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KAI1 reverses the epithelial-mesenchymal transition in human pancreatic cancer cells

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ABSTRACT

Background: Epithelial-mesenchymal transition (EMT) plays an important role in pancreatic cancer (PC). In the present study, we investigated the effects of KAI1 gene overexpression on the EMT of human PC cell lines, MIA PaCa-2 and PACN-1.

Methods: Plasmids overexpressing KAI1 and pCMV were transfected into MIA PaCa-2 and PACN-1 cells, respectively. After selection of differently transfected cells by G418, KAI1 protein levels were examined by Western blotting, and transfected cells were renamed as MIA PaCa-2-K, MIA PaCa-2-p, PACN-1-K and PACN-1-p. Wound healing and Transwell migration assays were then performed comparing the two groups of cells. EMT-related markers were analyzed by Western blotting.

Results: The percentage of wound closure significantly decreased in MIA PaCa-2-K cells compared with MIA PaCa-2-p and MIA PaCa-2 cells after 24, 48 and 72 h ($P < 0.05$). In PACN-1-K cells, the percentage of wound closure significantly decreased as well ($P < 0.05$). Numbers of invading MIA PaCa-2, MIA PaCa-2-p and MIA PaCa-2-K cells were determined as 48.0 ± 15.4, 50.0 ± 12.4, and 12.0 ± 3.8, respectively. The corresponding numbers of invading PACN-1, PACN-1-p and PACN-1-K cells were 29.0 ± 10.6, 31.0 ± 11.4, and 8.0 ± 4.2, respectively. KAI1 overexpression induced a significant upregulation of E-cadherin and also significant downregulation of Snail, vimentin, matrix metalloproteinase 2 (MMP2) and MMP9 (all $P < 0.05$) in PC cells.

Conclusions: KAI1 reversed EMT-related marker expression and inhibited migration and invasion of PC cells. Thus, KAI1 might represent a novel potential therapeutic target for PC.

Introduction

Pancreatic cancer (PC) is the seventh most common type of cancer worldwide, causing more than 300 000 deaths worldwide every year [1]. PC occurs most often in developed countries, which contributed about 70% of new cases in 2012 [2]. Current PC therapies, which include chemotherapy, surgical resection, and radiotherapy, are not satisfactory, and the risk of tumor recurrence is high. The mechanistic study of PC metastasis may identify new therapeutic targets and improve the prognosis of PC patients.

The epithelial-mesenchymal transition (EMT) is a process during which epithelial cells lose their epithelial characteristics (e.g., apical-basal polarity and cell-cell adhesion) and obtain mesenchymal characteristics (e.g., invasiveness, motility, and resistance to cell death) [3,4]. Increasing evidence suggests that the EMT plays a critical role in tumoral invasion, metastasis, and resistance to radiotherapy and chemotherapy [5]. EMT is essential for PC metastases [6–8]. During EMT, a number of molecular alterations occur, including upregulation of mesenchymal markers (e.g., N-cadherin and vimentin) and downregulation of epithelial markers (e.g., E-cadherin) [9].

KAI1 is a member of the transmembrane 4 super family which regulates many critical biological events, including cell signaling, differentiation, motility and fertilization [10–12]. Previous research [10–12] demonstrated that KAI1 is an important suppressor of metastasis in PC and that down-regulated expression of KAI1 is closely associated with PC metastasis. Clinicopathological studies showed that low KAI1 expression in PC tissue is closely correlated with the depth of PC cell invasion and the presence of lymph node metastasis [12]. Although the ability of invasion of PC cells is significantly attenuated by overexpression of KAI1, its

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potential role in EMT and the underlying mechanisms of KAI1 control over EMT in PC have not yet been studied. The present study aimed to evaluate the effects of KAI1 transfection on the EMT of MIA PaCa-2 and PACN-1 cells, and explore some of the related mechanisms. This may provide a rational for KAI1 as a new therapeutic target for PC.

Methods

Cell lines and cell culture condition

MIA PaCa-2 and PACN-1, human PC cell lines, were ordered from the Institute of Cell Biology of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (GE Healthcare Life Sciences, Logan, UT, USA), which was supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L), penicillin G (100 U/mL), and streptomycin (0.1 mg/mL) in an incubator (37 °C, 5% CO_2, and 95% humidity). Cell lysates were harvested after 24 h for Western blotting.

Plasmids and transfection

KAI1 cDNA was cloned into a pCMV-KAI1 plasmid vector obtained from Prof. Jin-Tang Dong (School of Medicine, Emory University, Atlanta, GA, USA) as a gift. The cells were transfected with plasmids according to our previous study [12]. PC cells with KAI1 overexpression and control cells were constructed by transfection with pCMV-KAI1 and an empty pCMV vector and renamed MIA PaCa-2-K and MIA PaCa-2-p, PACN-1-K and PACN-1-p, respectively.

Wound healing assay

The migratory ability of stably transfected cells was assessed by a wound healing assay. Cells were harvested and seeded at a density of 6×10^5 cells/well into a 6-well plate. Upon confluence, the cells were serum deprived for 6 h, and wound areas were then created using 200 μL pipette tips. Thereafter, the cultures were maintained in serum-free DMEM. Images of the wounds were recorded under a phase-contrast microscope (Olympus, Hamburg, Germany) at different time (e.g., 0, 24, 48, and 72 h) after wounding. Cells migrated into the wound areas were analyzed by the Image J software (Rawak Software, Inc. Germany).

Invasion assay

The invasive properties of the PC cells were demonstrated by an *in vitro* matrigel transwell assay (Corning, NY, USA). After transfection, 2×10^4 cells in 200 μL of FBS-free DMEM were planted into the upper chambers whereas the lower chambers were supplied with DMEM containing 20% FBS. The plates were then incubated for 24 h. Cells that invaded were fixed in 10% (vol/vol) formalin for the hematoxylin and eosin (HE) staining. Six random images of the invaded cells were recorded by a light microscope (Olympus, Hamburg, Germany) (40 × magnification) in the same focal plane. The number of invaded cells per image was counted using Image J software (Rawak Software, Inc. Germany).

Western blotting analysis

Cells were harvested in a lysis buffer. The concentrations of protein were quantified by the BCA protein assay kit (Solarbio Co., Ltd., Beijing, China). After denaturation, samples were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and proteins were separated. The proteins were wet transferred to a polyvinylidene fluoride (PVDF) membrane. Skimmed milk (5%) was used for membrane blockage. The membrane was incubated with primary antibodies (4 °C, overnight), and was rinsed by Tris-buffered saline containing 0.1% Tween 20 buffer three times (5 min each wash). The secondary antibody was then added at the recommended dilution. After rocking gently for 2 h at room temperature, the membrane was washed the same as the wash of the primary antibodies. Electro-Chemi-Luminescence kits (Solarbio Co., Ltd., Beijing, China) were used for signal detection. β-actin was used as the reference protein.

Antibodies

Rabbit polyclonal antibody against KAI1 (1:200 dilution), matrix metalloproteinase 2 (MMP2) (1:500 dilution), MMP9 (1:500 dilution), E-cadherin (1:500 dilution), Snail (1:500 dilution), vimentin (1:500 dilution), and β-actin (1:500 dilution) were all purchased from Santa Cruz, Inc. (Dallas, TX, USA).

Statistical analysis

Statistical analysis was performed by SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The experimental data were presented as the mean ± SD of at least three replicates and analyzed using the Student's paired *t*-test. A P value <0.05 was considered statistically significant.

Results

KAI1 decreased the migration ability of PC cell

The expression level of KAI1 was significantly increased in stably transfected MIA PaCa-2-K and PACN-1-K cells as compared to that of MIA PaCa-2-p, MIA PaCa-2 and PACN-1-p, PACN-1 cells ($P < 0.05$) (Fig. 1(A)). MIA PaCa-2-K and PACN-1-K cells did not undergo typical EMT processes, such as scattering and acquisition of a spindle-shaped, fibroblastic phenotype, but in contrast exhibited a slightly EMT phenotype compared with that of MIA PaCa-2-p, MIA PaCa-2, PACN-1-p and PACN-1 cells (Fig. 1(B)).

Regarding the effect of KAI1 on PC cell migration, our results (Fig. 2) showed that the percentage of wound closure were significantly decreased in MIA PaCa-2-K and PACN-1-K cells compared to that of MIA PaCa-2-p, MIA PaCa-2 and PACN-1-p, PACN-1 cells after 24, 48, and 72 h ($P < 0.05$).

KAI1 decreased PC cell invasion in vitro

The Matrigel transwell experiment showed that KAI1 overexpression influenced the invasion capacity of the MIA PaCa-2 and PACN-1 cell line (Fig. 3). Based on counts of three repeated experiments of cell invasion, the numbers of MIA PaCa-2 (control), MIA PaCa-2-p, and MIA PaCa-2-K cells were 48.0 ± 15.4, 50.0 ± 12.4, and 12.0 ± 3.8, the numbers of PACN-1, PACN-1-p, and PACN-1-K cells were 29.0 ± 10.6, 31.0 ± 11.4, and 8.0 ± 4.2, respectively. The MIA PaCa-2-K and PACN-1-K cells showed significantly reduced invasiveness as compared to that of MIA PaCa-2-p, MIA PaCa-2 and PACN-1-p, PACN-1 cells ($P < 0.05$). These data showed that KAI1 overexpression reduced the invasive capability of PC cells.

KAI1 regulated EMT-related factors in PC cells

To explore the possible mechanism of KAI1 in promoting the invasion and migration capacity of PC cells, the markers of EMT was detected. As shown in Fig. 4, KAI1 overexpression promoted E-cadherin expression, whereas expressions of Snail, vimentin, MMP2 and MMP9 expression (all $P < 0.05$) were reduced.

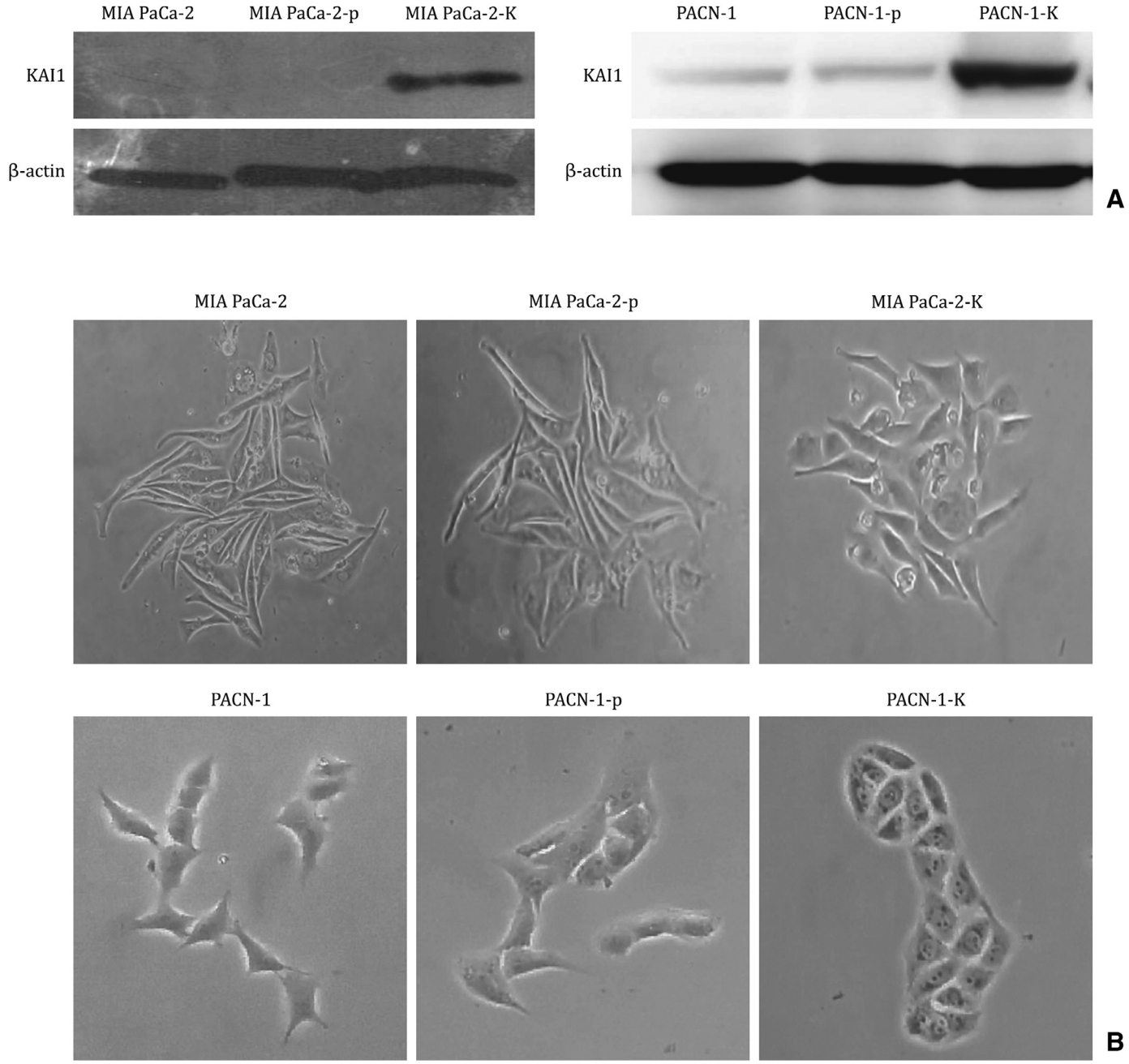

Fig. 1. (**A**): Western blotting analyses of KAI1 expression with no detectable KAI1 expression in MIA PaCa-2 and PACN-1 PC cells or pCMV transfected cells, but clearly positive expression of KAI1 protein in MIA PaCa-2 and PACN-1 cells transfected with pCMV-KAI1. (**B**): Phase-contrast photomicrographs demonstrated that MIA PaCa-2-K and PACN-1-K cells did not undergo EMT, compared with EMT-like morphology of MIA PaCa-2-p, MIA PaCa-2 and PACN-1-p, PACN-1 cells (original magnification × 200).

Discussion

The process of tumor metastasis includes four main steps: (i) loss of homotypic cell adhesion, which enhances the capacity of cells to invade neighboring tissue; (ii) invasion of the vascular or lymphatic tube wall of the circulatory system; (iii) adherence of tumoral cells to endothelial cells of vascular or lymphatic vessels; and (iv) proliferation in the new environment. Previous research concluded that activation of the EMT program results in the production of circulating tumor cells, which proliferates to form distant metastases [13]. Previous data also indicated that KAI1 inhibited cell migration in human PC [14]. In the present study, the EMT was clearly reversed in the human PC MIA PaCa-2 and PACN-1 cell lines overexpressing KAI1 as shown by EMT-like morphological changes, scratch assay and transwell chamber experiments. KAI1 knockdown experiments were not carried out in our study because MIA PaCa-2 and PACN-1 do not express KAI1 protein [12,14–17]. The effect of *KAI1* gene on the proliferation of PC cells in general remains unclear, but the overexpression of *KAI1* gene does not significantly affect the proliferation of PC cells [12]. The experimental conditions in this study were consistent with our previous study [12]. As such, we considered that slightly reduced proliferation of PC cells only moderately affected the scratch and transwell experiments.

The EMT process involves many molecules, which play an important role in the different stages of tumoral metastasis and

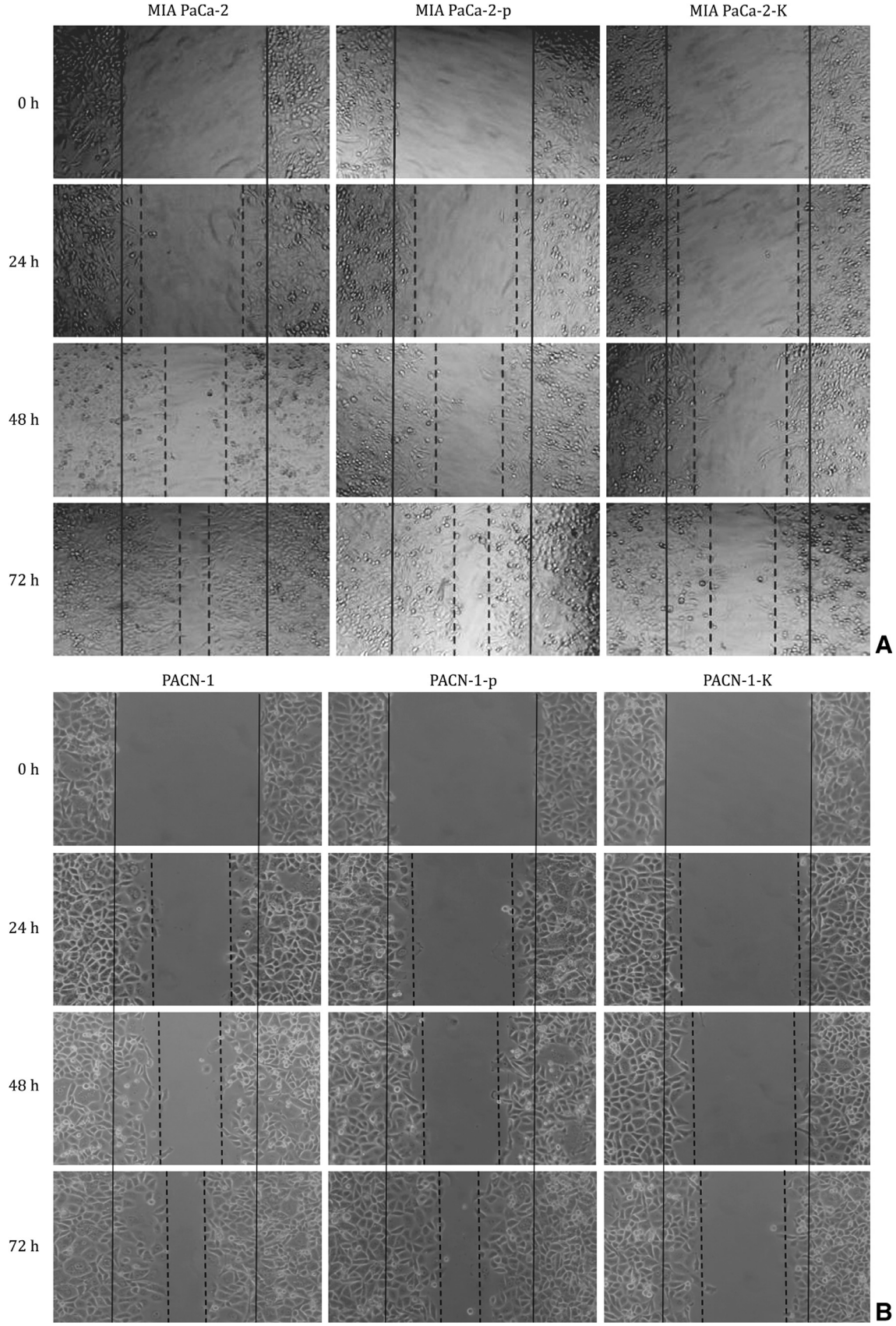

Fig. 2. Wound healing assay, showing a significant reduction in the cell count in the scratched area in the MIA PaCa-2-K and PACN-1-K group as compared with the MIA PaCa-2-p, MIA PaCa-2 (**A**) and PACN-1-p, PACN-1 groups (**B**) after 24, 48, and 72 h, respectively (original magnification × 100).

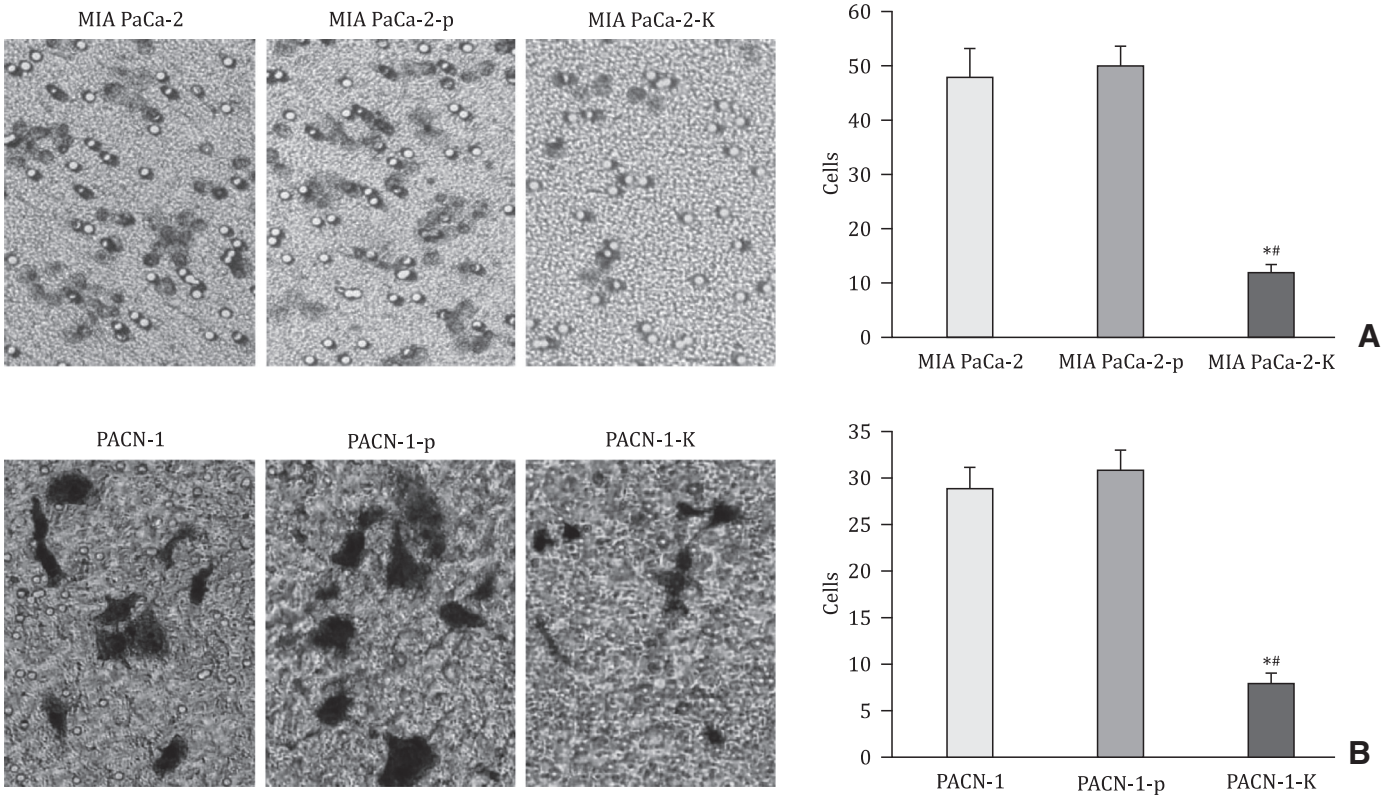

Fig. 3. In a transwell assay, cells that invaded were captured, stained, and counted after 24 h. Random horizons were selected from three replicates. The number of invaded cells in the MIA PaCa-2-K and PACN-1-K group was significantly lower than that in the MIA PaCa-2-p, MIA PaCa-2 (**A**) and PACN-1-p, PACN-1 (**B**) groups (original magnification × 40).
$^{*}P < 0.05$, compared to MIA PaCa-2 or PACN-1 group; $^{\#}P < 0.05$, compared to MIA PaCa-2-p or PACN-1-p group.

invasion in cancer, including PC [18]. E-cadherin is a calcium-dependent transmembrane glycoprotein with a main function of maintaining cell morphology, motility, and adhesion [19]. As one of the most important markers of EMT, E-cadherin can be found in both normal tissues and tumor tissues [20]. Low expression, uneven expression, and even absent expression of E-cadherin have also been reported in PC, with the expression intensity negatively correlated with the degree of tumoral differentiation [21]. E-cadherin is a key mediator in the EMT process of PC cells. First, downregulation of E-cadherin leads to the decrease of intercellular adhesion junction and polarity, the PC cell changes from epithelioid to interstitial, resulting in the release of PC cells from *in situ* carcinomas. Thus, the decrease in cell adhesion aids the invasion of tumoral cells into surrounding tissue. The present results revealed an increased E-cadherin protein expression in PC cells with high KAI1 expression. Previous studies reported low expression of KAIl was associated with low expression of E-cadherin in many kinds of tumor metastases [22–24] and indicated that KAI1 and E-cadherin may have a synergistic effect on inhibiting the metastasis of cancer cells. Another study indicated that KAI1 mediated the β-catenin signaling pathway, thereby enhancing the function of E-cadherin-mediated cell adhesion in cancer [25]. Therefore, regulating E-cadherin level may be one mechanism by which KAI1 reverse the EMT in PC.

Our study also indicated that KAI1 overexpression inhibited the expression of Snail, vimentin, MMP2 and MMP9 in MIA PaCa-2 and PACN-1 cells. The expression of Snail, a zinc finger DNA-binding protein, in PC is closely correlated with lymph node and distant metastases [26]. Its expression initiates EMT by activating the transcription of mesenchymal markers and inhibiting the expression of epithelial markers [27]. Vimentin, a type III intermediate filament protein, is normally expressed in mesenchymal- and mesodermal-derived cells but not in normal epithelial cells. Vimentin shows a highly positive expression rate in pancreatic ductal adenocarcinoma and a close relationship with the degree of PC differentiation and the prognosis of patients [28,29]. It plays an important role in the process of EMT [30]. MMPs, a group of Zn^{2+}-dependent proteolytic enzymes, belong to a protein family involved in the degradation of type IV collagenases [31]. Previous research reported high level of MMP2 and MMP9 in a number of highly metastatic tumor cells [32]. By degrading and reshaping the extracellular matrix, MMPs contribute to the EMT and infiltration of tumor cells into the normal tissues, including blood vessels and lymphatic vessels. Our present study demonstrated that KAI1 inhibited Snail, vimentin, MMP2 and MMP9 expression, thereby further confirming its role as a cancer suppressor gene, with multiple regulatory mechanisms in PC. However, regulatory mechanisms underlying the effects of KAI1 on EMT factors remain unclear. STAT3 phosphorylation is decreased by KAI1 in MIA PaCa-2 cells [15], and STAT3 overexpression enhances EMT [33], suggesting that the STAT3 pathway may be involved in the inhibitory effects of KAI1 on EMT in PC and should be assessed in future studies.

In conclusion, overexpression of KAI1 prohibits PC cell migration and invasion and regulates the expression of EMT-related factors in PC cells resulting in a less malignant phenotype. Targeting KAI1 may represent a previously unidentified strategy for PC.

Contributors

LX and GXZ proposed the study. LX and CJ performed the research and wrote the first draft. LX and LHY collected and analyzed

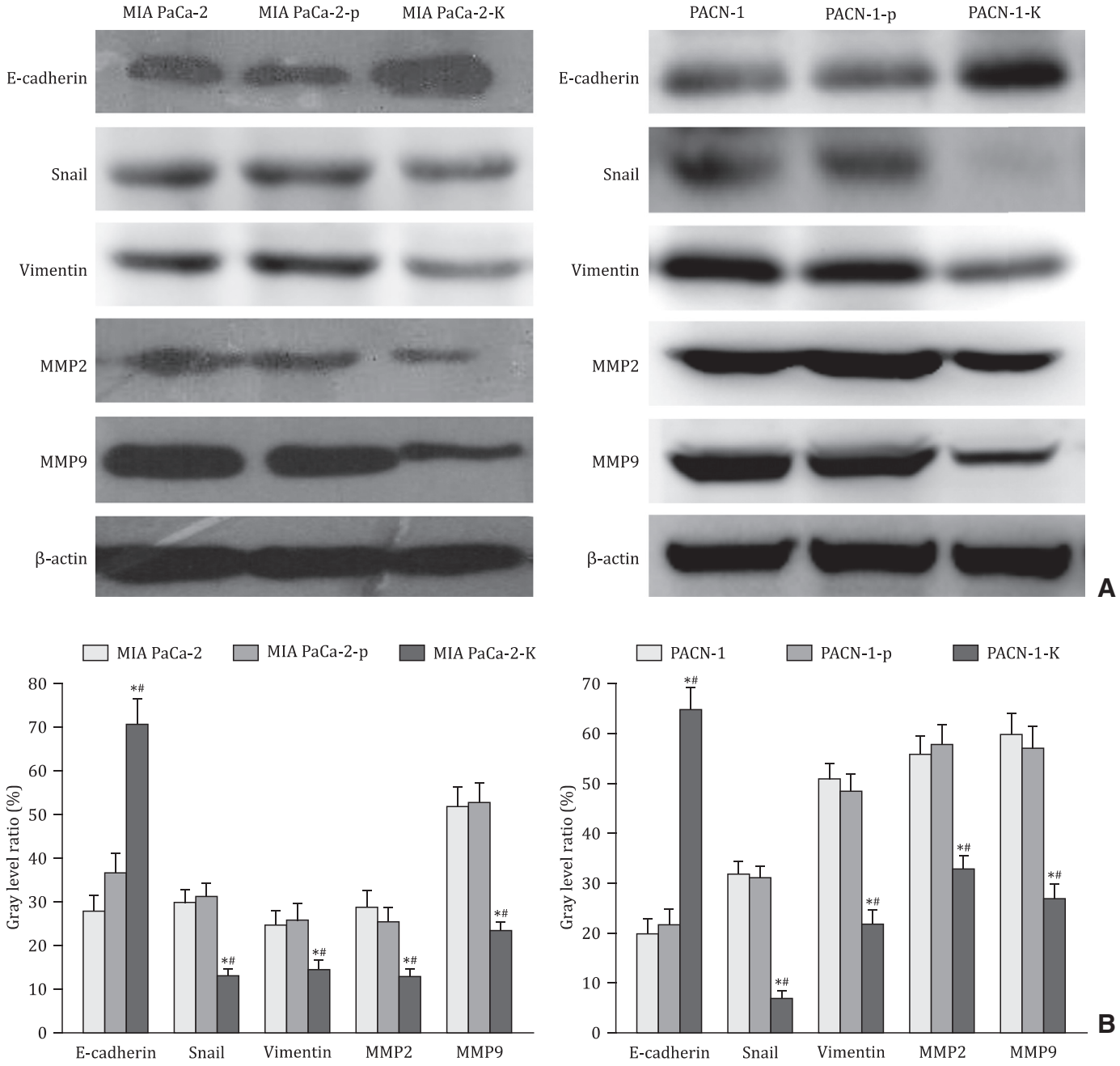

Fig. 4. Results of Western blotting, showing that overexpression of KAI1 altered the expression of EMT-related proteins. (**A**): The expression level of E-cadherin was increased, whereas those of Snail, vimentin, MMP2 and MMP9 were decreased in the MIA PaCa-2-K and PACN-1-K cells. (**B**): The expression of E-cadherin, Snail, vimentin, MMP2, and MMP9 were significantly different in the two respective groups of cells as assessed by densitometric analyses.
$^{*}P < 0.05$, compared to MIA PaCa-2 or PACN-1 group; $^{\#}P < 0.05$, compared to MIA PaCa-2-p or PACN-1-p group.

the data. All authors contributed to the design and interpretation of the study and to further drafts. GXZ is the guarantor.

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Ethical approval

Not needed.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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