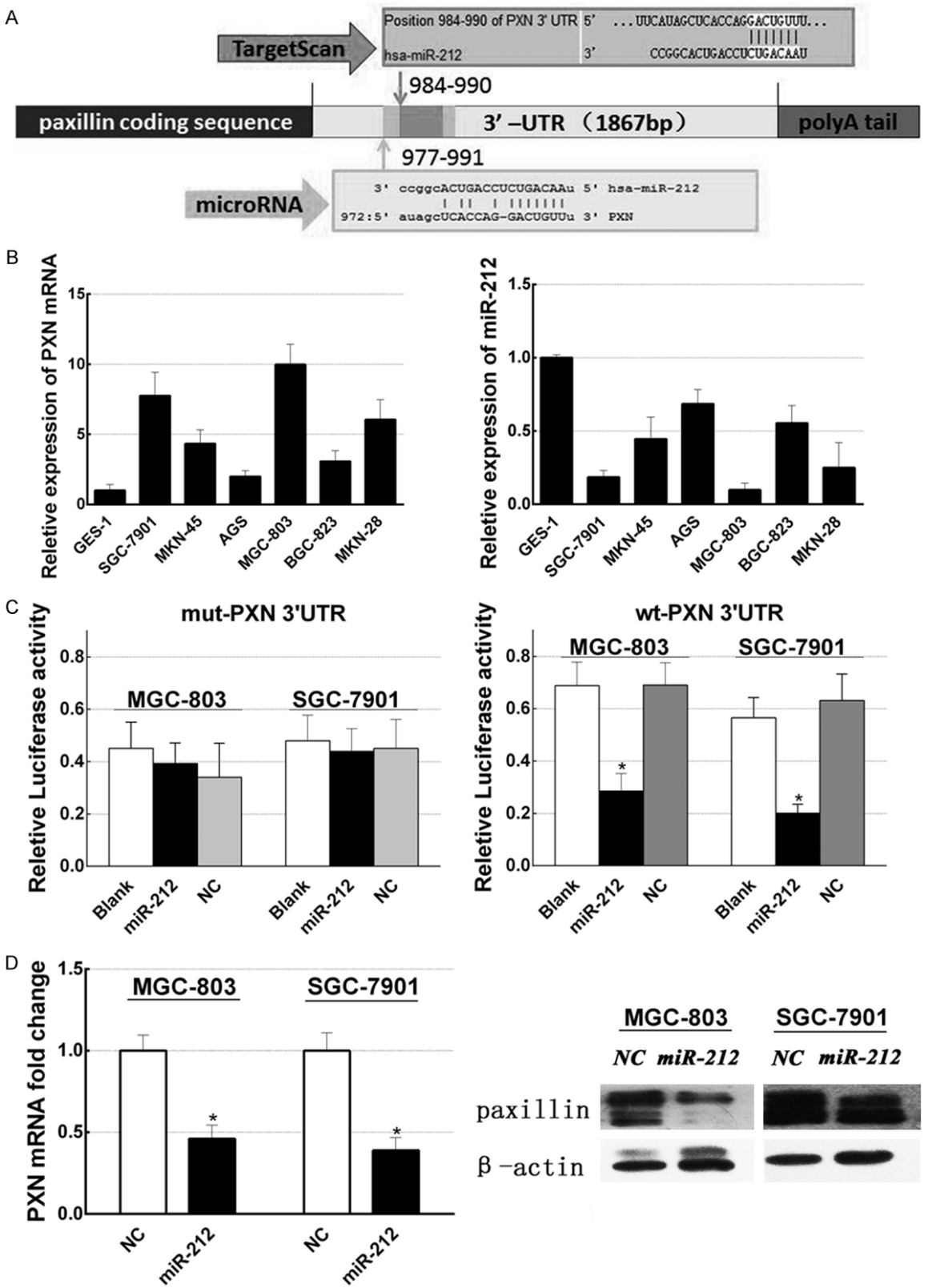

Figure 2. miR-212 directly target and down regulated PXN expression in GC cell lines. A. Schematic representations of predicted miR-212 binding sites in the PXN 3'-UTR by two representative predicted microRNA targets website micoRNA and TargetScan 6.2. The light-grey and dark-grey rectangle (977-991and 984-990) located in 3'-UTR shows the miR-212/PXN sequence alignment. B. Relative PXN mRNA (normalized to β-actin) and miR-212 (normalized to U6) expression levels were detected by real-time reverse transcription PCR in six GC cell lines and a normall gastric

epithelial cell lines GES-1. Data are presented as means ± SD. C. Luciferase activities of wild-type 3'UTR PXN and mutant 3'UTR PXN constructs in SGC-7901, MGC-803 cells after transfection of miR-212 precursor. Data are presented as means ± SD of three independent experiments (*P<0.05). D. The mRNA and protein levels of PXN in GC cell lines (SGC-7901 and MGC-803) treated with miR-212 mimics.

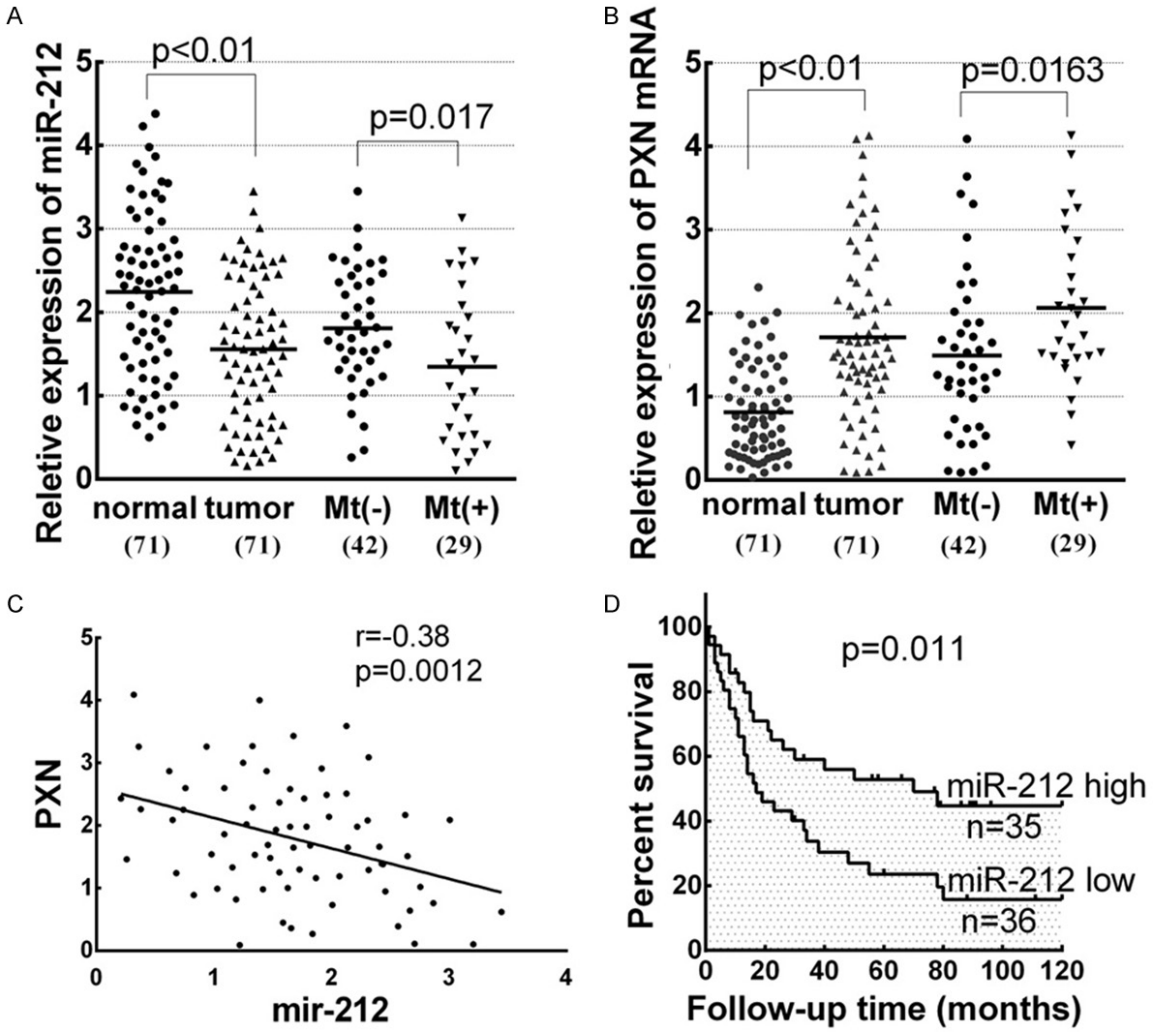

Figure 3. Correlations of miR-212 with PXN expression and clinical characteristics in GC tissues. A, B. Relative PXN mRNA and miR-212 expression levels in GC tissue samples. Relative PXN mRNA and miR-212 expression in normal (adjacent non-tumorous tissues), tumor (tumor tissues), Met (-) (non-metastatic tumor tissues) and Met (+) (metastatic tumor tissues) was determined by real-time PCR. The line in the middle indicates the mean value. C. PXN mRNA level was inversely correlated with miR-212 level in GC tissues. (Spearman's correlation analysis, r=-0.38, P=0.012). D. Kaplan-Meier analysis of overall survival based on miR-212 levels in GC patients.

report vector significantly reduced luciferase activity compare with the negative control or blank (P<0.5, **Figure 2C**), whereas such a suppressive effect of miR-212 on luciferase activity was not observed in both cells with mut-PXN 3' UTR (**Figure 2C**). The results make it evident that miR-212 effects PXN expression by directly binding to the 3'UTR region of PXN. To furether investigate whether miR-212 can downregulate endogenous PXN expression. We transfected synthenic miR-212 mimic or negative control (NC) into two human GC cell lines (MGC-803 and SGC-7901) and measured the levels of PXN mRNA and protein by RT-qPCR and western blot, respectively. As shown in **Figure 2D**, transfection of miR-212 results in a marked reduction of PXN mRNA levels in both cell lines compared with that of the NC cells (P<0.05). Consistent with this result, a significant downregulation of PXN protein levels was observed

Table 1. Comparision of expression levels and methylation of miR-212 with clinicopathological features in patients with gastric cancer

Characteristics	All cases (n=71)	miR-212 expression		*P* value§	miR-212 methylation		*P* value§
		Low no. case (%)	High no. case (%)		Methylation no. (%)	Unmethylation no. (%)	
Sex							
Male	40	18 (45.0)	22 (55.0)	0.275	30 (75.0)	10 (25.0)	0.938
Female	31	18 (58.1)	13 (41.9)		23 (74.2)	8 (25.8)	
Age (yr)							
≤55	32	15 (41.7)	17 (58.3)	0.559	22 (68.8)	10 (31.3)	0.301
>55	39	21 (48.6)	18 (51.4)		31 (79.5)	8 (20.5)	
Tumor size							
≤3.5	25	10 (40.0)	15 (60.0)	0.184	18 (72.0)	7 (28.0)	0.705
>3.5	46	26 (56.5)	20 (43.5)		35 (76.1)	11 (23.9)	
Differentiation status							
Well	16	6 (37.5)	10 (62.5)	0.456	9 (56.3)	7 (43.8)	0.056
Moderate	19	11 (57.9)	8 (42.1)		13 (68.4)	6 (31.6)	
Poor and other	36	19 (52.8)	17 (47.2)		31 (86.1)	5 (13.9)	
Luaren's grade							
Intestinal type	39	20 (51.3)	19 (48.7)	0.914	29 (74.4)	10 (25.6)	0.951
Diffuse type	32	16 (50.0)	16 (50.0)		24 (75.0)	8 (25.0)	
UICC staging							
0-I	18	5 (27.8)	13 (72.2)	0.024*	9 (50.0)	9 (50.0)	0.014*
II-IV	53	31 (58.5)	22 (41.5)		44 (83.0)	9 (17.0)	
Depth of invasion							
Tis-1	28	11 (39.3)	17 (60.7)	0.120	17 (60.7)	11 (39.3)	0.029*
T2-4	43	25 (58.1)	18 (41.9)		36 (83.7)	7 (16.3)	
0+N1	25	7 (28.0)	18 (72.0)	0.005*	14 (56.0)	11 (44.0)	0.008*
N2+N3	46	29 (63.0)	17 (37.0)		39 (84.8)	7 (15.2)	
Live metastasis							
Negative	53	21 (39.6)	32 (60.4)	0.001*	37 (69.8)	16 (30.2)	0.196
Positive	18	15 (83.3)	3 (16.7)		16 (88.9)	2 (11.1)	
Venous invasion							
Negative	48	22 (45.8)	26 (54.2)	0.236	34 (89.5)	14 (10.5)	0.218
Positive	23	14 (60.9)	9 (39.1)		19 (82.6)	14 (17.4)	
Peritoneal metastasis							
Negative	54	23 (42.6)	31 (57.4)	0.015*	36 (66.7)	18 (33.3)	0.015*
Positive	17	13 (76.5)	4 (23.5)		17 (100.0)	0 (0.0)	

§ χ^2 test, *Statistically significant.

in both cells at 48 h after miR-212 transfection.

Correlations of miR-212 with PXN expression and clinical characteristics in GC tissues

To further validate the correlation between miR-212 expression and endogenous PXN expression, we detected their levels by RT-qPCR in paired tumor tissues and adjacent non-cancerous tissues. PXN mRNA level was significantly higher in tumor tissues than in non-cancerous tissues. On the contrary, tumor tissues exhibit lower miR-212 expression than corresponding non-cancerous tissues. Furthermore, the PXN mRNA levels in patients with metastasis (n=29) were higher than those in patients without metastasis, whereas miR-212 level were lower in patients with metastasis than those without metastasis (**Figure 3A**, **3B**). Importantly, spearman's correlation coefficient-(r) revealed a significantly inverse correlation between miR-212 and PXN mRNA (r=-0.38,

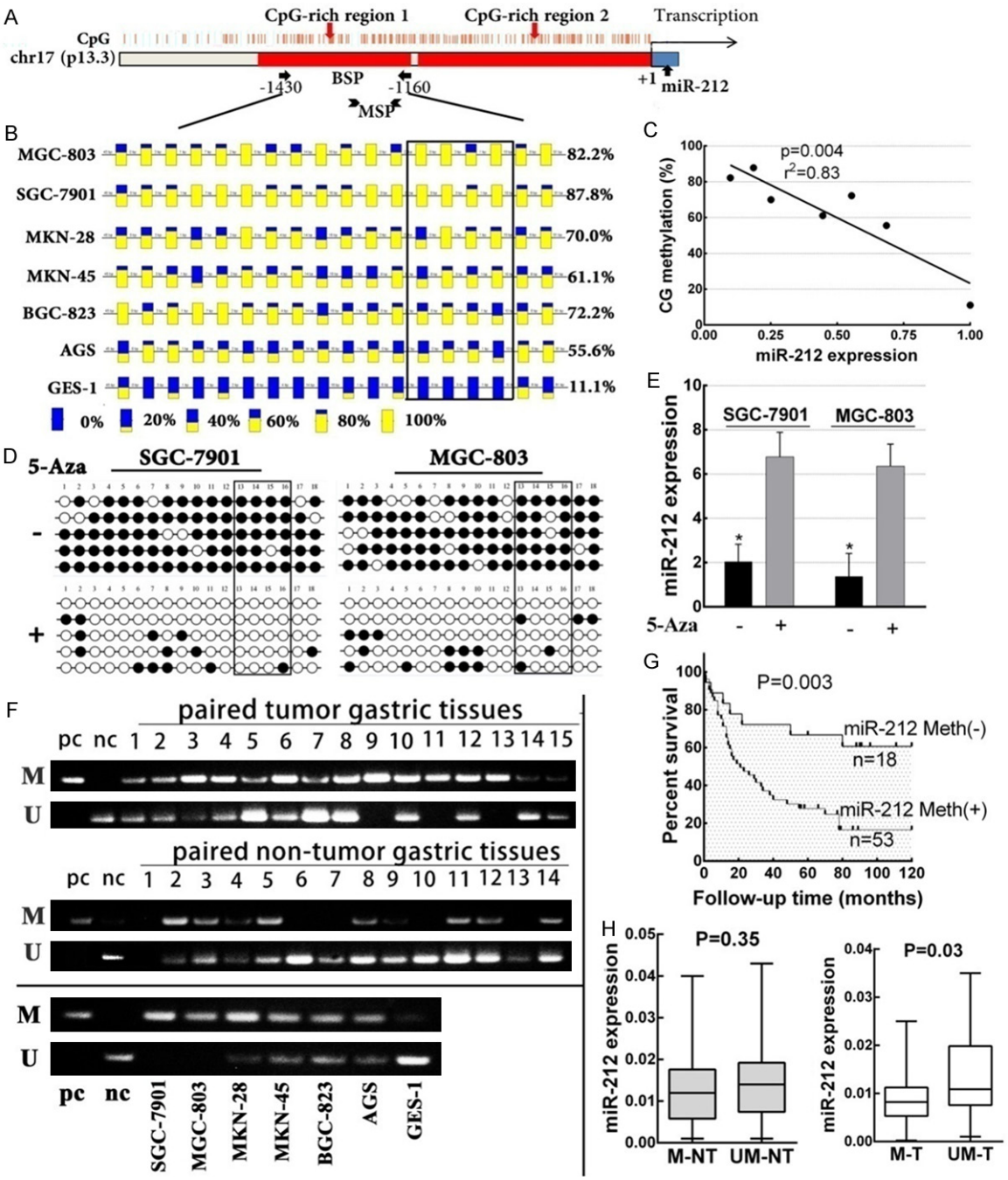

Figure 4. Analysis of miR-212 methylation and its correlation with clinicopathological characteristics of GC patients. A. Schematic illustration of the miR-212 CpG Island and the bisulfite sequencing and Methylation-specific PCR area. B. The bisulfite sequencing of the miR-212 upstream region in 7 cell lines analyzed by BIQ Analyzer. Each square represents a CpG site. Yellow squares represent methylated CpG dinucleotides whereas blue squares represent unmethylated CpG sites. If the methylation state of a CpG site could not be defined, it is represented as not present. The ratios of the filled area in the squares represent the methylation status in the CpG sites. The number of base pairs between each CpG dinucleotide is indicated at the top. Methylation-specific PCR area was demonstrated in the rectangle. C. CpG methylation status in cells as a function of miR-212 expression. D. The miR-212 methylation status in GC cell lines SGC-7901 and MGC-803 with and without 5-AZA treatment. Open and filled circles represent unmethylated and methylated CpG sites, respectively. Each horizontal row represents a single clone. E. RT-qPCR results for miR-212 in GC cell lines SGC-7901 and MGC-803 with and without 5-aza-CdR treatment. F. Representativ MSP analyses for miR-212 methylation in GC cell lines and GC tissues. U, unmethylated state; M, methylated state; PC: positive control (completely methylation) NC negative control (completely unmethylation); G. Comparison

of survival according to methylation of miR-212. There were significant differences between miR-212 with methylation (Meth+, n=18) and miR-212 without methylation (Meth-, n=53) (P=0.003). H. The expression levels of miR-212 were compared between methylated group and unmethylated group in tumor tissues (P=0.03) and non-tumor tissues (P=0.35).

P=0.0012) (**Figure 3C**). The relationship between the relative miR-212 expression and patient's clinical characteristics is shown in **Table 1**. The median value of miR-212 expression was selected as the cut-off point for separating tumors with low or high-level expression group, Chi-square test revealed that the lower expression of miR-212 was significantly associated with UICC staging, lymph node invasion, Live metastasis and peritoneal dissemination, Kaplan-Meier analysis of overall survival based on miR-212 expression in all 71 patients showed that patients with low miR-212 expression possessed with significantly poor overall survival compared with that of patients with high expression (P=0.011) (**Figure 3D**).

Analysis of miR-212 methylation and its correlation with clinicopathological characteristics of GC patients

Using the online tool MethPrimer with the default criteria, we found that miR-212 presented two clear CpG islands in its upstream chromosomal region (**Figure 4A**). To further confirm whether the CpG island hypermethylation of miR-212 promoter suppress its expression, we carried out methylation-specific (MSP) of CpG islands in a number of cell lines (**Figure 4F**). MSP analysis showed that the majority of GC cell lines displayed an increase in methylation relative to the normal gastric epithelial cell lines GES-1. In addition, detailed analysis via bisulfite sequencing consistent with MSP result (**Figure 4B**, **4D**), and revealed a strong inverse correlation between methylation of CpG island and miR-212 expression (correlation coefficient r^2=0.83, P=0.004) (**Figure 4C**). To test if promoter hypermethylation is sufficient to silence miR-212 expression, and also whether promoter hypermethylation and miR-212 are causally related, we treated MGC-803 and SGC-7901 cell with 5-aza-dexoxycytidine (5-aza). Inhibition of DNA methylation with 5-aza significantly reduce methylation levels and promoted endogenous miR-212 expression (**Figure 4D**, **4E**). To determine whether the methylation of miR-212 also occurs in primary GC tumor in a tumor-specific manner, the correlation between DNA methylation status and expression patterns of miR-212 in primary tissues and corresponding noncancerous tissues was examined using MSP and real-time PR-PCR analysis, respectively (**Figure 4F**). The result of MSP showed that the miR-212 promoter was methylated in 74.6% (53 of 71) cancer tissues and 46.5% (33 of 71) adjacent non-cancerous tissues (X^2= 11.79, P<0.01). In the RT-qPCR analysis, the expression levels of miR-212 in the 53 methylated tumors were reduced compared with 18 un-methylatede tumor tissues (P=0.03), whereas, no statistical significance was found in noncancerous group (**Figure 4H**). Meanwhile, we analyzed the correlation between DNA methylation and clinic-pathological featues. As shown in **Table 1**, ×2 (chi square test) revealed that the hypermethylation of miR-212 was significantly associated with tumor depth, lymph node, UICC staging and peritoneal dissemination, and Kaplan-Meier analysis showed that the overall survival of GC patients without miR-212 methylation was significantly longer than in those without methylation (**Figure 4G**). Interesting, those with hypermethylation exhibiting lower miR-212 expression. Those results suggest that miR-212 is frequently silenced by tumor-specific hypermethylation in GC.

Restoration of miR-212 expression contribute to demethylation induced PXN repression in GC cell lines

Based on the results that miR-212 directly targeted and repressed PXN expression, and itself was silenced by DNA methylation in GC cell lines. We wonder if demethylating treatment might indirectly downregulate PXN levels through restoring miR-212 expression. To answer this question, we treated MGC-803 and SGG-7901 GC cells with different concentrations of 5-aza (2.5 μmol/l, 5 μmol/l and 10 μmol/) and detected the levels of PXN mRNA and protein by means of RT-qPCR and westernblot, respectively. As exhibited in **Figure 5A**, **5B**, we observed that PXN mRNA and protein were downregulated in both cell lines. To further validated this speculation that posttranscriptional mechanisms such as miRNA-mediated regulation might involve in the 5-aza-induced PXN repression in both two GC cells, we transfecting the

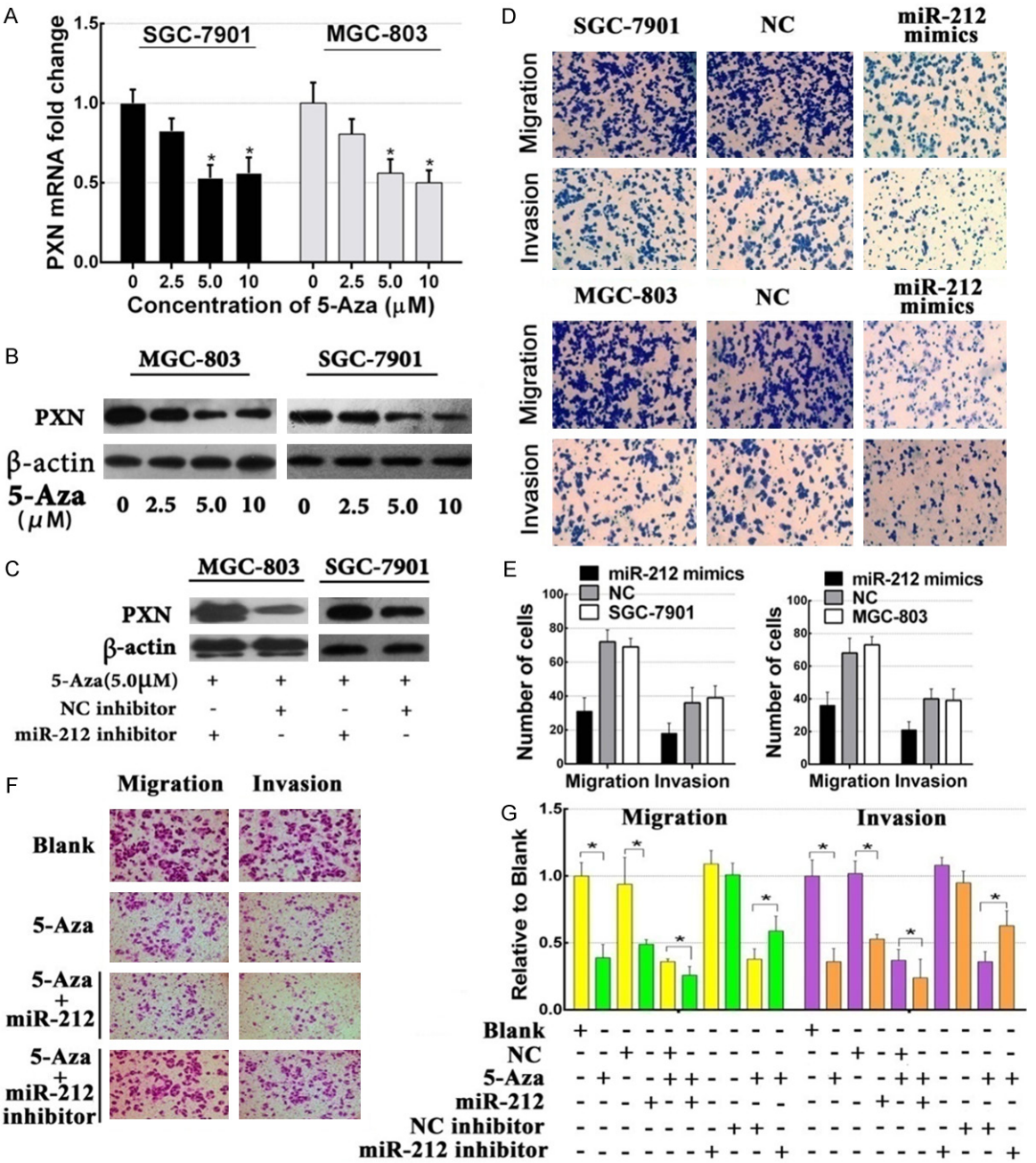

Figure 5. Restoration of miR-212 expression contributes to demethylation induced PXN repression in GC cell lines. Ectopic expression of miR-212 decreases the migration and invasion abilities in vitro. A. RTQ-PCR results for miR-212 in GC cell lines SGC-7901 and MGC-803 with different concentration 5-aza treatment (2.5 μmol/l, 5 μmol/l and 10 μmol/) for 72 h. (*P<0.05, compared with 0 μmol/l). B. Western blot results of PXN in GC cell lines SGC-7901 and MGC-803 with different concentration 5-aza treatment (2.5 μmol/l, 5 μmol/l and 10 μmol/) for 72 h. C. Western blot analysis of PXN expression in SGC-7901 and MGC-803 cells that treated with 5-aza (5 μmol/l) and miR-212 inhibitor or NC inhibitor for 48 h. 5-aza was added to the cell culture medium at 7 h after each transfection. D and E. Representative transwell migration and invasion assay of SGC-7901 and MGC-803 cells with and without miR-212 mimics treatment (*P<0.05, compared with NC). F. Representative transwell migration and invasion assay of SGC-7901 cell with combined treatment (miR-212 mimics or miR-212 inhibitor and 5-aza). G. Histograms show the transwell migration and invasion abilities of SGC-7901 GC cell with combined treatment for 48 h in three independent experiments. The results were independently calculated relative to those of blank cases, which are normalized to 1. *refers to statistical significance between cell lines (*P<0.05). SGC-7901 cells were plated for approximately 24 h before transfecting with with 100 nM synthetic miR-212mimics, NC, miR-212 inhibitor or NC inhibitor in the presence or absence of 5 μmol/l 5-aza for 48 h. The 5-aza was added to the cell culture medium at 7 h after each transfection.

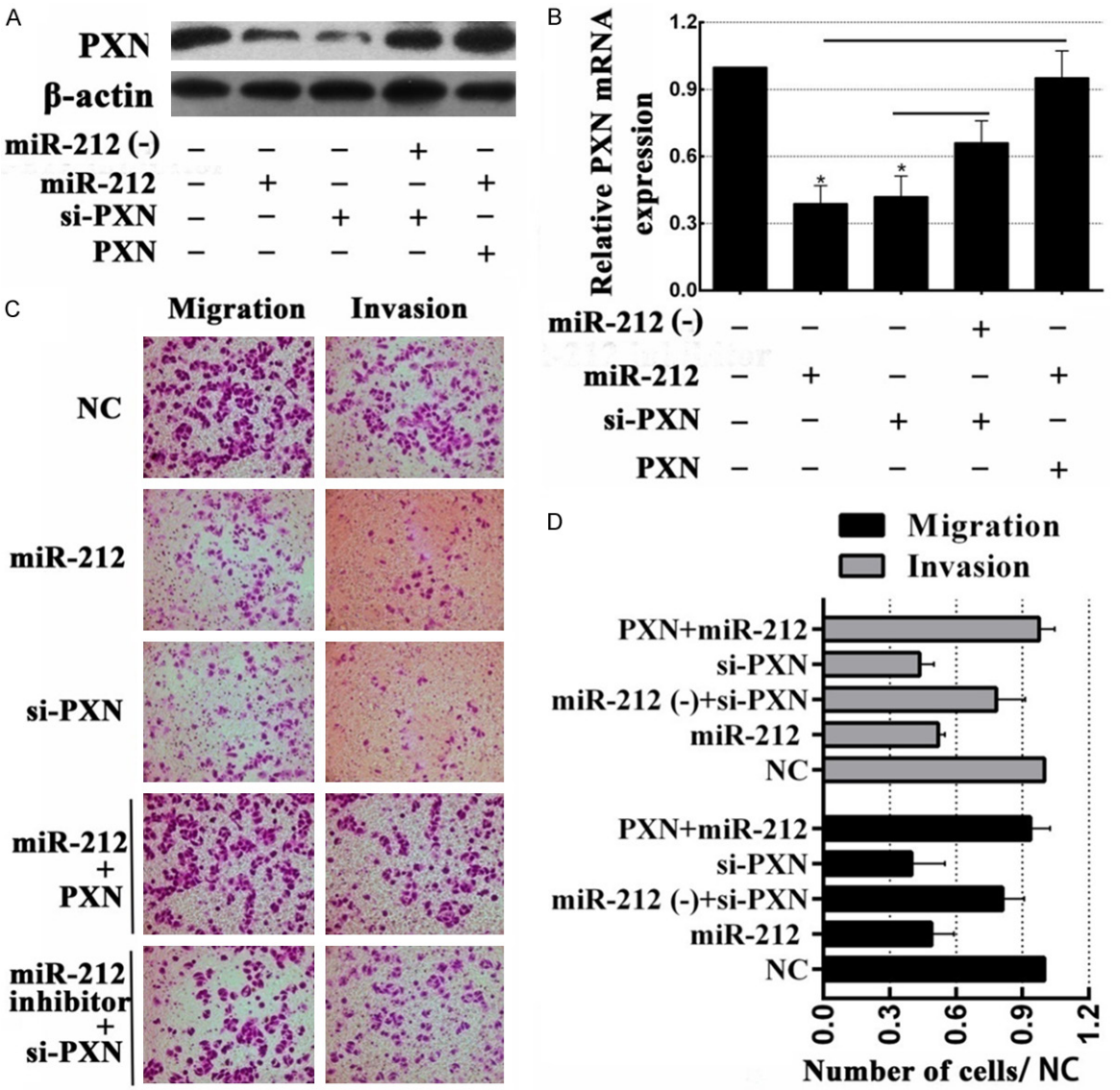

Figure 6. PXN mediates the role of miR-212 in suppression GC cell migration and invasion. A and B. Western blot and real-time PCR analysis were performed to evaluated the efficiency of ectopic miR-212 expression, inhibition of miR-212, PXN knockdown and PXN reintroduction in SGC7901 cells (*P<0.050). C and D. Transwell assays were performed to evaluated the efficiency of ectopic miR-212 expression, inhibition of miR-212, PXN knockdown and PXN reintroduction in SGC7901 cells. NC: control group, miR-212 (-): miR-212 inhibitor.

5-aza-treated MGC-803 and SGC-7901 with synthetic miR-212 inhibitor or NC inhibitor and found that a significant upregulation of PXN protein in the 5-aza-treated cells after miR-212 inhibitor transfection (**Figure 5C**).

Ectopic expression of miR-212 decrease the migration and invasion abilities in vitro

A function assay was carried out to ascertain whether miR-212 could functionally target PXN, thereby regulating invasion and metastasis in GC. We transfected MGC-803 and SGC-7901 GC cell lines with miR-212 mimics or NC mimics, and then examinated the migration and invasion abilities of cell lines. A shown in **Figure 5D**, **5E**, compared with that of NC and without treated cells, the migration and invasion ability were markedly reduced in both cells which were transfected with miR-212 mimics (P<0.05). In addition, AGS cells that possess a relatively high level of miR-212 and low level of PXN were transfected with miR-212 inhibitor, the result showed that suppression of miR-212 could apparently promote the migration and invasion of AGS cells (**Figure 7A**, **7B**). As shown above, 5-aza could restore the expression of miR-212

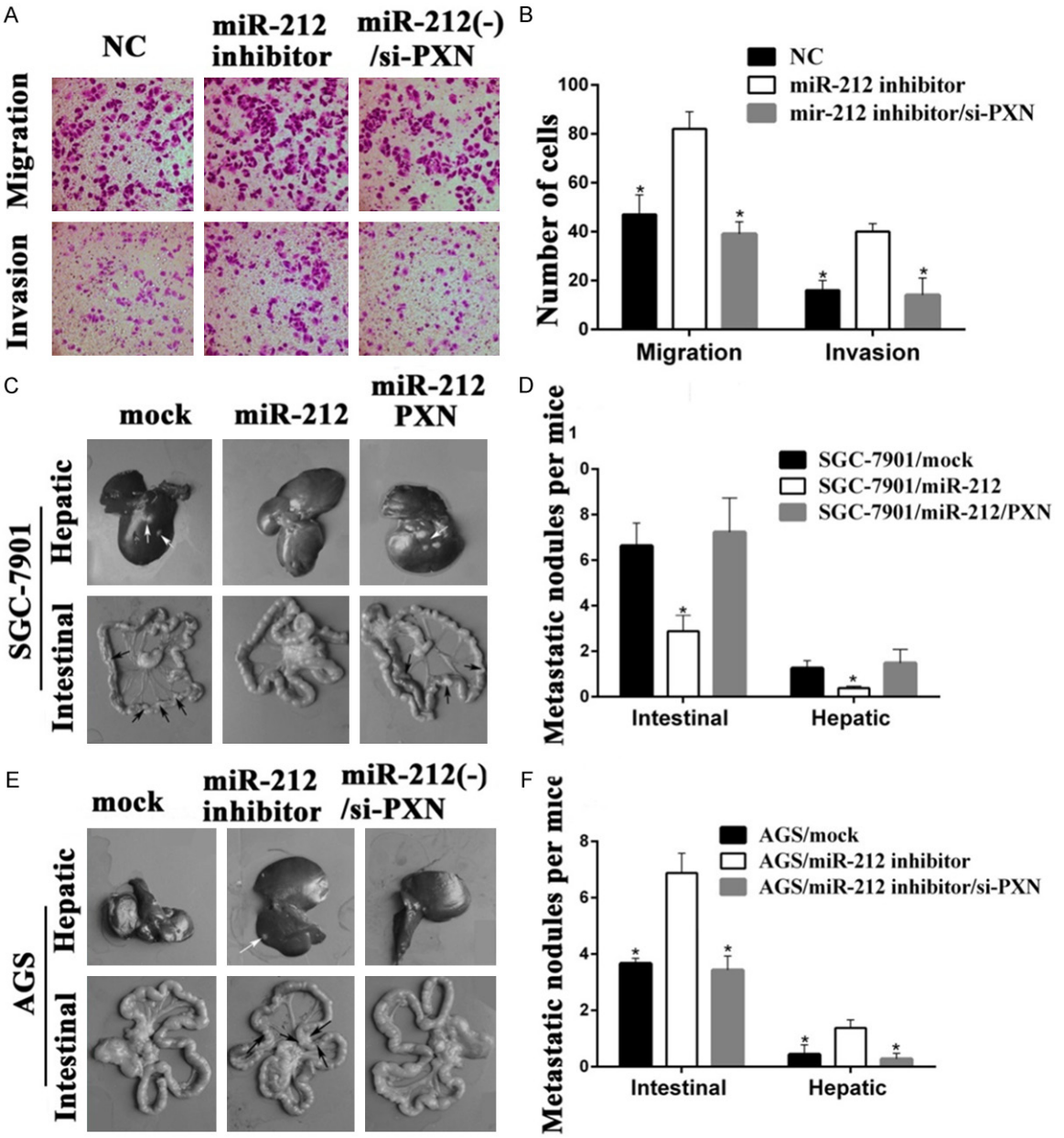

Figure 7. A and B. Representative transwell migration and invasion assay of AGS with miR-212 inhibitor treatment and with miR-212 and PXN knockdown treatment. Results represent the means of the values from three independent experiments. Bars indicate s.d. *Refers to statistical significance between groups (P<0.050). (miR-212 inhibitor compared with NC, miR-212 inhibitor/si-PXN compared with miR-212 inhibitor). C. Representative Images of metastasis lesions in mice inoculated into SGC-7901/miR-212 cells, SGC-7901/miR-212/PXN cells, or control cells (n=8). D. Number of metastatic hepatic or intestinal nodules in per mice. Results represent the means of the values. Bars indicate s.d. *Refers to statistical significance between groups (P<0.050). (compared with SGC-7901/mock). E. Representative Images of metastasis lesions in mice inoculated into miR-212 inhibitor and miR-212 inhibitor/si-PXN and control cells (n=8). F. Number of metastatic hepatic or intestinal nodules in per mice. Results represent the means of the values. Bars indicate s.d. *Refers to statistical significance between groups (P<0.050). (miR-212 inhibitor compared with NC, miR-212 inhibitor/si-PXN compared with miR-212 inhibitor).

and proceed to downregulated PXN levels, therefore, it was conceivable that inhibited the 5-aza-induced miR-212 reexpression could promote the migration and invasion abilities of GC cell lines. To test this hypothesis, firstly, we studied the migration and invasion abilities of SGC-7901 cell line in the presence or absence of 5-aza and found 5-aza can curb the abilities (**Figure 5F**, **5G**), To further validated this hypothesis, we analyzed the biologic effect of 5-aza in

SGC-7901 cell lines with and without miR-212 inhibitor or miR-212 mimics. The results exhibited that the combined treatment of 5-aza with miR-212 inhibitor brought about partial reversal of the migration and invasion abilities induced by 5-aza in SGC-7901 GC cell lines. On the contrary, the combined treatment with miR-212 mimics markedly enhanced the curb effect treated only by 5-aza (**Figure 5F**, **5G**).

PXN mediates the role of miR-212 in suppression GC cell migration and invasion

Previous studies have reported that PXN is closely associated with tumor metastasis and invasion in GC. Usually, a microRNA has many direct targets depending on its seed sequence and cellular environments and considering the aforementioned results, we investigated whether miR-212 exerted its effects through the regulation of PXN. The restoration of PXN expression in cells stably expressing miR-212 (SGC-7901/miR-212) was able to counteract the inhibitory effects of miR-212 in the gastric cancer cells (**Figure 6A**, **6B**). Moreover, we performed a loss-of-function experiment, siRNA targeting PXN (si-PXN) was transfected into SGC-79001 cells to knockdown endogenous PXN expression, and real-time PCR and Western blot analyses were carried to confirm that the PXN levels reduced (**Figure 6A**, **6B**). Cell migration and invasion analysis exhibited PXN knockdown inhibited GC cell migration and invasion, which resembled the suppressive effects of miR-212 overexpression in GC Cells (**Figure 6C**, **6D**). In addition, we transfected miR-212 inhibitor into GC cell SGC-7901 of PXN knockdown and found that miR-212 inhibitor could partially eliminate the effects of si-PXN that restore the PXN expression as well as the invasion capacity in SGC-7901 cells (**Figure 6A-D**). In addition, AGS cells that possess a relatively high level of miR-212 and low level of PXN were transfected with miR-212 inhibitor and si-PXN, as shown in **Figure7A**, **7B**, knockdown the PXN can eliminate the effects of microRNA inhibitors which promote the invasion and migration ability.

miR-212 suppresses GC metastasis through PXN in vivo

Finally, we evaluate the effect of miR-212 inhibited GC metastasis through PXN in vivo, the peritoneal cavities of nude mice were inoculated into SGC-7901/miR-212 cells, SGC-7901/miR-212/PXN cells, or control cells and the intestine and liver metastatic lesions were analyzed (**Figure 7C**, **7E**). The metastatic liver and intestine nodules was found in SGC-7901/miR-212 cells, SGC-7901/miR-212/PXN cells, or control cells were 12.5% (1 of 8) and 25% (2 of 8), 62.5% (5 of 8) and 100% (8 of 8), 75% (6 of 8) and 87.5% (7 of 8), respectively. Compared with SGC-7901/miR-212, the number of intestinal or hepatic metastatic lesions in SGC-7901/miR-212/PXN and SGC-7901 mock was reduced. ($P<0.05$) (**Figure 7D**). To further investigate whether miR-212 can suppress GC metastasis through PXN in vivo, the AGS cells, which have a relatively high level of miR-212 and low level of PXN were transfected with miR-212 inhibitor and miR-212 inhibitor/si-PXN, and then implanted into mice, the metastatic lesions in the liver and intestine was exhibited in AGS/mock, AGS/miR-212 inhibitor cells, AGS/miR-212 inhibitor/si-PXN were 0% (0 of 8) and 37.5% (3 of 8), 50% (4 of 8) and 87.5% (7 of 8), 0% (0 of 8) and 37.5% (3 of 8), the total number of hepatic or intestinal metastatic lesions in mice injected with AGS/miR-212 inhibitor was increased compared with miR-212/mock, but repression of PXN in AGS/miR-212 inhibitor could apparently eliminate the effects of microRNA inhibitors (**Figure 7F**). Based on these results it would be reasonable to draw the conclusion that miR-212 suppress the tumor metastasis in vivo by silencing PXN.

Discussion

On account of DNA methylation-associated silencing of some tumor suppressor miRNAs has been recognized as a crucial component of the mechanism underlying tumorigenesis and development [15, 28], the similar molecular mechanism might also play a role in the aberrant expression of paxillin in GC. The present study was designed to identify the tumor suppressor miRNAs which directly target paxillin but were silenced by tumor-specific DNA methylation in GC. It was previously reported that miR-218 [4, 29] and miR-137 [11] directly target paxillin and repress its expression. In this study, we firstly report that epigenetically silenced miR-212 nullify its suppression on abnormal paxillin activity, and then promote the migration and invasion in GC.

To screen for epigenetically silenced miRNAs gene targeting paxillin, we firstly performed computational searches of miRNA target databases, and selected miR-216b, miR-137, miR-

132, miR-145, miR-21 as possible candidate genes. Then by using RT-PCR and genome database, miR-137 and miR-212 were confirmed for the frequent down-regulation in GC cell lines and located on (with 1000 bp) CpG islands. In consideration of previous studies that miR-137 is not silenced its "promoter" methylation [27]. We, therefore, further analyzed miR-212. To further investigate whether miR-212 directly target paxillin and regulated endogenous PXN expression. A luciferase activity assay was performed, which confirmed that miR-212 could directly target the 3'UTR of PXN. Enhanced miR-212 expression markedly reduced endogenous PXN mRNA and protein levels in GC cell lines. Moreover, it was observed that downregulated miR-212 expression have a significantly inverse correlation with PXN expression level in clinical tissues samples and GC cell lines. These results strongly suggested that PXN is negatively regulated by miR-212 in GC. Previous studies have shown that miR-212 can target Retinoblastoma binding protein 2 (RBP2) and methyl-CpG-binding protein MeCP2 in GC [30, 31], here, we added PXN as a new target of miR-212 in GC.

To date, miR-212 have been studied to play an import role in varety of human tumor, just as increased chronic lymphocytic leukemia proliferation upon IgM stimulation is sustained by the upregulation of miR-212 [32]. MiR-212 may improve the current prognostic risk stratification of acute myeloid leukemia with cytogenetic and molecular abnormalities [33]. Downregulation of miR-212 has been found in colorectal cancer, lung cancer and GC, head and neck squamous cell carcinoma [30, 34-37]. In this study, we confirmed that miR-212 leves were dramatically downregulated in GC cell lines and primary GC tissues, especially in those with metastasis, these result is analogous to previous study [38]. Meanwhile, we firstly analyzed its correlation with clinicopathological factors and prognosis in GC tissues, which revealed that downregulation of miR-212 is significantly associated with poor overall survival, and with aggressive and invasive phenotype (tumor stage, lymph node invasion, live metastasis, peritoneal dissemination. Recent studies have also shown that upregulated PXN play an important role in proliferation and invasion in GC [6, 12], and as shown above, miR-212 can directly target PXN and suppress its expression, which demonstrate that miR-212 may act as a metastasis suppressor in GC by interfering PXN. In addition, the expression of miR-212 may act as an independent prognosis factor that is significantly associated with overall survival rates of gastric cancer patients.

Epigenetic mechanisms have been shown to be key mediators underlying the downregulation of miRNA expression [39, 40]. We evaluated miR-212 mehylation level in a panel of gastric cell lines and primary tissues by MSP and BSP, by comparison, we identified that the miR-212 promoter had higer methylation levels in GC. Importantly, the miR-212 expression has a significantly inverse correlation with methylation level not only in GC cell lines but also in GC tissues and demethlating treatment result in a markedly induction of miR-212 expression in GC cell lines, which suggested that the miR-212 was transcriptionally regulated by DNA methylation. To our knowledge, epigenetically silencing of miR-212 is not an isolated incident in GC, it has been reported in lung cancer and colorectal tumor [36, 41]. Moreover, the analysis about correlation between methylation levels of miR-212 and clinicopathological feather and prognosis revealed that hypermethylation of miR-212 is significantly associated with tumor depth, tumor stage, lymph node invasion and peritoneal dissemination. Considering the role of downregulation of miR-212 played in GC, we further confirmed that methylation at the promoter of miR-212 suppress its expression, which in turn activate tumorigenesis and development by weaking its effect on target gene, just as PXN. Futhermore, the results obtained by this study combined with our own observation, could correlate miR-212 hypermethylation status into the development of screening marker for GC detection.

Previous studies have demonstrated that paxillin was overexpressed in GC and involved in its progression and metastasis [6]. In this study, restoring miR-212 expression in GC cell lines by synthetic miR-212 transfection markedly reduced endogenous PXN mRNA and protein, which in turn inhibited cell migration and invasion in vitro. This is in line with past reports that indicated miR-212 could inhibit cell invasion in colorectal cancer [36], osteosarcoma [42] and prostate cancer [43]. In addition, using real-time RT-PCR and western blot, we observed that pro-oncogene PXN was downregulated in cell treated with 5-aza, suggesting reexpressing of endogenous miRNAs can exert similar effects by transfection of exogenous miR-212 mimics. Not only in our study, it has been

reported that 5-aza can induced miR-34b/c expression and downregulated its target gene CDK4, CCNE2 and CCNA2 in GC [20], and restored miR-193a expression and in turn reduced c-kit in acute myeloid leukemia [44]. Furthermore, 5-aza-induced PXN reduction could be partially blocked by the synthetic miR-212 inhibitor leading to a reversal of metastasis and invasion inhibition effects of 5-aza on SGC-7901 and MGC-803 GC cell lines which implied that there are other potential target gene of this demethylating treatment have involved in.

Usually, a microRNA has many direct targets depending on its seed sequence and cellular environments [45, 46]. In this study, we performed a rescue experiment which overexpressing PXN in miR-212 mimics treated cell line (SGC-7901), which should counteract the effects of the miR-212, and a loss-of-function experiment that knockdown of the target gene PXN in cells (SGC-7901) treated with miR-212 inhibitors, which should eliminate the effects of miR-212 inhibitors. Furthermore, AGS cells that possess a relatively high level of miR-212 and low level of PXN were transfected with miR-212 inhibitor and miR-212 inhibitor/si-PXN, the result showed that suppression of miR-212 could apparently promote the migration and invasion of AGS cells and PXN knockdown blocks GC cell migration and invasion in the presence miR-212 inhibitors. In addition, the experiment in vivo had shown the same result. These results support the conclusion that PXN mediates the role of miR-212 in gastric tumor metastasis and invasion.

In summary, we show a novel regulatory mechanism of paxillian expression in GC wherein DNA hypermethylation of miR-212 silenced its expression, which weaken its effect on its direct target gene PXN, in turn regulating GC invasion and metastasis of GC cells. Downregulated miR-212 and its hypermethylation are associated with aggressive tumor phenotypes and adverse overall survival. Knockdown of PXN by reactivation of miR-212 mimic and demethylation treatment inhibits tumor migration and invasion in GC cell lines. Our studies therefore suggest that methylation of miR-212 or miR-212 expression levels could serve as a promising tumor marker and that restoration of its express could be an effective anticancer therapeutic strategy for gastric and other human cancers.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. The primer sequence for RTQ-PCR, BSP and MSP in this reasearch

Primer		Primer sequence (5'-3')
miR-216b	F	CAGGCACACACTTACCCGTA
	R	GCAGGGTCCGAGGTATTC
miR-137	F	CAAGGCTTGTTAACACTGTAAC
	R	TCTGTCAATGTCTGAATAAATG
miR-132	F	TGGATCCCCCCCAGTCCCCGTCCCTCAG
	R	TGAATTCGGATACCTTGGCCGGGAGGAC
miR-145	F	GTGCTTTGTGGAGGGTACTTCAT
	R	CGACGGTCCAGTTTTCCCA
miR-212	F	CGCTAACAGTCTCCAGTC
	R	GTGCAGGGTCCGAGGT
β-actin	F	TGGATCAGCAAGCAGGAGTA
	R	TCGGCCACATTGTGAACTTT
U6	F	CTCGCTTCGGCAGCACA
	R	AACGCTTCACGAATTTGCGT
PXN	F	ACGTCTACAGCTTCCCCAACAA
	R	AGCAGGCGGTCGAGTTCA
miR-212 BSP primer*	F	TTATTTTTTTTATTTTTATTTTTGG
	R	CTCCTCAATTCCTAAACCTAAC
miR-212-U	F	GAAGGTGTTGGTGTTTTAGGAGAT
	R	AAAAAACAAAAATAAACATCCATC
miR-212-M	F	GTGTTGGCGTTTTAGGAGAC
	R	AAAAACGAAAATAAACGTCCGT
miR-212 inhibitor		GGCCGUGACUGGAGACUGUUA
NC inhibitor		UUCUCCGAACGUGUCACGUTT
miR-212 mimics	F	UAACAGUCUCCAGUCACGGCC
	R	CCGUGACUGGAGACUGUUAUU
NC mimic	F	UUCUCCGAACGUGUCACGUTT
	R	ACGUGACACGUUCGGAGAATT

RTQ, quantitative real-time; PCR, BSP bisulfite sequence-PCR; MSP, methylation-specific PCR; F, forward primer; R, reverses primer; U, unmethylated primer; M, methylated primer; NC, negative control; *this BSP product contain 18 CpG site.