临床细胞生物学

蛋白质精氨酸甲基转移酶1促进胰腺癌细胞的增殖和迁移能力

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摘要 该文探讨了蛋白质精氨酸甲基转移酶1(protein arginine methyltranserase 1, PRMT1)对胰腺癌细胞迁移和增殖的影响。在数据库中分析了PRMT1在正常胰腺组织和胰腺癌组织中的表达。用Western blot和Real-time PCR实验检测不同胰腺癌细胞中PRMT1的基础表达量。在PaTu8988和BxPC3细胞中干扰*PRMT1*, Western blot、qPCR检测shRNA干扰效率; CCK-8检测细胞增殖活性; 平板克隆法检测细胞克隆形成能力; Transwell迁移实验检测细胞迁移能力; Western blot检测细胞EMT(epithelial-mesenchymal transition)相关蛋白变化。相反, 在SW1990细胞中过表达*PRMT1*, 并做上述同样的实验进行检测。结果显示, PRMT1在胰腺癌组织中的表达明显高于正常胰腺组织。在胰腺癌细胞中下调*PRMT1*的表达, 可以抑制细胞的增殖和迁移能力, 增高上皮标志物E-钙黏蛋白的水平, 降低间质标志物N-cadherin钙黏蛋白的水平。上调*PRMT1*的表达得到相反的结果。 结果表明, PRMT1能促进胰腺癌细胞的增殖和迁移能力, 并影响EMT标志物蛋白质水平。该研究为进一步研究体内PRMT1的作用提供基础。

关键词 蛋白质精氨酸甲基转移酶1(PRMT1); 胰腺癌; 增殖; 迁移; 上皮间质转化(EMT)

Protein Arginine Methyltransferase 1 Promotes the Abilities of Proliferation and Migration in Pancreatic Cancer Cells

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Abstract The aim of the present study is to investigate the effects of protein arginine methyltransferase 1 (PRMT1) on cell proliferation and migration in pancreatic cancer cells. We analyzed the *PRMT1* gene expression in pancreatic non-tumor tissues and pancreatic cancer tissues of few databases. We then measured the levels of PRMT1

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mRNA and protein in pancreatic cancer cells by Real-time PCR and Western blot. We knocked down *PRMT1* in PaTu8988 and BxPC3 cells and detected the abilities of proliferation and migration by CCK-8 assay, colony-forming assay and migration assay. Epithelial-mesenchymal transition (EMT) markers were examined by Western blot. Conversely, we overexpressed *PRMT1* in SW1990 cells and conducted the above-mentioned experiments again. The results showed that *PRMT1* gene expression was higher in pancreatic cancer tissues than that in pancreatic non-tumor tissues at both mRNA and protein levels. The data *in vitro* revealed that *PRMT1* knockdown inhibited the abilities of proliferation and migration, while *PRMT1* overexpression promoted the above behaviors in pancreatic cancer cells. Further studies indicated that PRMT1 knockdown remarkably decreased the level of mesenchymal marker N-cadherin, and increased the level of epithelial marker E-cadherin. Conversely, *PRMT1* overexpression resulted in the opposite effects. Our work suggested that PRMT1 promoted the ability of proliferation and migration in pancreatic cancer cells, and might provide a new therapeutic target for the clinical treatment of pancreatic cancer.

Keywords PRMT1; pancreatic cancer; proliferation; migration; EMT

Pancreatic cancer is one of the most lethal cancers among all malignances, with a median overall survival of <1 year and a 5-year survival of ~5%[1]. The health burden of pancreatic cancer in China is increasing, and the annual mortality rates almost equal to incidence rates[2]. Most of the pancreatic cancer-related deaths are due to metastatic disease. Therefore, it is necessary for us to explore the specific marker that promotes the progress of pancreatic cancer.

The transdifferentiation of epithelial cells into motile mesenchymal cells, a process known as epithelial-mesenchymal transition (EMT), plays an important role in tumor progression. A hallmark of EMT is a decrease in the expression of E-cadherin[3] and an increase in N-cadherin[4]. Previous studies have proven that epigenetic mechanisms including DNA methylation and histone modifications are involved in the regulation of EMT-related genes during EMT[5]. Arginine methylation is an abundant covalent modification that regulates diverse cellular processes, including transcription, translation, DNA repair and RNA processing. The enzymes that catalyze these marks are known as the protein arginine methyltransferases (PRMTs)[6]. This family currently consists of nine characterized members in higher eukaryotes. The majority of arginine methylation is catalyzed by PRMT1, and loss of PRMT1 expression is not compatible with life[7]. PRMT1 is the predominant PRMT in mammalian cells, and performs over 80% of PRMT activity in cell. PRMT1 is known to methylate histone H4R3 and regulate different cellular functions such as cell proliferation and survival, transformation, resistance to DNA damaging agents and cell invasion[8-9]. Moreover, several studies had demonstrated that *PRMT1* was up-regulated in breast cancer[10], lung cancer[11], colon cancer[12], bladder cancer[13] and acute myeloid leukemia[14]. However, the role of PRMT1 in pancreatic cancer cells remains to be clarified.

In this study, we found that PRMT1 expression had a great difference in pancreatic cancer tissues, and was critical for the ability of proliferation and migration in pancreatic cancer cells *in vitro*. Furthermore, it was found that PRMT1 promoted EMT in pancreatic cancer cells. All these findings for the first time proved that PRMT1 might be a promoter in pancreatic cancer progression.

1 Materials and methods

1.1 Cells and cell culture

The pancreatic cancer cell lines, PaTu8988, SW1990 and BxPC3, were kindly provided by Second Military Medical University in Shanghai, and have been tested and authenticated by short tandem repeat analysis. The cells were cultured with DMEM (Multicell, China) supplemented with 10% FBS, in humidified 5% CO_2 incubator at 37 °C.

1.2 Western blot

The cultured cells were rinsed with cold PBS

before treated with 1×SDS loading buffer at 100 °C for 10 min. Then the mixture was centrifuged at 12 000 r/min for 5 min. About 10 μL of protein was loaded each lane, and separated by 10% SDS-PAGE and then transferred to the PVDF membrane. The membrane was blocked by 5% non-fat milk powder for 1 h at room temperature and then incubated with primary antibodies at 4 °C overnight. The membranes were washed with TBS-T buffer (10 mmol/L Tris-HCl, pH7.4, 150 mmol/L NaCl, 0.05 % Tween 20) for 15 min and incubated with HRP-labeled secondary antibodies. The antibodies were mouse anti-PRMT1 (Santa Cruz Biotechnology, sc-166963), mouse anti-β-Tubulin (Cell Signaling, CAT 6181), mouse anti-Flag (SIGMA, CAT F1804), rabbit anti-N-cadherin (Cell Signaling, CAT 13116), rabbit anti-E-cadherin (Cell Signaling, CAT 3195).

1.3 Real-time PCR

Total RNA was isolated by RNAiso Plus (TaKaRa). Reverse transcription was performed by Revert Aid First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer's recommendations. The SYBR green-based Real-time PCR was then performed in triplicate by CFX-96 Sequence Detection System (Bio-Rad). The primer pair has been used for the amplification of the human *PRMT1* gene was as follows: forward primer, 5′-CAT GGA GGA CTA CCT GAC-3′, and reverse primer, 5′-GTT GTT CTT GGC GTT GGG-3′. As an internal standard, a fragment of human *GAPDH* was amplified by PCR using the following primers: forward primer, 5′-GGT GAA GGT CGG TGT GAA CG-3′, and reverse primer, 5′-CTC GCT CCT GGA AGA TGG TG-3′. The relative fold change in RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

1.4 Cell Counting Kit-8 assay

The measurement of viable cell mass was performed with Cell Counting Kit-8 (Promega) according to manufacturer's instructions. Briefly, 1 000 cells/well were seeded in a 96-well plate and grew in an incubator (37 °C, 5% CO_2). 10 μL CCK-8 was added to each well respectively in the first five days, and cells were incubated at 37 °C for 2 h and the absorbance was finally determined at 490 nm.

1.5 Colony-forming assay

The cells were harvested, resuspended in medium and transferred to the 6-well plate (500, 1 000, 2 000 cells/well) for 10~14 days until large colonies were visible. Colonies were fixed in 4% paraformaldehyde for 30 min and then stained with 0.05% crystal violet for 30 min, and the number of colonies was counted or photomicrographs were taken under phase-contrast microscope.

1.6 Wound healing assay

The cells have grown to confluence in complete cell culture medium. At time 0, a scrape wound was created across the diameter with a 10 μL pipette tip followed by extensive washes with medium to remove dead and floating cells. The distance was recorded at 0, 24, and 48 h. Images were captured by an inverted microscope equipped with a digital camera.

1.7 Migration assay

For assessing cell migration, 1×10^5 cells in serum free media were seeded into the transwell inserts (Corning) containing 8 μm permeable pores and were allowed to migrate toward 10% FBS-containing medium. 24-36 h later, the migrated cells on the bottom of the insert were fixed with 4% paraformaldehyde solution followed by crystal violet (1%) staining. Pictures were taken after washing the inserts three times with PBS. Five independent fields were counted for each transwell and the average numbers of cells/field were represented in the graphs.

1.8 Statistical analysis

All data are presented as mean±S.D. from at least three independent experiments. Differences between groups were assessed by ANOVA or Student's *t*-test using GraphPad Prism5 software. $P<0.05$ was considered as statistical significant.

2 Results

2.1 PRMT1 is up-regulated in clinical pancreatic cancer tissues

To confirm the clinical relevance of *PRMT1* gene expression, we first analyzed the mRNA levels

of *PRMT1* from oncomine (www.oncomine.org), Pei Pancreas datasets[15] (Fig.1A), and found significant overexpression of *PRMT1* in pancreatic cancer tissues compared to pancreatic non-tumor tissues [pancreatic non-tumor tissue (n=16); pancreatic cancer (n=36); P<0.001, Fig.1A]. To evaluate protein levels of *PRMT1* in pancreatic tissues, we analyzed the human protein atlas datasets[16] (www.proteinatlas.org). We observed strong *PRMT1* staining in pancreatic cancer tissues, but very weak staining in pancreatic non-tumor tissues (Fig.1B). These data indicated that *PRMT1* gene expression is significantly up-regulated in clinical pancreatic cancer tissues.

2.2 PRMT1 expression is profiled in pancreatic cancer cells

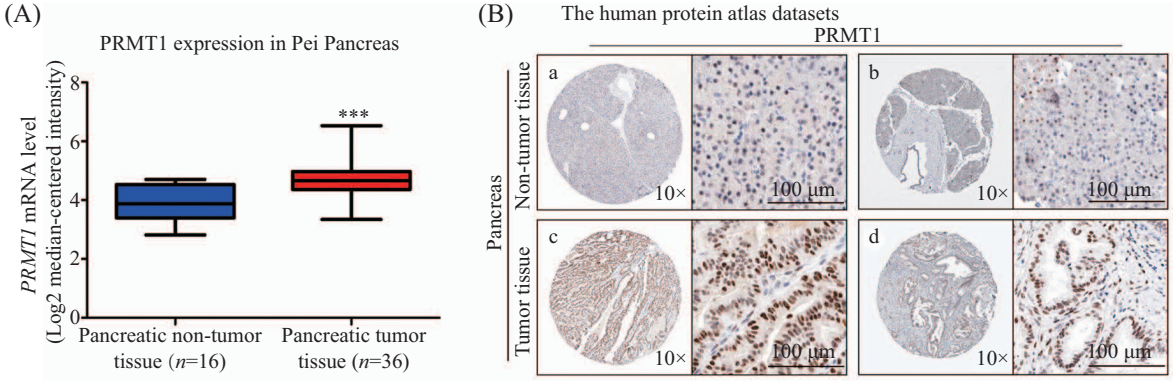
To determine the role of PRMT1 in pancreatic cancer cells, we first examined the level of PRMT1 expression in PaTu8988, SW1990 and BxPC3 cells by Real-time PCR and Western blot. The results showed that PRMT1 expression was higher in PaTu8988 and BxPC3 cells than that in SW1990 cells at both mRNA and protein levels (Fig.2A). Subsequently, we constructed sh-PRMT1 and Flag-PRMT1 plasmids to investigate the roles of PRMT1 in pancreatic cancer cells, sh-EGFP or Vector as a control, respectively. After transfection, the mRNA and protein levels of PRMT1 significantly reduced in sh-PRMT1 group compared with sh-EGFP group (Fig.2B). Vector or Flag-PRMT1 was transferred into SW1990 cells, and then PRMT1 overexpression was confirmed at both mRNA and protein levels (Fig.2C).

2.3 *PRMT1* promotes the ability of proliferation in pancreatic cancer cells

Next, we examined the ability of proliferation by colony-forming assay in pancreatic cancer cells. PaTu8988 cells expressing sh-PRMT1 showed reduced colony number compared with the cells expressing sh-EGFP (263±12 *vs* 156±15, Fig.3A). On the contrary, overexpression of *PRMT1* in SW1990 cells promotes the ability of cell colony formation (252±18 *vs* 489±21, Fig.3B). Similarly, CCK-8 assays were also used to follow cell growth over time. *PRMT1* depletion caused a significant reduction in the cell proliferation in PaTu8988 cells (Fig.3C). Conversely, *PRMT1* overexpression led to a meaningful increment of cell proliferation in SW1990 cells (Fig.3D). These results revealed that PRMT1 promotes the ability of proliferation in pancreatic cancer cells.

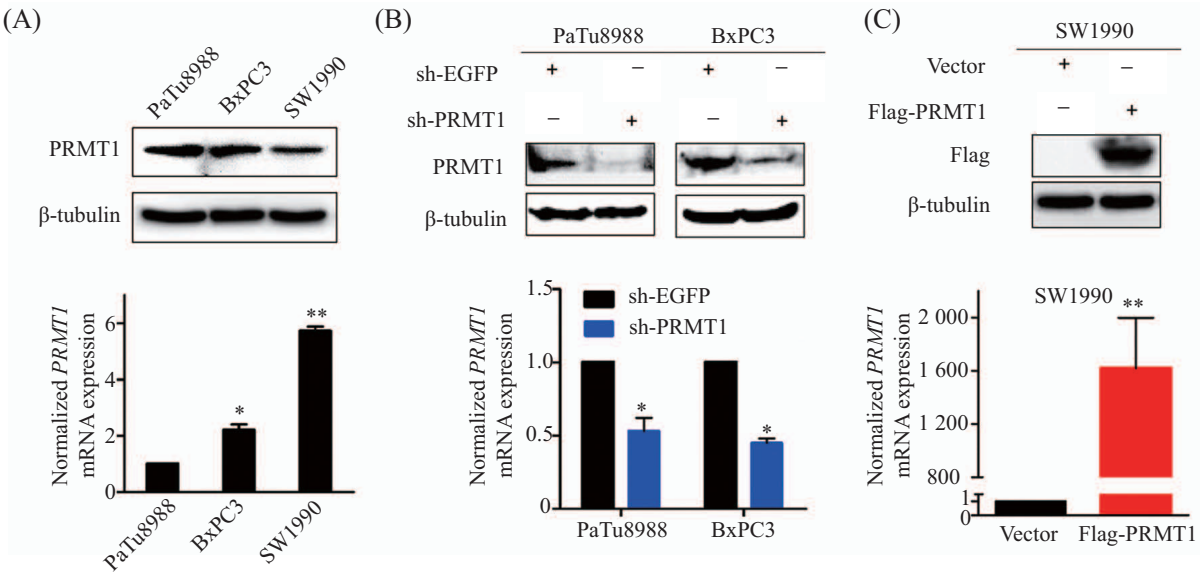
2.4 *PRMT1* promotes the migration ability of pancreatic cancer cells

Subsequently, we examined the ability of migration by wound healing assays and migration assay. PaTu8988 cells expressing sh-PRMT1 displayed reduced motility in comparison to PaTu8988 cells



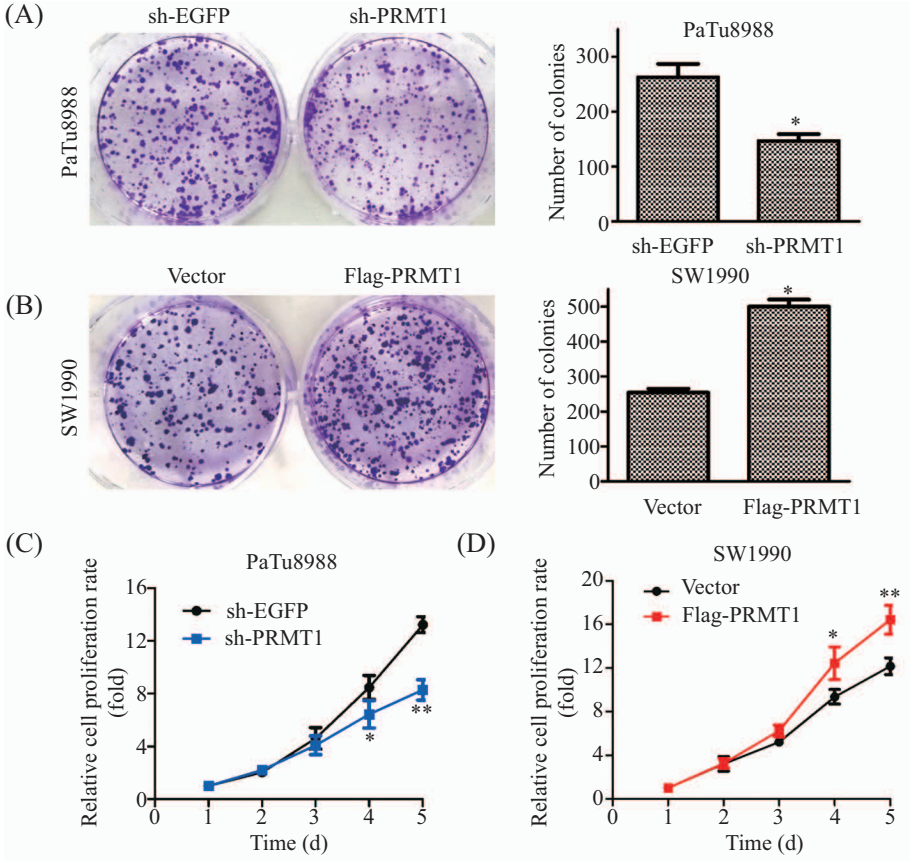
A: oncomine analysis of the Pei Pancreas database indicates elevated *PRMT1* mRNA level in pancreatic cancer tissues compared with pancreatic non-tumor tissues (n=16 for pancreatic non-tumor tissues group, and n=36 for pancreatic tumor tissues group. ***P<0.001 *vs* pancreatic non-tumor tissues group). B: PRMT1 protein levels in pancreatic tumor tissues and pancreatic non-tumor tissues were analyzed through the human protein atlas. The tissue (a) and (b) were obtained from pancreatic non-tumor tissues, and PRMT1 was not detected in these tissues. The tissue (c) was obtained from a 63 years old male patient with pancreas adenocarcinoma, and the PRMT1 protein level was high in this tissue. The tissue (d) was obtained from a 64 years old female patient with pancreas adenocarcinoma, and the PRMT1 protein level was medium in this tissue.

Fig.1 PRMT1 is up-regulated in clinical pancreatic cancer tissues



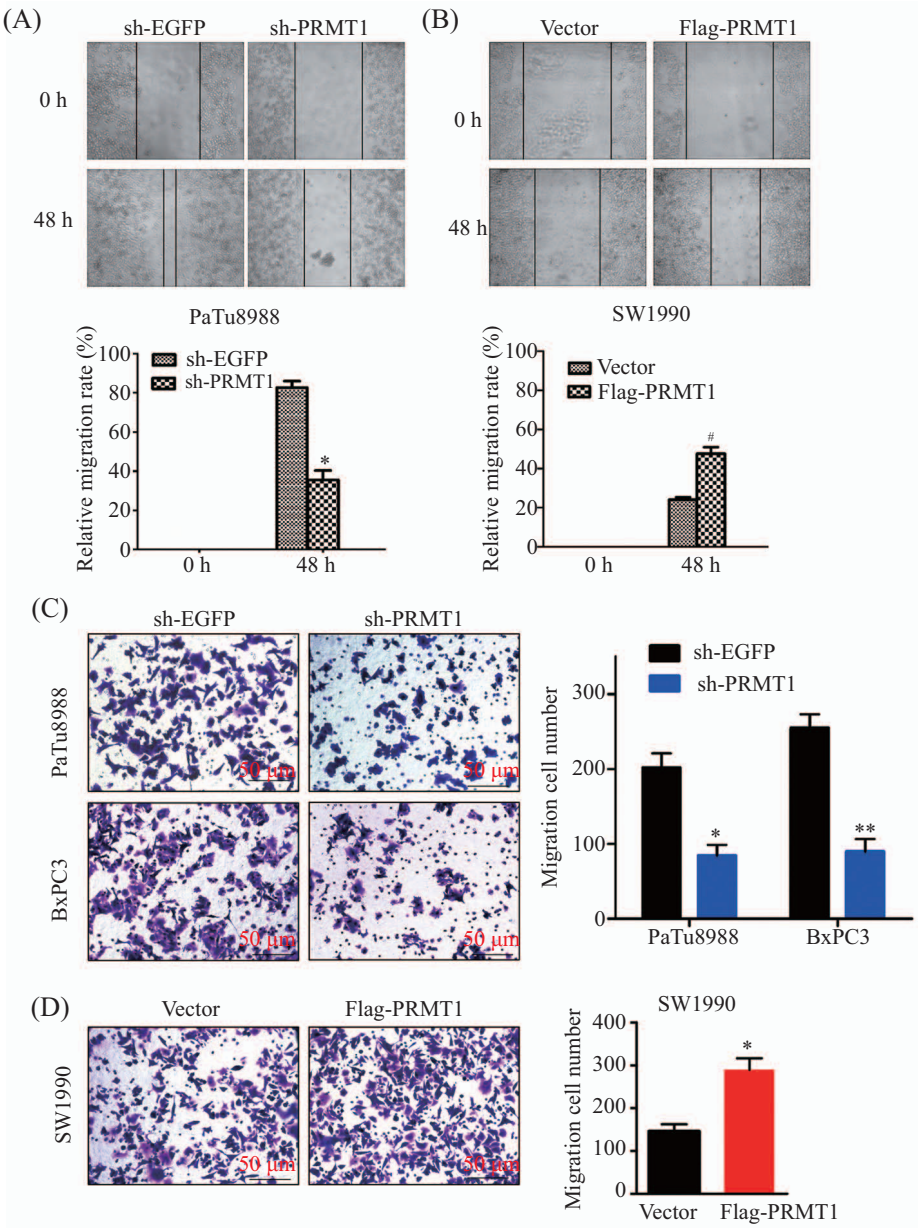
A: relative levels of PRMT1 protein and mRNA were assessed in SW1990, PaTu8988 and BxPC3 cells. *P<0.05, **P<0.01 *vs* SW1990 group. B: PRMT1 protein and mRNA levels were reduced in sh-PRMT1 PaTu8988 and BxPC3 cells. **P<0.01 *vs* shEGFP group. C: PRMT1 protein and mRNA levels were increased in SW1990 cells transfected with Flag-PRMT1. **P<0.01 *vs* Vector group.

Fig.2　PRMT1 expression is profiled in pancreatic cancer cells



A: the effect of knockdown of *PRMT1* on the growth of PaTu8988 cells examined by colony-forming assay. *P<0.05 *vs* sh-EGFP group. B: the effect of overexpression of *PRMT1* on the growth of SW1990 cells examined by colony-forming assay. *P<0.05 *vs* Vector group. C: the effect of knockdown of *PRMT1* on the proliferation rate of PaTu8988 cells examined by CCK-8 assay. *P<0.05, **P<0.01 *vs* sh-EGFP group. D: the effect of overexpression of *PRMT1* on the proliferation rate of SW1990 cells examined by CCK-8 assay. *P<0.05, **P<0.01 *vs* Vector group.

Fig.3　*PRMT1* promotes the ability of proliferation in pancreatic cancer cell lines



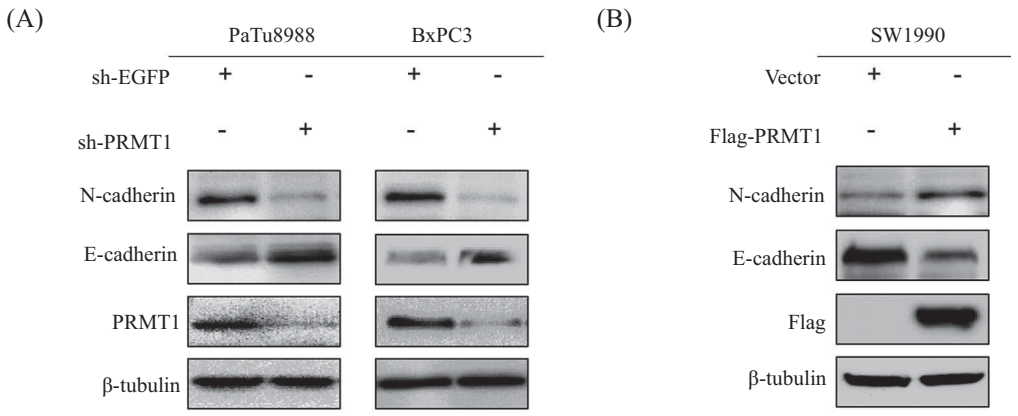
A-B: a scrape wound was created in confluent cultures of PaTu8988 cells with the expression of either sh-EGFP or sh-PRMT1, and SW1990 cells with the expression of either vector or Flag-PRMT1. The distance of cell migration was recorded and the relative rate of migration was calculated. **P*<0.05 *vs* sh-EGFP group. #*P*<0.05 *vs* vector group. C: PRMT1 knockdown results in reduced cell migration in PaTu8988 and BxPC3 cells. **P*<0.05, ***P*<0.01 *vs* sh-EGFP group. D: PRMT1 promotes the ability of cell migration in SW1990 cells. **P*<0.05 *vs* Vector group.

Fig.4 *PRMT1* enhances the ability of migration in pancreatic cancer cells

expressing sh-EGFP (Fig.4A). PaTu8988 and BxPC3 cells expressing sh-PRMT1 showed reduced migration in compared with the cells expressing sh-EGFP, the migrated cell numbers were 203±23 *vs* 86±14 and 246±22 *vs* 87±20, respectively (Fig.4C). To confirm the above results, we assessed the ability of migration in SW1990 cells transfected with Vector or Flag-PRMT1 plasmid. SW1990 cells expressing Flag-PRMT1 showed increased motility in comparison to SW1990 cells expressing Vector (Fig.4B). The numbers of migrated cells were 107±9 and 36±5 in Vector and Flag-PRMT1 SW1990 cells (Fig.4D). These results suggested that PRMT1 promotes the ability of migration in pancreatic cancer cells.

2.5 *PRMT1* promotes EMT in pancreatic cancer cells

A hallmark of EMT is a decrease in the expression of E-cadherin[3] and an increase in the expression of



A: knockdown of *PRMT1* in PaTu8988 and BxPC3 cells and the EMT markers N-cadherin and E-cadherin were detected. B: overexpression of PRMT1 in SW1990 cells and the EMT markers N-cadherin and E-cadherin were detected.

Fig.5　The effect of PRMT1 on EMT markers at protein levels in pancreatic cancer cells

N-cadherin[4]. We detected the EMT markers at protein levels by Western blot. Our data suggested that *PRMT1* knockdown resulted in up-regulation of E-cadherin and down-regulation of N-cadherin in PaTu8988 and BxPC3 cells (Fig.5A). In contrast, PRMT1 overexpression led to down-regulation of E-cadherin and up-regulation of N-cadherin in SW1990 cells (Fig.5B). These data suggested that PRMT1 promoted EMT in pancreatic cancer cells.

3　Discussion

In this study, we described that both the mRNA and protein levels of PRMT1 were up-regulated in the clinical tissues of pancreatic cancer. Also, *PRMT1* was found to promote the proliferation and migration ability of pancreatic cancer cells in the functional study. Moreover, we found that PRMT1 affected EMT marker at protein levels in pancreatic cancer cells, which further confirmed the oncogenic roles of PRMT1 in the malignancies.

Previous studies have determined that *PRMT1* expression is up-regulated in non-small cell lung cancer[11], bladder cancer[13], esophageal squamous cell carcinoma[17], ovarian cancer[18] and gliomas[19], indicating that elevated *PRMT1* expression is a potential marker of the carcinogenesis process. In our data, the expression of PRMT1 was different between pancreatic non-tumor tissues and pancreatic tumor tissues, which indicated that PRMT1 might be a potential biomarker in the pathological examination of human pancreatic cancer. In addition, *PRMT1* knockdown suppressed the proliferation and migration of both PaTu8988 and BxPC3 cells while *PRMT1* overexpression promoted the proliferation and migration of SW1990 cells. Combined with previous studies, our results further confirmed the critical functions of PRMT1 for the progression of cancers.

As we know, EMT is a complex phenomenon and an important driver of tumor invasion, tumor progression, and tumor metastasis. Loss of E-cadherin expression is considered as a key event during the induction of EMT[3]. In the current study, we identify that PRMT1 affects EMT marker at protein levels, as PRMT1 knockdown leads to an up-regulation of E-cadherin and PRMT1 overexpression causes a down-regulation of E-cadherin in pancreatic cancer cells. Previous studies have proved that the process of EMT is mediated by key transcription factors, including Snail, zincfinger E-box-binding (ZEB) and basic helix-loop-helix transcription factors[20]. Several signaling pathway such as TGF-β (transforming growth factor beta) signaling, the Wnt signal, and the Notch pathway are also proved to mediate EMT[21]. Recently, Twist1 (Twist-related protein 1)[11], ZEB1[10] and FAM98A (family with sequence similarity 98 member A)[18] are identified as new substrates of PRMT1, which is required for the process of EMT and tumor progression. Our next work is to find the substrate of

PRMT1 or the signaling pathway which it influences.

In summary, our study provides a strong evidence for the involvement of PRMT1 in proliferation, migration and the protein levels of EMT markers in pancreatic cancer cells. All these findings suggest that PRMT1 might represent a novel anti-cancer strategy for pancreatic cancer in future.

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