PNN促进肾癌的发展并抑制舒尼替尼诱导的细胞凋亡

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摘要 桥粒相关蛋白与肿瘤的关系是目前的研究热点之一。传统观点认为, 桥粒相关蛋白PNN(pinin)能够促进上皮细胞间的黏附和RNA的选择性剪接; 而新近研究发现, PNN在肝癌、乳腺癌等肿瘤的发生发展中扮演着重要角色; 但其是否参与肾透明细胞癌(ccRCC)的发生, 尚需深入研究。在该研究中,首先发现PNN在肾癌组织和细胞中的表达显著性高于对照组; 且其升高程度与肾癌的病理分级密切相关。其次, 降低肾癌细胞中PNN的表达后, 细胞增殖被显著抑制, 细胞周期被阻滞在G_0/G_1期。最后, 降低细胞内PNN的表达能显著增强靶向药物舒尼替尼的细胞毒性, 增加凋亡细胞的数量, 此作用与PI3K/AKT途径密切相关。因此, PNN能激活PI3K/AKT通路促进靶向药物舒尼替尼对肾癌细胞的毒性作用; PNN有望成为肾癌耐药靶向治疗的潜在靶点。

关键词 桥粒相关蛋白PNN; 细胞增殖; 舒尼替尼; 肾透明细胞癌

Pinin Contributes to ccRCC Progression and Resistance to Sunitinib-Induced Apoptosis

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Abstract The expression and bio-function of desomosome-related proteins have been gradually disclosed in various human cancers. PNN (pinin) is a desmosome-associated molecule that has been well studied in epithelial cell-cell adhesion and RNA alternative splicing, which suggests its involvement in cancer progression. However, little is known about the association between PNN expression and ccRCC (clear cell renal cancer cell) tumorigenesis. This study reported that the expression of PNN was significantly increased in ccRCC tissues and cells, and the elevated level of PNN was closely associated with pathological grade of patients with ccRCC. Suppression of PNN expression inhibited cell proliferation and cell viability, inducing G_0/G_1 cell cycle arrests. Furthermore, si-PNN treatment significantly enhanced the cytotoxic effect of sunitinib and the apoptotic cell number compared with cells underwent sunitinib treatment only. The molecular signals for this phenomenon involved the PNN-mediated activation of PI3K/AKT pathway. In conclusion, these results reveals that PNN contributes to ccRCC progression and resistance to targeted drug-induced apoptosis via maintaining PI3K/AKT activation and may become a potential therapeutic target for ccRCC.

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RCC (renal cell carcinoma) has a rising incidence and represents the third most prevalent urologic malignancy[1-2]. Compared with other tumours, there are few effective biomarkers for RCC, especially in ccRCC, the most common subtype. Many patients who have metastasized ccRCC at diagnosis may be resistant to conventional chemotherapy, radiotherapy and immunotherapy[3-4]. Therefore, it is urgent to identify specific biomarkers that can be used to develop novel therapies for the patients with ccRCC.

PNN is originally identified and characterized as a desmosome-associated molecule[5]. Desmosomes are intercellular junctions that tether IF (intermediate filaments) to the plasma membrane[6-8]. The presence of PNN within the desmosome is correlated with highly organized, perpendicular bundles of keratin filaments, and primarily stabilizing the desmosome-IF complex and reinforcing epithelial cell to cell adhesion[9-10]. The postulated role of a desmosome-associated molecule, such as PNN, is to reinforce epithelia cell-cell adhesion, so that loss of its expression increases the transition from benign to highly invasive tumours[11-13]. Therefore, it seems that desmosome-associated molecules could be considered as tumour suppressors. However, recent reports have demonstrated that PNN may be implicated in chemotherapy resistance in breast cancer cell[14] and act as an oncogene to facilitate proliferation as well as metastasis of hepatocellular carcinoma[15] and colorectal cancer cells[16]. These conflicting results stimulate the current research on the role of PNN in ccRCC.

1 Materials and methods

1.1 Tissue samples

Pair-matched ccRCC tumour and adjacent non-tumour tissues were received from the Pathology Department, Ningbo Urology and Nephrology Hospital. Ethics and scientific committee approvals were obtained from our institution for this study and informed consents were obtained from all participants.

1.2 Cell culture and transfections

Human renal cancer cell lines (Caki-1, ACHN, OS-RC-2 and 786-O), and immortalized proximal tubule epithelial cell line HK-2 used in the study were purchased from American Type Culture Collection (Rockville, MD). OS-RC-2 and 786-O cells were cultured in RPMI (Gibco, ThermoFisher, USA). ACHN and HK-2 cells were cultured in DMEM (Gibco, USA). Caki-1 cells were cultured in McCoy's 5A medium (Gibco, USA), supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China). All cell cultures were carried out in a humidified chamber at 37 °C with an atmosphere of 5% CO_2.

siRNAs for PNN: si-PNN 254 (si-PNN1), sence: 5′-GGU AGA GGA CGU GGU AGU UTT-3′, antisence: 5′-AAC UAC CAC GUC CUC UAC CTT-3′; si-PNN 465 (si-PNN2), sence: 5′-GCA CAC GUA GAG ACC UUA UTT-3′, antisence: 5′-AUA AGG UCU CUA CGU GUG CTT-3′; and siRNA for negative control (siNC), sence: 5′-UUC UCC GAA CGU GUC ACG UTT-3′, antisence: 5′-ACG UGA CAC GUU CGG AGA ATT-3′ were purchased from GenePharma. Transfection of si-PNN or siNC was performed using Lipofectamine 2000 transfection reagent (Invitrogen, USA).

1.3 Cell proliferation assay

Cell proliferation was measured using Cell Titer 96® Aqueous One Solution Reagent (PROMEGA, Madison, USA). In brief, 2 000 cells were plated in each well of a 96-well plate. After cells were transfected with si-PNN or siNC, 20 μL of reagent was added to each well. Absorbance was read at 490 nm after incubation for 4 h.

1.4 RNA preparation, reverse transcription, and qRT-PCR (quantitative real-time PCR) analysis

Total RNA was isolated from cells and tissues using the Trizol reagent (Invitrogen, USA). Total RNA was quantified using spectrophotometric. RNA was reversely transcripted into cDNA using a reverse transcription system (Thermo, USA). Real-time fluorescence quantitative PCR was performed using the

LightCyclerTM (Roche Molecular Biochemicals, USA) and SYBR Green I (Roche, US). qPCR (quantitative real-time PCR) was performed in duplicate with special primers (*PNN* sence: 5′-CCT GTA AAG CAG TCT CAA GCC-3′, antisence: 5′-CGA ATG TTC TCA TCC ACG TTC T-3′; *GAPDH* sence: 5′-ACC CAC TCC TCC ACC TTT GAC-3′, antisence: 5′-TGT TGC TGT AGC CAA ATT CGT T-3′).

1.5 Western blot analysis

For Western blot analysis, 50 μg of each sample were processed as described[17]. Incubate the membrane and specific antibody (1:500) overnight at 4 °C, and then incubate with the secondary antibody (1:2 000) for 1 h at 37 °C. The following antibodies were used: anti-PNN, GAPDH (Mouse, Abcam, Hong Kong China), anti-Cyclin A, Cyclin B1, Cyclin E, CDK2, AKT, p-AKT (Ser473), PI3K, p-PI3K, β-actin (Rabbit, Cell Signaling Technology, MA, USA) and CDK4, Cyclin D1 antibody (Mouse, Cell Signaling Technology, MA, USA). The secondary antibodies were coupled to horseradish peroxidase (Boster, Wuhan, China) and detected by chemiluminescence.

1.6 Immunohistochemistry staining and evaluation

The surgical specimens were fixed in 10% buffered formaldehyde solution, embedded in paraffin according to standard procedures and then cut into 4 μm thick sections in direct series. Histopathological examination was performed using standard hematoxylin-eosin staining. The paraffin-embedded tissue sections were subjected to immunostaining with the use of anti-PNN antibodies (1:500) at 4 °C overnight. The secondary antibody was horseradish peroxidase-goat anti-rabbit IgG (Zhongshan Jinqiao, Beijing, China). Sections were lightly counterstained with hematoxylin and photographs were taken using fluorescence microscopy. According to the percentage of positive cells, less than 5% was 0, between 5% and 25% was I, between 25% and 50% was II, between 50% and 75% was III, and greater than 75% was IV[18].

1.7 Apoptosis analysis

Apoptosis rates were detected using a FITC-labeled Annexin V/PI (propidium iodide) apoptosis detection kit (V13241, Invitrogen, Shanghai, China) following the standard procedure. Briefly, cells were harvested and washed with PBS, resuspended in binding buffer, and incubated with annexin V-FITC/PI in dark for 15 min. Flow cytometry analysis was performed by flow cytometry, and results were determined using Flowjo 7.6 software.

1.8 Fluorescent immunocytochemistry

The stainings of Ki67 and nucleus were observed under confocal microscopy at an excitation of 488 and 405 nm respectively. Cells were fixed with 4% (*W/V*) paraformaldehyde (Aladdin, California, US), permeabilized with 0.1% Triton X-100 in PBS, blocked by 0.5% BSA, incubated with ki67 rabbit mAb conjugated to Alexa Flour 488 (1:500, Cell signaling Technology, MA, USA) in 1% BSA overnight, and DAPI (1 μg/mL) (Sigma, St Louis, MO, USA) was added before the end of incubation. Finally, coverslips were washed twice with PBS and photographed using confocal scanning microscopy.

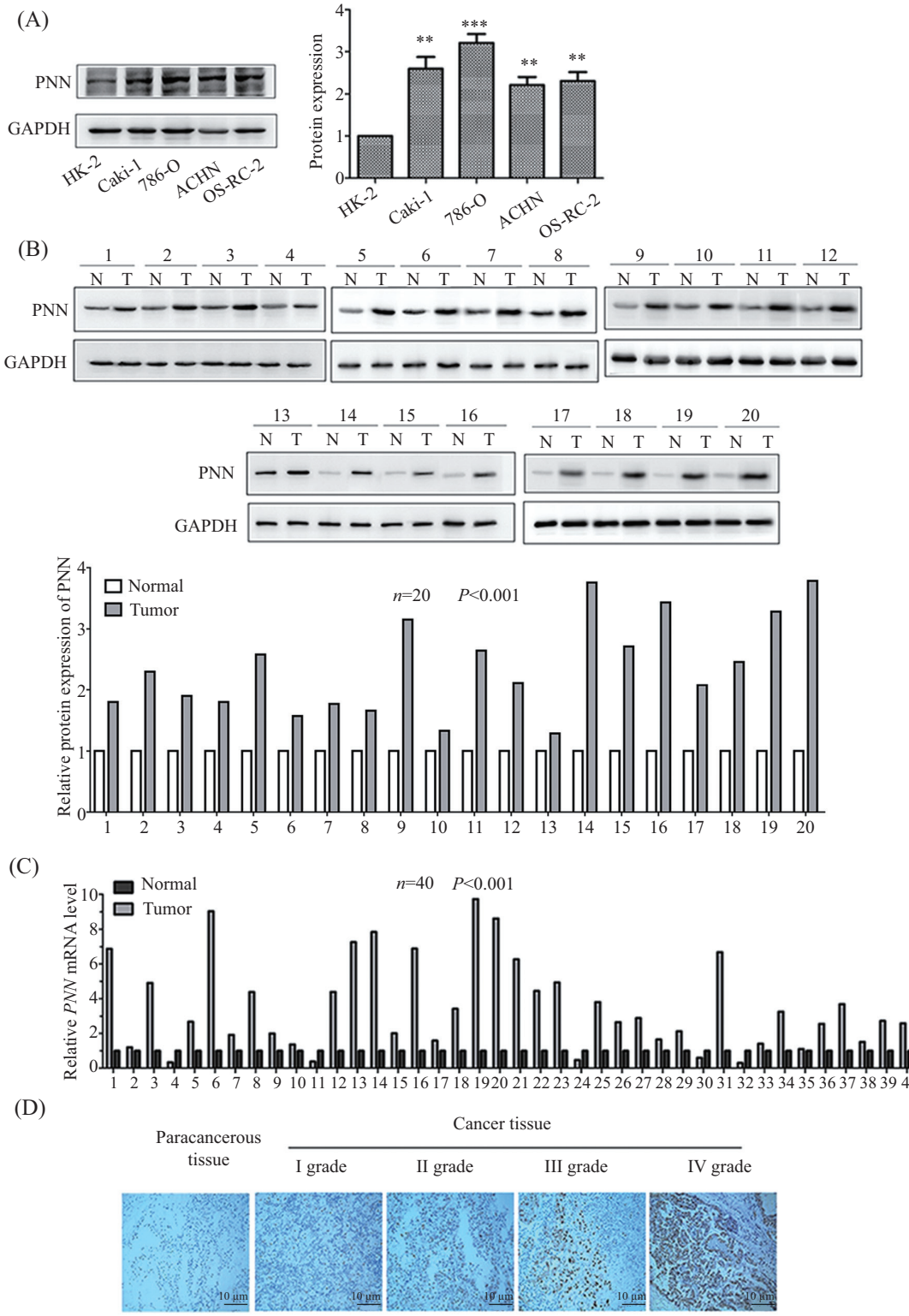
1.9 Statistical analysis

Statistical analyses were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The values between the two groups in Fig.5A was compared with Student's *t*-test. Multiple group comparison was analyzed using One-Way ANOVA with a post-hoc test for subsequent individual group comparisons. Data in Table 1 were analyzed using the χ^2 test. The data were showed as $\bar{x}\pm s$. $P<0.05$ was considered to be statistical significantly.

2 Results

2.1 PNN is overexpressed in human ccRCC cell lines and tissues

To investigate differential expression of PNN between RCC cells and non-carcinoma cells, four carcinoma cell lines (Caki-1, ACHN, OS-RC-2 and 786-O) and immortalized proximal tubule epithelial cell line HK-2 were analyzed. We found that PNN protein expression level was markedly increased in all the RCC cell lines, most notably in Caki-1 and 786-O lines (Fig.1A). Hence 786-O cell line was used in subse-



A: Western blot实验检测正常肾细胞和肾癌细胞(Caki-1、ACHN、OS-RC-2 and 786-O)中PNN蛋白表达情况, ***P*<0.01, ****P*<0.001, 与HK-2细胞比较; B: Western blot实验检测20对肾癌和癌旁组织中PNN蛋白表达水平; C: qRT-PCR实验检测40对肾癌组织和癌旁组织中*PNN* mRNA的表达水平; D: 免疫组化染色实验检测肾癌和癌旁组织中PNN表达水平, 根据表达情况将其分为I-IV期。结果以均值±标准差表示, ****P*< 0.001, 与癌旁组织比较(B、C)。

A: PNN protein expression in normal and RCC cell lines (Caki-1, ACHN, OS-RC-2 and 786-O) was detected by Western blot, ***P*<0.01, ****P*<0.001 compared with HK-2; B: PNN protein expression in tumour tissues and adjacent benign renal tissues from 20 RCC patients; C: *PNN* mRNA expression levels in 40 paired RCC and adjacent benign renal tissues was evaluated by qRT-PCR; D: representative IHC images of benign and cancer renal tissues according to tumour Grade I-IV. Data is represented as $\bar{x}\pm s$, ****P*<0.001 compared with adjacent benign renal tissues (B,C).

图1 PNN在RCC细胞和组织中高表达

Fig.1 Overexpression of PNN in human RCC cell lines and tissues

quent experiments.

Increased expression of PNN protein was detected in 20 paired ccRCC and adjacent benign renal tissues (Fig.1B) and 85% ccRCC samples had higher *PNN* mRNA levels (Fig.1C), compared with adjacent benign renal tissues (n=40).

Performing immunohistochemical analyses in 95 human ccRCC samples, 62 cases (65.3%) showed overexpression of PNN in ccRCC tissues compared with the corresponding benign paracancerous tissues (Table 1). In these clinical samples, moderate to strong cytoplasmic staining was observed according to tumour grade I-IV (Fig.1D).

2.2 PNN promotes ccRCC cell proliferation and cell viability

In order to evaluate the importance of PNN in regulating biological processes in ccRCC cells, we firstly suppressed its expression in 786-O cells using specific siRNAs of PNN. Inhibition of PNN with si-PNN2 significantly suppressed cell proliferation in a time-dependent manner, evident at incubation from 48 to 96 h (Fig.2A). Cell proliferation was examined by immunofluorescent staining using specific antibodies, after si-PNN2 treatment, and the number of Ki-67 positive cell were decreased by 50% compared with mock and siNC group (Fig.2B).

2.3 *PNN* knockdown induces G_0/G_1 arrest

To identify the mechanism underlying the decreased proliferation of RCC cells with *PNN* knockdown, we analyzed cell cycle by flow cytometry in 786-O cells. We observed that si-PNN2 treatment induced cell cycle arrest at G_0/G_1 phase. Cell number in G_0/G_1 phase was increased from 45.26% to 65% (Fig.3A), and Cyclin D1, Cyclin E, CDK2 and CDK4 were significantly decreased, whereas, no marked changes were observed in the expression of Cyclin A and Cyclin B1 (Fig.3B).

2.4 *PNN* knockdown increases chemotherapy drug sunitinib-induced apoptosis in ccRCC cells

As PNN is involved in drug resistance in breast cancer, we tested whether PNN is also involved in the response of 786-O cells to sunitinib. The inhibitory effect of sunitinib on 786-O cell proliferation, although significant, was only slight (Fig.4A), interestingly, highly significant reduction of cell proliferation was shown in cells treated with the combination of sunitinib and si-PNN2 (Fig.4B).

Next, we examined cell apoptosis after the treatment of si-PNN2 and/or sunitinib. After 24 h, the cell percentage showing apoptosis was significantly higher in the si-PNN2 treatment group than in control or siNC group. Indeed, the combination of si-PNN2 and sunitinib further increased the apoptotic cell percentage (P<0.001) (Fig.4C). These results suggested that PNN might reverse the resistance of ccRCC cells to sunitinib.

2.5 PNN activates PI3K/AKT signaling pathway

Previous work suggested that the PI3K/AKT pathway was constitutively activated and played a critical roles in mediating survival and growth in cancer cells. To determine whether the PI3K/AKT signaling pathway was involved in the effect of PNN, we examined AKT phosphorylation, a direct downstream target of PI3K activation. Our data showed that the level of phosphorylated AKT (p-AKT) and PI3K (p-PI3K) was significantly increased in four ccRCC tissues compared with paired benign adjacent renal tissues (Fig.5A). p-AKT and p-PI3K were also markedly decreased in si-

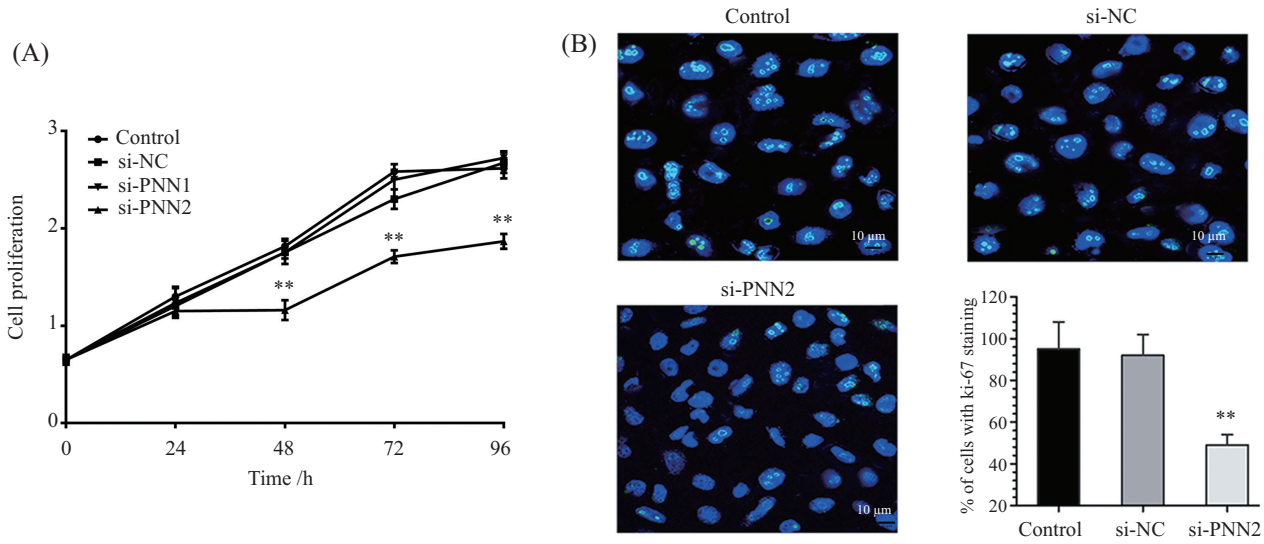
表1 ccRCC组织样本中PNN的表达

Table 1 Expression of PNN in ccRCC samples

类别 Types	高表达 High expression	低表达 Low expression	*P*值 *P* value
Carcinoma	62 (65.3%)	33 (34.7%)	0.001
Paracancerous	39 (41.1%)	56 (58.9%)	

PNN在ccRCC组织中高表达。

PNN is significantly upregulated in ccRCC cancer tissues.

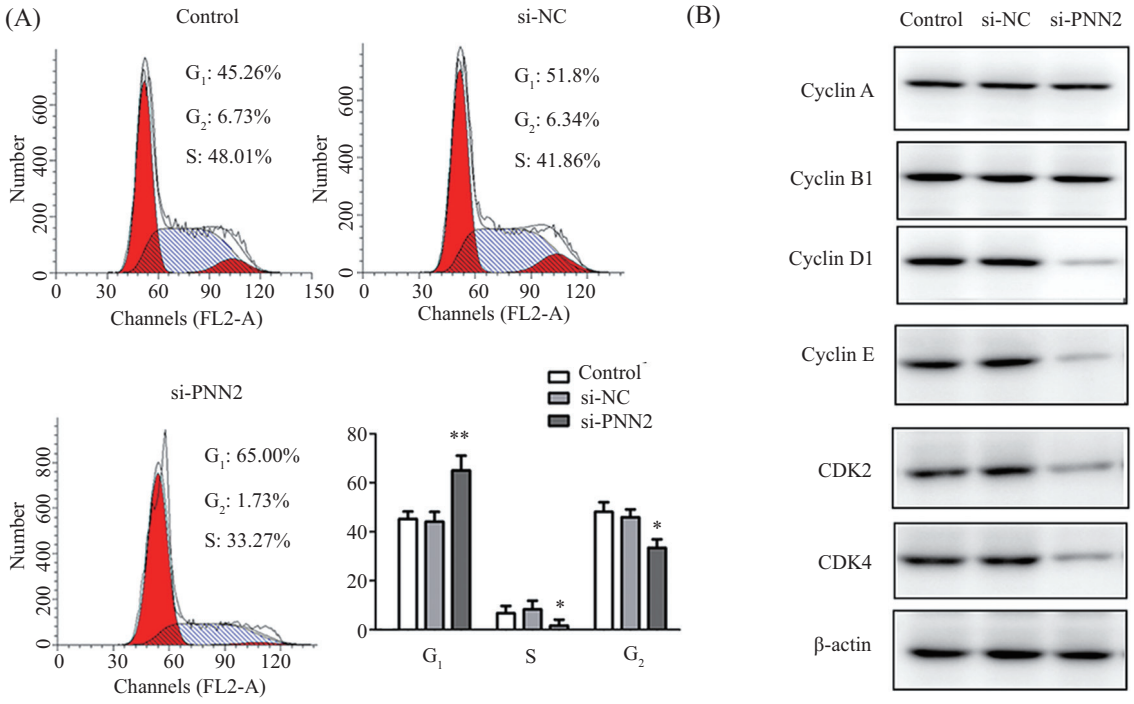


A: 降低PNN水平抑制肾癌细胞增殖; B: 降低PNN表达, 减少Ki-67阳性表达细胞数。结果以均值±标准差表示; **P<0.01与空白组比较。

A: decreasing PNN expression suppressed cell proliferation; B: decreasing PNN expression suppressed the number of Ki-67 positive cells. Data is represented as $\bar{x}\pm s$. **P<0.01 compared with control group.

图2 降低PNN水平抑制786-O细胞增殖

Fig.2 PNN suppression suppresses 786-O ccRCC cell proliferation



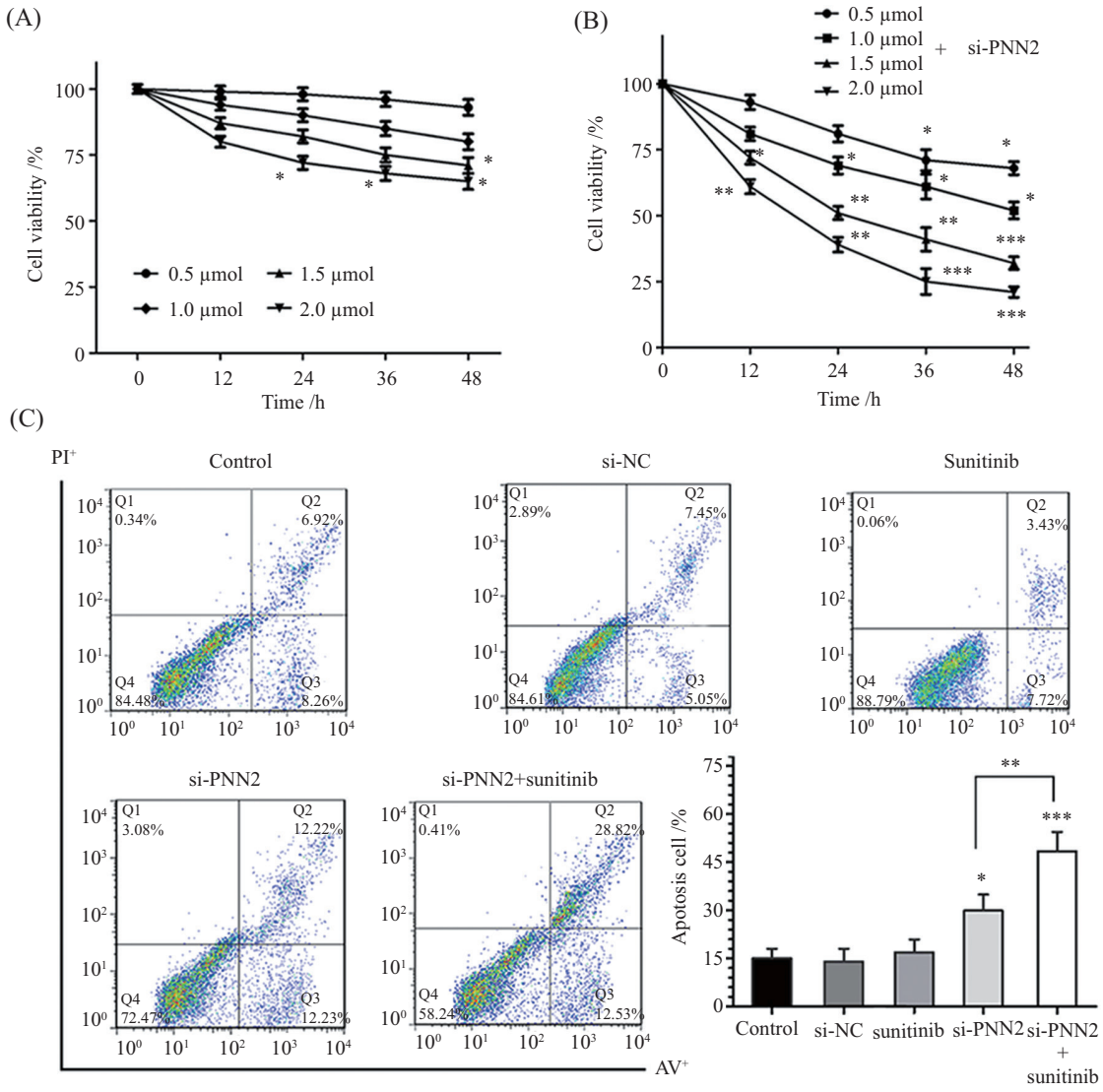
A: si-PNN2处理, 诱导786-O细胞周期G_0/G_1期阻滞; B: si-PNN2处理, 抑制Cyclin D1、Cyclin E以及CDK2、CDK4蛋白的表达。结果以均值±标准差表示。**P<0.05, **P<0.01, 与空白组比较。

A: si-PNN2 treatment induced cell number increased in G_0/G_1 phase. B: si-PNN2 treatment decreased Cyclin D1, Cyclin E, CDK2 and CDK4 protein expression. Data is represented as $\bar{x}\pm s$. *P<0.05, **P<0.01 compared with control group.

图3 降低*PNN*水平诱导786-O细胞周期G_0/G_1期阻滞

Fig.3 Knockdown of *PNN* induces G_0/G_1 arrest in 786-O cells

PNN2 treated 786-O cells. Meanwhile, we used SC79, a chemical specifically activating AKT phosphorylation, to treat the 786-O cells, and found that it reversed the expression of p-AKT and p-PI3K (Fig.5B). And addition of SC79 (10 μmol) also reversed the si-PNN2-induced inhibition of proliferation (Fig.5C) and apop-



A: 舒尼替尼处理, 抑制786-O细胞增殖; B: si-PNN2处理后显著提高舒尼替尼对786-O细胞增殖的抑制作用; C: si-PPN2和1.5μmol舒尼替尼共处理24 h后细胞凋亡率显著高于单独用si-PNN2或舒尼替尼处理。结果以均值±标准差表示。*P<0.05, **P<0.01, ***P<0.001, 与0 h(A、B)或空白组(C)比较。

A: 786-O cell proliferation was inhibited by sunitinib; B: addition of si-PNN2 greatly enhanced the inhibitory effects of sunitinib on 786-O cell proliferation; C: after cells were treated with si-PNN2 and/or 1.5 μmol sunitinib for 24 h, the number of apoptosis cells were significantly higher in cells undergoing both si-PNN2 transfection and sunitinib treatment than those undergoing si-PNN2 or drug treatment alone. Data is represented as $\bar{x}\pm s$. *P<0.05, **P<0.01, ***P<0.001 compared with 0 h (A,B) and control (C).

图4 降低*PNN*水平, 提高786-O对舒尼替尼的敏感

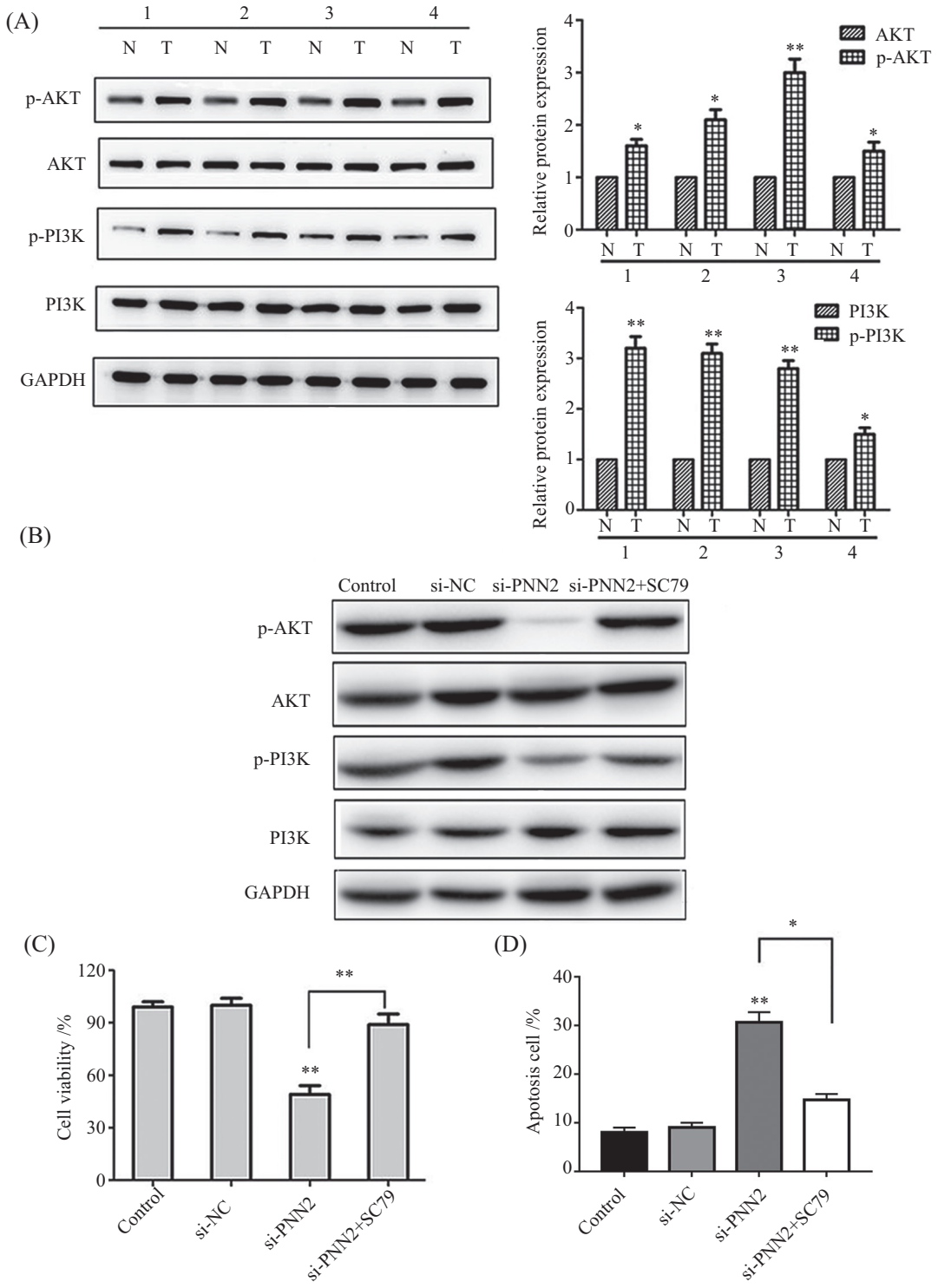
Fig.4 Knockdown of *PNN* sensitizes 786-O cells to sunitinib

tosis (Fig.5D), and similar results were observed in the cell cycle (data not shown).

Therefore, these data suggested that the PI3K/AKT pathway played a critical role in mediating PNN effects on RCC cell proliferation.

3 Discussion

PNN is a dual-location protein found both in the nucleus regulating mRNA alternative splicing[9,17,19] and in desmosomes mediating cell-cell adhesions[20-23].The biological functions of PNN have been mainly discovered in the context of epithelial cell-cell adhesion[24-25] and RNA alternative splicing[26], indicating that it may play a role in cancer progression. In various human cancers, the expression and functions of PNN have been gradually disclosed. PNN is observed to promote cell growth and survival via upregulating BCL-xL expression in human breast cancer cells[27], and PNN



A: ccRCC组织(T)中PI3K/AKT磷酸化水平(p-AKT和p-PI3K)显著高于其配对癌旁组织(N); B: 降低*PNN*水平抑制786-O细胞中的PI3K/AKT磷酸化, 而加入SC79可逆转此现象; C: SC79(10 μmol)升高p-AKT的水平逆转了si-PNN2诱导的细胞增殖抑制; D: SC79(10 μmol)升高p-AKT水平可逆转si-PNN2诱导的细胞凋亡抑制。结果以均值±标准差表示。*P<0.05, **P<0.01, 与癌旁组织(A)或与空白组(B、C)比较。

A: PI3K/AKT phosphorylations (p-AKT and p-PI3K) were significantly increased in ccRCC tumour tissues (T) compared with benign adjacent tissues (N); B: *PNN* knockdown using si-PNN2 suppressed PI3K/AKT phosphorylation in ccRCC 786-O cells, and the addition of SC79 reversed this phenomenon; C: elevating the level of p-AKT by SC79 (10 μmol) reversed si-PNN-induced inhibition of cell proliferation; D: elevating the level of p-AKT by SC79 (10 μmol) reversed si-PNN-induced inhibition of cell apoptosis. Data are represented as $\bar{x}\pm s$. *P<0.05, **P<0.01 compared with adjacent benign renal tissue (A) or control group (B,C).

图5 PNN通过PI3K/AKT通路调控ccRCC细胞增殖

Fig.5 PNN activates ccRCC cell proliferation via PI3K/AKT signaling pathways

contributes to growth of HCC (hepatocellular carcinoma) and resistance to glucose-deprivation-induced apoptosis via maintaining ERK1/2 activation, which can be considered as a potential therapeutic target in HCC[15]. Furthermore, PNN is also found to function as an oncogene in CRC (colorectal cancer)[16].

Herein, we explored and elucidated the function of PNN in ccRCC cell proliferation. When the expressions of PNN in ccRCC tumour tissues and matched benign renal tissue were evaluated, we found higher PNN protein and mRNA expressions in ccRCC tissues. Moreover, in ccRCC 786-O cells, *PNN* knockdown inhibited cell proliferation, causing G_0/G_1 cell cycle arrest. These results were consistent with the previous reports that PNN promoted the proliferation and migration of tumour cells[7,15-16].

In earlier research, to investigate whether *PNN* gene expression was affected in primary tumours and tumour cell lines, *PNN* mRNA levels in four epithelial-derived RCC tumours and a panel of RCC cell lines were examined. Northern blot analyses revealed depressed-levels of *PNN* mRNA levels in two RCC tissues as compared with their corresponding normal tissue controls[28]. In contrast, they also found that in a RCC tumour sample, an aberrant over-expression of PNN with large cytosolic accumulation of immunoreactive material in the undifferentiated tumour cells, while epithelial cells within collecting ducts exhibited normal PNN staining. In a panel of cancer cell lines (promyelocytic leukemia HL-60, HeLa cell S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549 and melanoma G361 cells), depressed *PNN* mRNA level was shown in two of these eight cell types (Burkitt's lymphoma Raji and melanoma G361 cells)[28]. Therefore, it seemed that PNN gene and protein expressions may be different in tumorigenesis of certain cancers. Especially, the relationship between PNN and RCC subtype needs further exploration.

Aberrent activity of PI3K/AKT pathway plays a key role in RCC cell proliferation[29-33], however, very limited information is available regarding the upstream molecular mechanisms causing constitutive PI3K activation[34-36]. In the present study, we provided molecular evidences that PNN was a regulator of PI3K/AKT phosphorylation, and elevation of AKT phosphorylation could rescue both the inhibition of cell proliferation and increased apoptosis induced by *PNN* knockdown.

Collectively, our results clearly identified the high expression of PNN in ccRCC tumour tissues. PNN upregulation promotes proliferation and reduces sunitinib sensitivity in ccRCC by activating the PI3K/AKT signaling pathway. As PNN is a cell-surface molecular which can be easily targeted by antibodies, our data suggested that PNN is a potential therapeutic target for ccRCC treatment.

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