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S100A14 promotes progression and gemcitabine resistance in pancreatic cancer

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ABSTRACT

S100 calcium binding protein A14 (S100A14) plays an important role in the progression of several types of cancer. However, its roles in pancreatic ductal adenocarcinoma (PDAC) are largely unexplored. Here, we characterized the functional roles of S100A14 in the progression and chemoresistance of PDAC. Gene expression microarray identified that S100A14 was significantly highly expressed in four pairs of human PDAC tumor compared with corresponding non-tumor tissues genes. Quantitative reverse transcription PCR (qRT-PCR), western blotting and immunohistochemical staining (IHC) showed that S100A14 was frequently overexpressed in PDAC cell lines and tissues. Moreover, expression level of S100A14 was positively correlated to advanced cancer stages. Further, Kaplan-Meier survival analysis suggested that PDAC patients with low S100A14 expression had longer overall survival in TCGA PDAC datasets. Transient overexpressing of S100A14 promoted cell proliferation, anchorage-independent colony formation, cell migration and invasion in cell lines with low endogenous S100A14 levels, while transient silencing of S100A14 inhibited cell proliferation, anchorage-independent colony formation, cell migration and invasion in cell lines with high endogenous S100A14 levels. Persistent knockdown of S100A14 by transducing shRNAs carrying lentivirus inhibited subcutaneous tumor formation in nude mice, and sensitized the PDAC cells to gemcitabine treatment. Taken together, S100A14 exhibited oncogenic properties by promoting cell proliferation, transformation, migration and invasion, and enhanced in vivo tumor growth. More importantly, inhibition of S100A14 could effectively abrogate the cancerous properties of the PDAC cells. Our study indicated that S100A14 was a valuable target for the development of therapeutic strategy, as well as a diagnostic and prognosis biomarker for PDAC patients.

Introduction

Pancreatic cancer is the fourth leading cause of death from malignancies in the United States, with 5-year survival rate below 5%. The overall benefit of therapeutic treatment against pancreatic cancer has no improvement for more than 30 years [1,2]. Pancreatic ductal adenocarcinoma (PDAC) accounts for 85–90% of all pancreatic neoplasms. Its poor outcomes are partly owing to the delay in disease diagnosis [3]. As a result, the development of early detection for PDAC is urgently needed.

S100 proteins, which belong to a large family of the EF-hand calcium-binding proteins, can modulate cellular responses by functioning both as intracellular and extracellular factors [4]. They exhibit a large number of functions by interacting with specific target proteins responsible for the regulation of tumorigenic processes such as cell proliferation, metastasis, angiogenesis and immune evasion [4–6]. Dysregulated expression of S100 proteins has been reported in various cancers [7–9]. For instances, S100 family members S100A4, S100A16 and S100P have been involved in tumor invasion and metastasis in melanoma, prostate cancer and colon adenocarcinoma [10–12].

S100A14, located on chromosome 1q21, is another S100 calcium-binding protein reported to be frequently associated with cancer development [13]. It has been proved that S100A14 was differentially expressed in various cancer types. For example, it was overexpressed in breast [14], liver [15] and cervical cancer [16], meanwhile it was found downregulated in rectal, kidney, colon and esophageal malignancies [13]. Studies showed that S100A14

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induced the deregulation of critical signaling pathways in tumors. Overexpression of S100A14 in breast cancer functioned as a modulator of HER signaling [14]. S100A14 also played vital roles in epithelial ovarian tumors through the regulation of PI3K/AKT pathway [17]. Overexpression of S100A14 was associated with poor prognosis of ovarian cancer [17], lung cancer18 and breast cancer [19]. S100A14 expression strongly correlated with poor differentiation, metastasis, stage, smoking, and EGFR mutation in lung cancer [18]. It could be secreted by cells of different origin, and it was reported that elevated S100A14 secretion contributed to cancer development. The differential secreted level of S100A14 was observed in non-small cell lung cancer cells, and high serum S100A14 level was related to distal metastasis [18]. However, the expression and roles of S100A14 in PDAC were still largely unknown. In this study, we aimed to study the expression pattern of S100A14 and characterize the functional roles of S100A14 in the progression and chemoresistance of PDAC.

Material and methods

Clinical samples and cell culture

Ten pairs of PDAC tumor tissues and surrounding normal tissues were obtained from patients who underwent pancreatic resection at the Prince of Wales Hospital. All specimens of PDAC patients were fixed and embedded into paraffin. Cell lines: Capan2, Panc1, CFPAC1, SW1990 and 293T were maintained in our laboratory. They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) or RPMI-1640 (Sigma) supplemented with 100 unit/ml penicillin/streptomycin (Invitrogen, Carlsbad, California, USA) and 100 mL/L fetal bovine serum (Hyclone®, Perbio, Thermo Fisher Scientific, Cramlington, UK) in a humidified incubator at 37 °C supplied with 50 mL/L CO_2. Human pancreatic ductal epithelial (HPDE) cell line was a gift from Dr Ming-Sound Tsao (University Health Network, Ontario Cancer Institute and Princess Margaret Hospital Site, Toronto, CA) and was maintained according to the publications [20,21]. Gemcitabine (Sigma) was dissolved in water to have a stock solution of 10 mM. Cells were treated with gemcitabine at designated doses and durations.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) and was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Invitrogen). qRT-PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, ABI, USA). GAPDH was used to normalize the quantity of cDNA. Each assay was performed in triplicate and expressed as the mean ± SD. The primers used for the PCR analysis were as follows: for S100A14 forward 5′-CCTCATCAAGAACTTTCACCAGTA-3′, S100A14 reverse 5′-GGTTGGCAATTTTCTCTTCCAGG-3′, GAPDH forward 5′-TGCCTCCTGCACCACCAACT-3′, GAPDH reverse 5′-CCCGTTCAGCTCAGGGATGA-3'. The relative gene expression in cells was determined using the comparative delta-delta CT method ($2^{-\Delta\Delta Ct}$).

Immunohistochemical staining (IHC) in tissue microarray

IHC staining of S100A14 was conducted on tissue microarray consisting of 175 PDAC cases and 27 normal cases. IHC staining was carried out using Histostain-Plus kit according to manufacturer's protocol. Briefly, antigen retrieval was conducted by heating the sections in boiling sodium citrate buffer for 20 min. After 3% hydrogen peroxide and BSA blocking, the tissues were incubated with anti-S100A14 (Proteintech, Chicago, IL) at 4 °C overnight. After washing, the tissues were incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). IHC signal was developed by DAB substrate, and counter-stained by haematoxylin. Three random fields at 200x magnification were captured per sections for evaluation. Scoring of the IHC was based on the percentage of positive cells and staining intensity under a light microscope. Four categories (0, 1+, 2+ and 3+) were denoted as negative, weak, moderate and strong respectively, and final score was calculated by averaging the score of three fields.

Western blot analysis

Cells were lysed in 1x RIPA buffer with 1 mM PMSF and 1x complete protease inhibitor cocktail (Roche, Basel, Switzerland). Western blottings were conducted under standard procedures. Briefly, Protein lysates were separated by SDS-PAGE, and electrophoretically transferred to PVDF membrane from MilliporeTM with nominal pore size of 0.22 μm. After blocking, the membrane was incubated at 4 °C overnight with anti-S100A14 (Proteintech) or β-Actin (Santa Cruz Biotechnology) diluted in non-fat dry-milk (Santa Cruz Biotechnology), followed by HRP-labeled secondary (Santa Cruz Biotechnology) antibody incubation. Chemiluminescence signal was developed by ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Munich, Germany).

S100A14 overexpression and knockdown

For S100A14 overexpression, full-length S100A14 cDNA was cloned into pcDNA3.1 to construct the S100A14 expression vector pcDNA3.1-S100A14. The empty pcDNA3.1 vector was used as control. CFPAC-1 and Panc1 Cells ($1–2 \times 10^5$ cells/well) were transfected with pcDNA3.1 or pcDNA3.1-S100A14 using Lipofectamine™ 3000 reagent (Invitrogen) following the manufacturer's protocol.

For S100A14 knockdown, the S100A14 shRNA and scrambled control shRNA were inserted into human H1 promoter–containing pBluescript SK(+) plasmid (pH1) after annealing. The sense sequence of S100A14 shRNA was 5′-GATCTATGGGAAATGATTTGAATAATCGAAATTATTCAAATCATTTCCCATTTTTG-3′ and the antisense was 5′-AATTCAAAAATGGGAAATGATTTGAATAATTTCGATTATTCAAATCATTTCCCATA-3′. We then subcloned the H1-shRNA cassettes into the lentiviral vector pLUNIG. Lentiviral particles expressing S100A14 shRNAs were packaged for permanent knockdown of S100A14 according to our protocol [22]. Capan2 and SW1990 cells were transduced with lentivirus containing shRNAs targeting S100A14 (sh-S100A14) in the presence of 8 μg/ml polybrene (Sigma). Lentivirus with scramble shRNA (sh-SCR) was used as negative control.

For transient knockdown of S100A14, CAPAN2 and SW1990 cells ($1–2 \times 10^4$ cells/well) plated in 12-well plates were transfected with siRNAs targeting S100A14 (GenePharma, Shanghai, China) using Lipofectamine™ 3000 reagent (Invitrogen) following the manufacturer's protocol. The sequence of S100A14 siRNAs were si-S100A14(1) sense: 5′-AACGCAGAGGAUGCUCAGGAAdTdT-′3/antisense: 3′-dTdTUUCCUGAGCAUCCUCUGCGUU-5′; si-S100A14(2) sense:5′-AUGGGAAAUGAUUUGAAUAAUdTdT-3′/antisense: 3′-dTdTAUUAUUCAAAUCAUUUCCCAU-5′ (GenePharma, Shanghai, CN). A negative control siRNAs duplex were transfected as a control.

Cell proliferative assay

Cell proliferative rate was measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. Cells were plated on 96-well plates ($2–5 \times 10^3$ cells/well). At designated time point, cells were then incubated with 2 mg/ml of MTT solution for 2 h and the resulting formazan product was dissolved in DMSO for O.D. measurement at 570 nm. The experiment

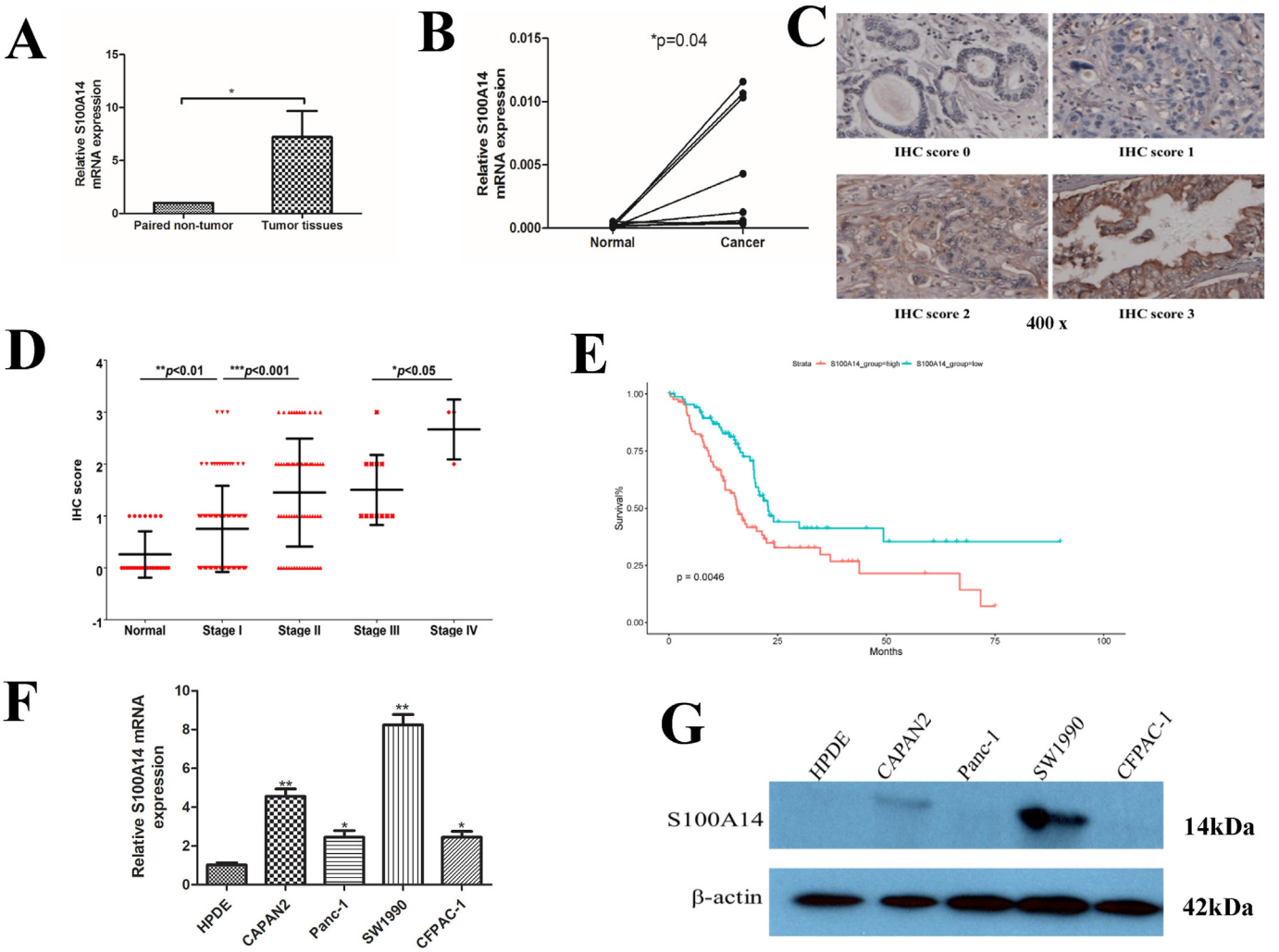

Fig. 1. S100A14 was highly expressed in human pancreatic tissue specimens and cancer cells, and its expression correlated with increase of cancer stages of disease. **A**: Global gene expression microarray analysis in 4 pairs of PDAC tumor and corresponding non-tumor tissues. S100A14 was upregulated in all 4 tumor tissues. **B**: S100A14 mRNA was higher in human PDAC tumor tissues compared to matched non-tumor tissues by qRT-PCR. **C**: S100A14 protein level was higher in PDAC tissues compared to non-tumor tissues by IHC. Moreover, the abundance of S100A14 was positively correlated to the increase of cancer stages. **D**: Overall-survival (OS) from PDAC patients in TCGA with high and low levels of S100A14 was analyzed by Kaplan-Meier. **E**: S100A14 protein level was higher in PDAC cell lines compared to HPDE cell line by Western blot. *P < .05; **P < .01; ***P < .001.

was performed in triplicate. Clonal expansion rate was studied by colony formation assay. Briefly, cells were seeded into a 6-well culture plate ($0.5–1 \times 10^3$ cells/well) and cultured for 2–3 weeks. Colonies formed in each well were stained with 0.05% crystal violet and then counted. The experiment was performed in triplicate.

Soft agar assay

1.5 ml 0.5% agar in DMEM was added onto each well of a 6-well culture plate. Cells ($2–5 \times 10^3$) were resuspended in 1.5 ml 0.35% agar in DMEM and added on top of the solidified 0.5% agar, and the cells were allowed to growth for 2–4 weeks. After incubation, the agar layers were stained with 0.05% crystal violet solution for 10 min s. Agars were destained with phosphate-buffered saline prior to photo-capturing. Only cell mass with more than 50 cells was counted as a colony and representative photos of the assay were shown in result section.

Cell migration and invasion assays

Cell migration and invasion assays were carried out according to the manufacturer's protocols. To measure cell migration, transwell chambers were used to observed cultured cells inserts (Transwell chamber; 8-μm pore size; Corning, New York, USA). PDAC cells ($2–4 \times 10^4$ cells) per well were added to the upper chamber and cultured with serum-free DMEM medium, whereas the lower chamber was filled with complete medium (contain 10% FBS). After 48–72 h of incubation, the cells in the upper chamber were carefully removed with a cotton swab and those cells that had migrated through the membrane to the lower surface were stained with 0.05% crystal violet. The number of cells that had migrated through the pores was quantified by counting five independent visual fields under the microscope (Olympus, USA) using a 20 × objective. For invasion assays, the procedures were the same except the membrane in the transwell were covered with matrigel (BD Biosciences, California, USA). Three independent assays were performed.

Animal studies

All animal experiments conducted in this study were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. Four to six weeks old male athymic nude mice were used for the establishment of PDAC xenografts. SW1990 cells (1×10^6), which were suspended in 100 μl PBS with 10%

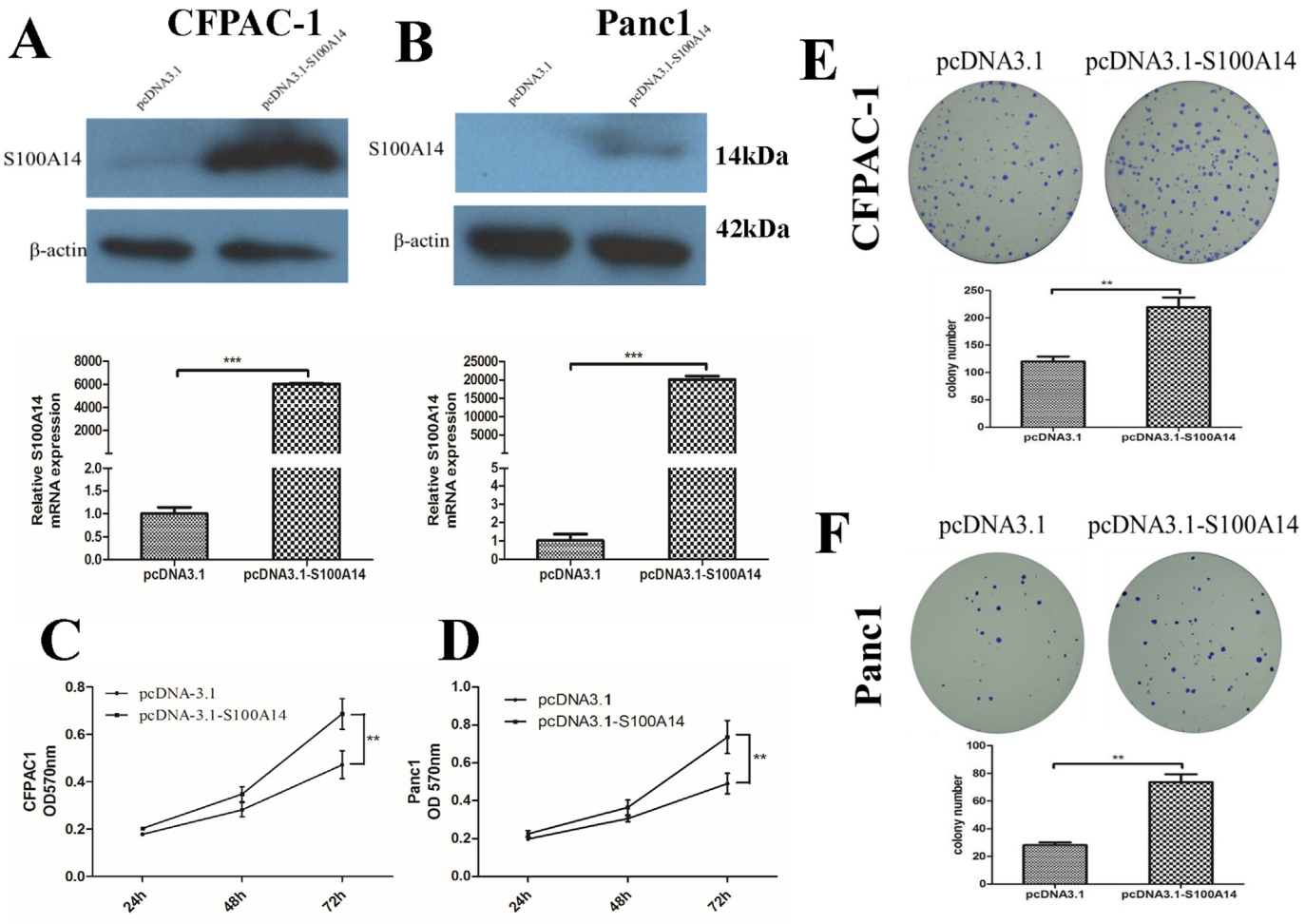

Fig. 2. Overexpression of S100A14 expression increased cell proliferation and clonal expansion ability in PDAC cells after overexpression of S100A14. **A** and **B**: Both S100A14 protein and mRNA expression were successfully overexpressed in (A) CFPAC1 and (B) Panc1 cell lines after transfection of pcDNA3.1-S100A14 by Western blot (upper panels) and qRT-PCR (lower panels), respectively. **C** and **D**: MTT assays showed that rates of cell proliferation in **(C)** CFPAC-1 and **(D)** Panc1 were significantly increased after transfection of pcDNA3.1-S100A14. **E** and **F**: Clonal expansion ability in **(E)** CFPAC-1 and **(F)** Panc1 were significantly increased after transfection of pcDNA3.1-S100A14. **P < .01; ***P < .001.

matrigel, were inoculated into the nude mice by subcutaneous injection. Mice were randomly allocated into groups consisted of 5 individuals. Tumor growth was monitored three times a week using a digital caliper, and tumor volume was calculated as: [(Length × Width [2]) ÷ 2].

In vitro chemosensitivity assay

CAPAN2 cells transduced with sh-S100A14 or control sh-SCR were seeded into 96-well plates (2000 cells/well) and treated with gemcitabine (Sigma) at various concentrations. After 72 h, MTT assay was used to detect the cell viability. The calculative formula for suppression rate was: cell suppression rate = (1 − ODtreated/ODcontrol) × 100%. The dose–response curve at different concentrations was charted to calculate the half maximal inhibitory concentration (IC50) using a probit regression model.

Statistical analysis

All data were analyzed using the software GraphPad Prism 5 and expressed as mean ± SD. Differences between groups were compared by one-way analysis of variance and *t*-test. A P-value of P < .05 was considered statistically significant.

Results

High S100A14 expression was correlated with the increase of cancer stages in pancreatic cancer

Global gene expression microarray was conducted in 4 pairs of PDAC tumor and adjacent non-tumor tissues. Analysis of the microarray showed that the transcription level of S100A14 had an average of 7.22-fold increase in four tumor tissues (p = .0151) (Fig. 1A). We then measured the S100A14 mRNA expression in human PDAC tissues and matched non-tumor tissues by qRT-PCR and showed that the levels of S100A14 mRNA were overexpressed in human PDAC tumor tissues (p = .0356) (Fig. 1B). In order to study the protein expression in pancreatic tissues, we measured the level of S100A14 protein by immunohistochemical staining (IHC) on a tissue microarray consisting of PDAC tissues and non-tumor pancreatic tissues. IHC staining showed that the protein level of S100A14 was higher in PDAC tissues (n = 175) compared to non-tumor tissues (n = 27) (p < .001). In addition, the abundance of S100A14 was positively correlated to the advancement of PDAC, in which the level of S100A14 increased with the increase of cancer stages (Stage I vs normal: P < .01; Stage II vs Stage I: P < .001; Stage IV vs Stage III: P < .05) (Fig. 1C). Further, Kaplan-Meier survival analysis suggested that PDAC patients with low S100A14

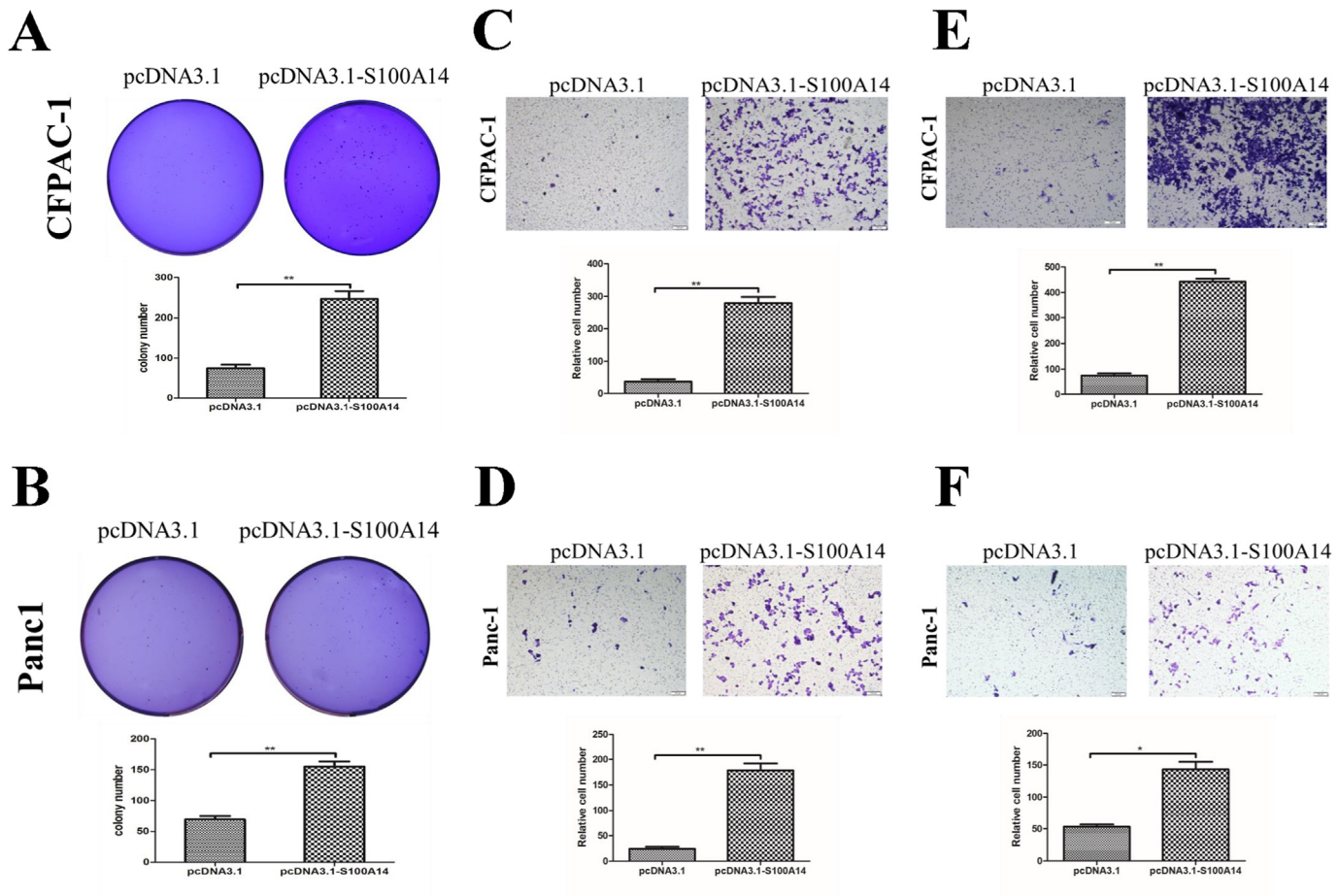

Fig. 3. Overexpression of S100A14 expression increased cell transformation, migration and invasion in PDAC cells after transfection of pcDNA3.1-S100A14. **A** and **B**: Cell transformation ability in **(A)** CFPAC-1 and **(B)** Panc1 were significantly increased after transfection of pcDNA3.1-S100A14 by Soft agar assay. **C** and **D**: Rates of cell migration in **(C)** CFPAC-1 and **(D)** Panc1 were significantly increased after transfection of pcDNA3.1-S100A14 by Transwell assay. E and F: Rates of cell invasion in **(E)** CFPAC-1 and **(F)** Panc1 were significantly increased after transfection of pcDNA3.1-S100A14 by Transwell assay. **P < .01; ***P < .001.

expression had longer overall survival in TCGA PDAC datasets. In this analysis, a total of 178 PDAC cases from TCGA are separated into low (green) and high (red) expressing group (Fig. 1D, Log-rank test, P < .01). S100A14 protein expression level in PDAC cells was also measured by Western blot, and we observed that S100A14 protein level was higher in PDAC cell lines compared to HPDE cells (Fig. 1E). These results suggested that S100A14 was highly expressed in human pancreatic cancer tissue specimens and cell lines, its expression was correlated with the increase of cancer stages in human PDAC.

Overexpression of S100A14 expression increased cell proliferation, colony formation, migration and invasion in PDAC cells

As the tumorigenic roles of S100A14 in human PDAC were largely unexplored, we attempted to investigate the biological functions of S100A14 in the development of a malignant phenotype in PDAC cells by modulating intracellular S100A14 expression. For experiments involving the overexpression of S100A14, we employed CFPAC-1 and Panc1 cell lines which had low levels of endogenous S100A14. Transfection of pcDNA3.1-S100A14 plasmid effectively induced an overexpression of S100A14 proteins in CFPAC-1 and Panc1 cells (Fig. 2A-B upper panels). Quantitative RT-PCR also showed that S100A14 mRNA expressions were upregulated in S100A14-expressing cells (Fig. 2A-B lower panels). We then studied the effect of S100A14 overexpression to the cell proliferative rate and clonal expansion rate in CFPAC-1 and Panc1 using MTT assay and colony formation assay respectively. In all S100A14-overexpressing cell lines, MTT assay showed that the rates of cell growth were significantly higher than in empty vector-expressing controls (Fig. 2C-D). Colony formation assay showed that the overexpression of S100A14 could also promote the clonal expansion rates of both cell lines (Fig. 2E-F). In turn, we performed soft agar assay to study the role of S100A14 in transformation, and found that CFPAC-1 and Panc1 transformation potential was greatly enhanced by the overexpression of S100A14 via the transfection of pcDNA3.1-S100A14. The number of anchorage independent colonies was increased in S100A14-overexpressed cells (Fig. 3A-B).

Furthermore, we investigated the effect of S100A14 overexpression to the rate of migration and invasion in PDAC cells by carrying out the transwell migration and invasion assays. Overexpression of S100A14 in CFPAC-1 and Panc1 cells resulted in the increases of migration (Fig. 3C-D) and invasion rates (Fig. 3E-F) when compared to empty vector-expressing cells. Taken together, we showed that the overexpression of S100A14 could promote the growth and enhance the aggressiveness of PDAC cells. S100A14 could potentially play an oncogenic role during the development and progression in human PDAC.

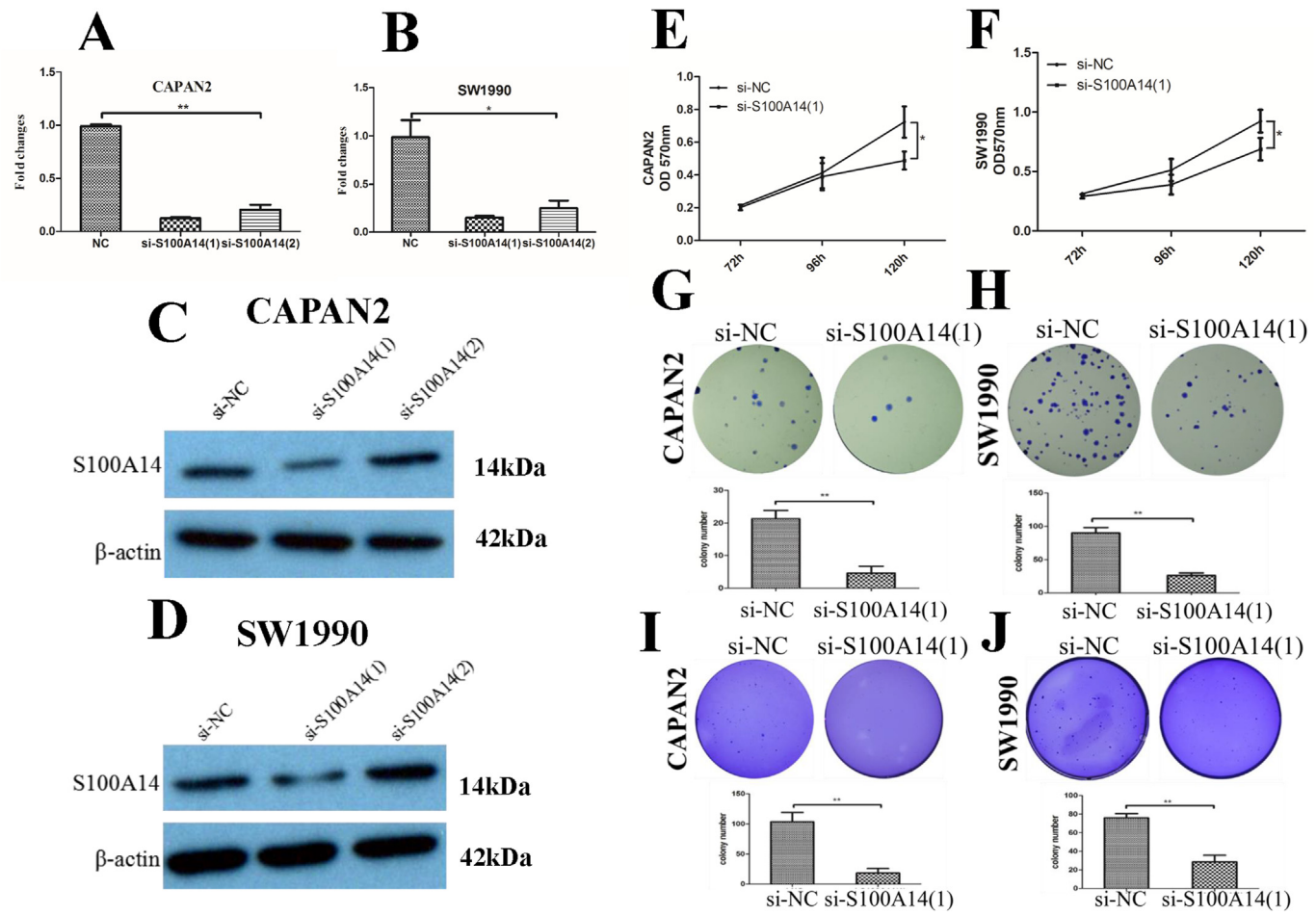

Fig. 4. Knockdown of S100A14 expression decreased cell proliferation and transformation in PDAC cells after transfection of si-S100A14(1). **A** and **B**: S100A14 mRNA expression was successfully knockdown in **(A)** CAPAN2 and **(B)** SW1990 cell lines after transfection of si-S100A14(1) or si-S100A14(2) by qRT-PCR. **C** and **D**: S100A14 protein expression was successfully knockdown in **(C)** CAPAN2 and **(D)** SW1990 cell lines after transfection of si-S100A14(1) while not si-S100A14(2) by Western blot. Therefore, we used si-S100A14 (1) for following assays. **E** and **F**: Rates of cell short-term proliferation in **(E)** CAPAN2 and **(F)** SW1990 were significantly decreased after transfection of si-S100A14 (1) by MTT assay. **G** and **H**: Rates of cell long-term proliferation in **(G)** CAPAN2 and **(H)** SW1990 were significantly decreased after transfection of si-S100A14 (1) by Colony formation assay. **I** and **J**: Cell transformation ability in **(I)** CAPAN2 and **(J)** SW1990 were significantly decreased after transfection of si-S100A14(1) by Soft agar assay. *P < .05; **P < .01.

Knockdown of S100A14 expression decreased cell proliferation, transformation, migration and invasion in PDAC cells

For S100A14 knockdown experiments, we used CAPAN2 and SW1990 cell lines that had high levels of endogenous S100A14. Firstly, we transfected the cells with small interfering RNA (siRNA) and found that S100A14 mRNA level was decreased after transfection of si-S100A14(1) or si-S100A14(2) in CAPAN2 and SW1990 (Fig. 4A-B). Western blotting showed that only si-S100A14(1) could successfully knockdown S100A14 in CAPAN2 and SW1990 (Fig. 4C-D) cell lines, but si-S100A14(2) failed to achieve a successful knockdown of S100A14. Therefore, we used si-S100A14 (1) for all the subsequent assays. MTT assay showed that the knockdown of S100A14 by the transfection of si-S100A14(1) could significantly reduce the rates of cell proliferation in both CAPAN2 and SW1990 cells (Fig. 4E-F). In turn, we showed that knockdown of S100A14 could significantly inhibit the number of colonies formed in the colony formation assay and the soft agar assay. It suggested that inhibiting S100A14 could hinder the clonal expansion ability (Fig. 4G-H) and transformation potential (Fig. 4I-J) of CAPAN2 or SW1990 cells.

In order to validate the impact of S100A14 knockdown in PDAC cells, we used lentiviral-mediated shRNA for knockdown experiments. We found that both protein and mRNA levels of S100A14 were decreased in CAPAN2 and SW1990 cells after the transduction of lentivirus carrying S100A14 shRNAs transgene sh-S100A14(2), but not that of sh-S100A14(1) (Fig. 5A-B). Therefore, we adopted sh-S100A14(2) for all the subsequent experiments involving shRNAs knockdown. MTT assay showed that knockdown of S100A14 could decrease the rates of cell growth when compared to sh-SCR control group (Fig. 5C-D). Inhibition of S100A14 could also inhibit the number of colonies formed in both cell lines (Fig. 5E-F). Soft agar assays again showed that inhibition of S100A14 greatly prohibited the transformation ability of PDAC cells, as the number of anchorage dependent colonies was significantly reduced (Fig. 6A-B). In turn, we demonstrated that the knockdown of S100A14 resulted in the decrease of cell migration (Fig. 6C-D) and invasion (Fig. 6E-F) of CAPAN2 and SW1990 cells when compared to sh-SCR control group. Taken together, we demonstrated that S100A14 played an important role in cell growth, transformation, migration and invasion in human PDAC.

S100A14 knockdown in SW1990 cells decreased xenografted tumor growth in nude mice

To explore whether S100A14 could affect tumor growth in vivo,

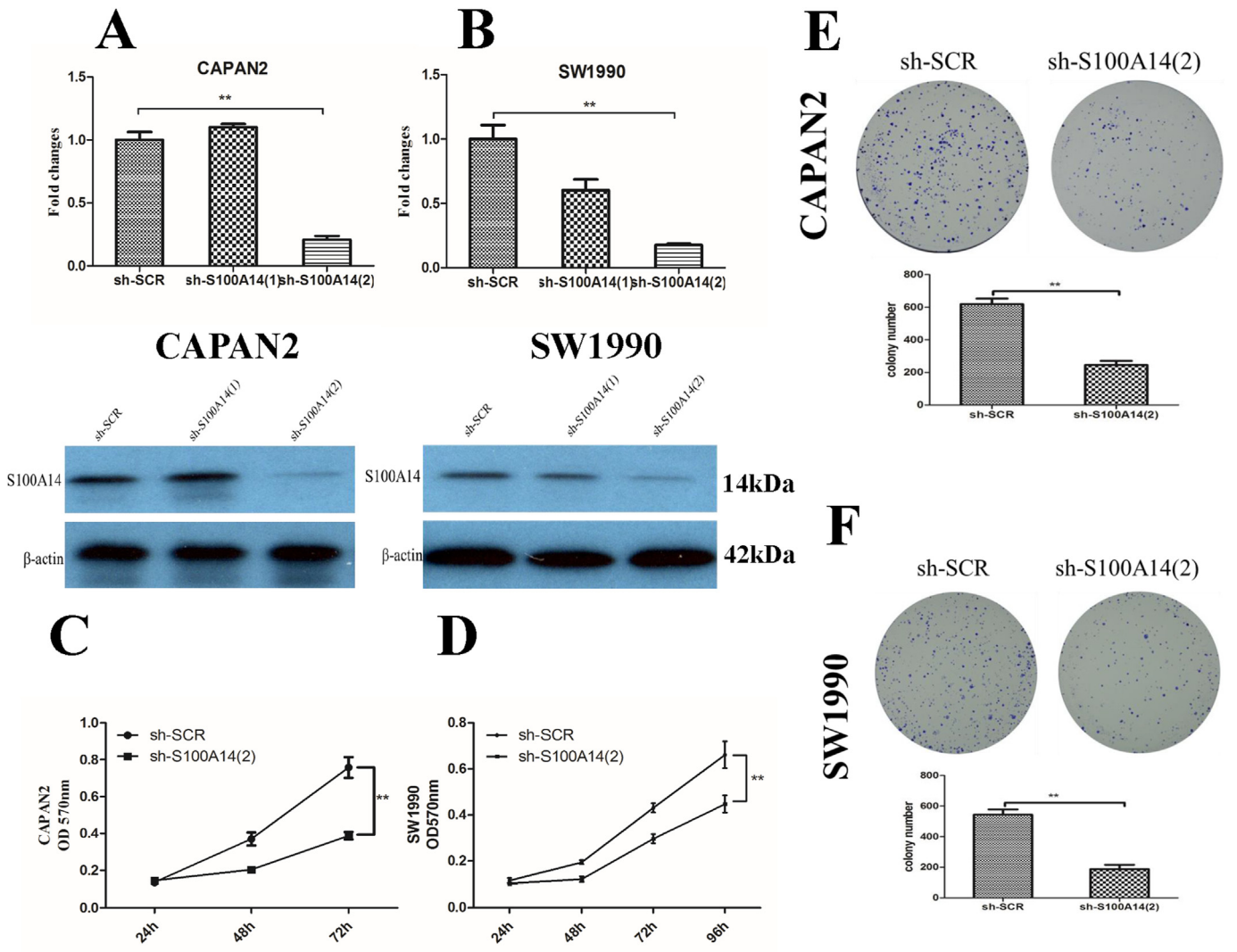

Fig. 5. Knockdown of S100A14 expression decreases cell short-term and long-term proliferation in PDAC cell lines after infection of sh-S100A14. **A** and **B**: Both S100A14 protein and mRNA expression was successfully knockdown in **(A)** CAPAN2 and **(B)** SW1990 cell lines after infection of sh-S100A14(2) while not sh-S100A14(1) by Western blot (upper panels) and qRT-PCR (lower panels), respectively. Therefore, we used sh-S100A14(2) for functional experiments. **C** and **D**: Rates of cell short-term proliferation in **(C)** CAPAN2 and **(D)** SW1990 were significantly decreased after infection of sh-S100A14(2) by MTT assay. **E** and **F**: Rates of cell long-term proliferation in **(E)** CAPAN2 and **(F)** SW1990 were significantly decreased after infection of sh-S100A14(2) by Colony formation assay. **P < .01.

SW1990 cells transduced with lentiviral-mediated sh-S100A14(2) were subcutaneously injected into nude mice, and tumors were allowed to grow for ~3 weeks. Tumor volume was measured regularly. Mean tumor volume at day 19 was significantly smaller for tumor derived from S100A14-inhibited cells (413.0 ± 124.3 mm^3) in contrast to those derived from shRNA-control group (1024.4 ± 289.0 mm^3) (Fig. 7C). In addition, mean tumor weight at day 19 of S100A14-inhibited tumor (0.56 ± 0.12 g) were significantly smaller than the shRNA-control tumor (1.41 ± 0.29 g) (Fig. 7D). These data indicated that S100A14 knockdown could inhibit tumor growth in vivo, and further supported our hypothesis that S100A14 contributed to the malignant transformation and development of PDAC. More importantly, inhibition of S100A14 could effectively attenuate the oncogenic properties of PDAC cells, which might represent a novel therapeutic approach against human PDAC.

S100A14 knockdown enhanced the chemosensitivity of human pancreatic cancer cells to gemcitabine in vitro

Innate or acquired resistance to chemotherapy is a hallmark of PDAC. Considering the effect of S100A14 on cell proliferation, transformation, cell migration and invasion above, we further tested whether inhibition of S100A14 could attenuate the resistance of PDAC cells to gemcitabine. CAPAN2 cells infected with lentiviral mediated sh-S100A14(2) and sh-SCR control were exposed to 0.1, 1, and 10 μM gemcitabine for 72 h, and IC50 values were calculated using the MTT assay. Knockdown of S100A14 promoted the growth inhibitory effect of gemcitabine in PDAC cells. Upon gemcitabine treatment, the cell survival rate of S100A14-inhibited cells was significantly reduced when compared to CAPAN2 control cells. In addition, a lower IC50 of gemcitabine treatment could be achieved when the PDAC cells were having their

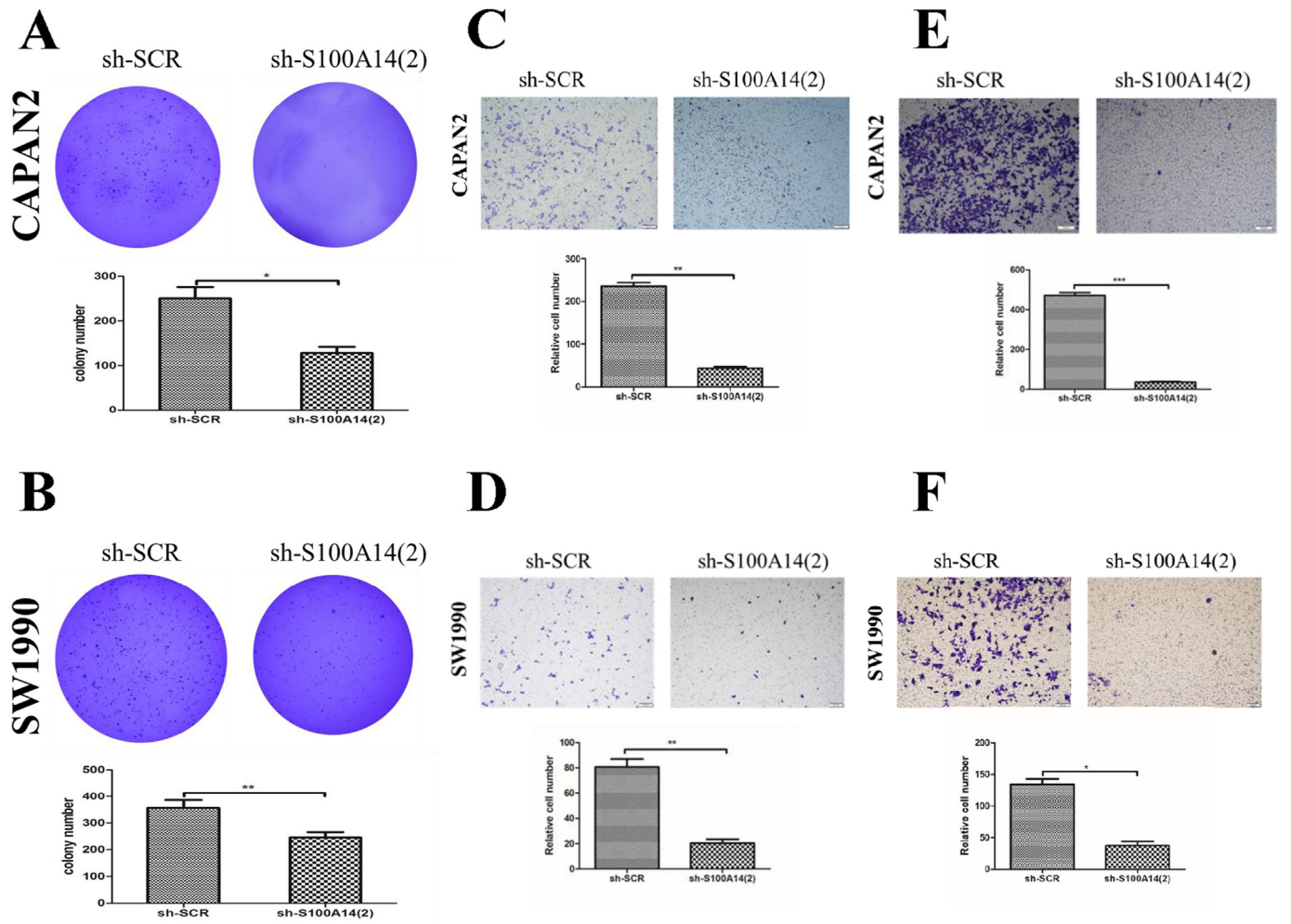

Fig. 6. Knockdown of S100A14 expression decreases cell transformation, migration and invasion in PDAC cells after infection of sh-S100A14. **A** and **B**: Cell transformation ability in **(A)** CAPAN2 and **(B)** SW1990 were significantly decreased after infection of sh-S100A14 (2) by Soft agar assay. **C** and **D**: Rates of cell migration in **(C)** CAPAN2 and **(D)** SW1990 were significantly decreased after infection of sh-S100A14(2) by Transwell assay. **E** and **F**: Rates of cell invasion in **(E)** CAPAN2 and **(F)** SW1990 were significantly decreased after infection of sh-S100A14(2) by Transwell assay. *P < .05; **P < .01; ***P < .001.

S100A14 inhibited (1.063 ± 0.138 μM vs 4.468 ± 0.212 μM) (Fig. 7E and F). It suggested that knockdown of S100A14 might inhibit chemoresistance and sensitize the PDAC cells to gemcitabine treatment.

Discussion

The extremely poor prognosis of PDAC is partly owing to the delay of disease diagnosis [3], so identification and development of biomarkers for pancreatic cancer is urgently required. The benefit will be largely increased if the target can be detected by a simple non-invasive blood test for the early identification of pancreatic cancer patients. Previous studies have already shown that S100A14 was secreted by cancer cells and exert extracellular functions [20]. In addition, recent studies have shown that altered expression of S100A14 in various cancers were heavily linked with disease progression which might also predict patient outcome [14,16,17]. Measuring the level of serum S100A14 could be a promising approach in diagnosis and prognosis of cancer. In this study, we demonstrated that S100A14 was overexpressed in PDAC clinical samples and cell lines. The abundance of S100A14 was positively correlated with the increase of cancer stages. Future studies can be conducted to study the changes of S100A14 plasma levels in pancreatic cancer patients prior to or following surgery or chemotherapy to further analyze the diagnostic and prognostic value of S100A14 in pancreatic cancers.

Accumulating evidence suggests the S100 family plays a vital role in cell growth, migration, invasion and metastasis. Indeed, multiple members of S100 family, including S100A2, S100A4, S100A6, S100A11 and S100P, are closely related to pancreatic cancer. S100A2 is a good predictor of the response to pancreatectomy for pancreatic cancer, and a marker for metastatic phenotype [21]; S100A4 is an independent prognostic marker of pancreatic cancer which can differentiate pancreatic cancer from lymph node metastasis [22]; high level of S100A6 protein expression in the nucleus often indicates poor prognosis [23]; high expression of S100A11 is associated with tumor differentiation and lymph node metastasis [24,25]; S100P expression levels in patients with pancreatic cancer and intraductal papillary mucinous neoplasm (IPMN) were significantly higher than those in patients with pancreatitis [26]. S100P and S100A11 analysis in the pancreatic juice may allow the early detection of pancreatic cancer [27,28]. Recent studies revealed that S100A14 was also associated with pancreatic cancer. A study showed that the expression of S100A14 could be induced by the sonic-hedgehog −Gli1 signaling pathway in pancreatic cancer [29].

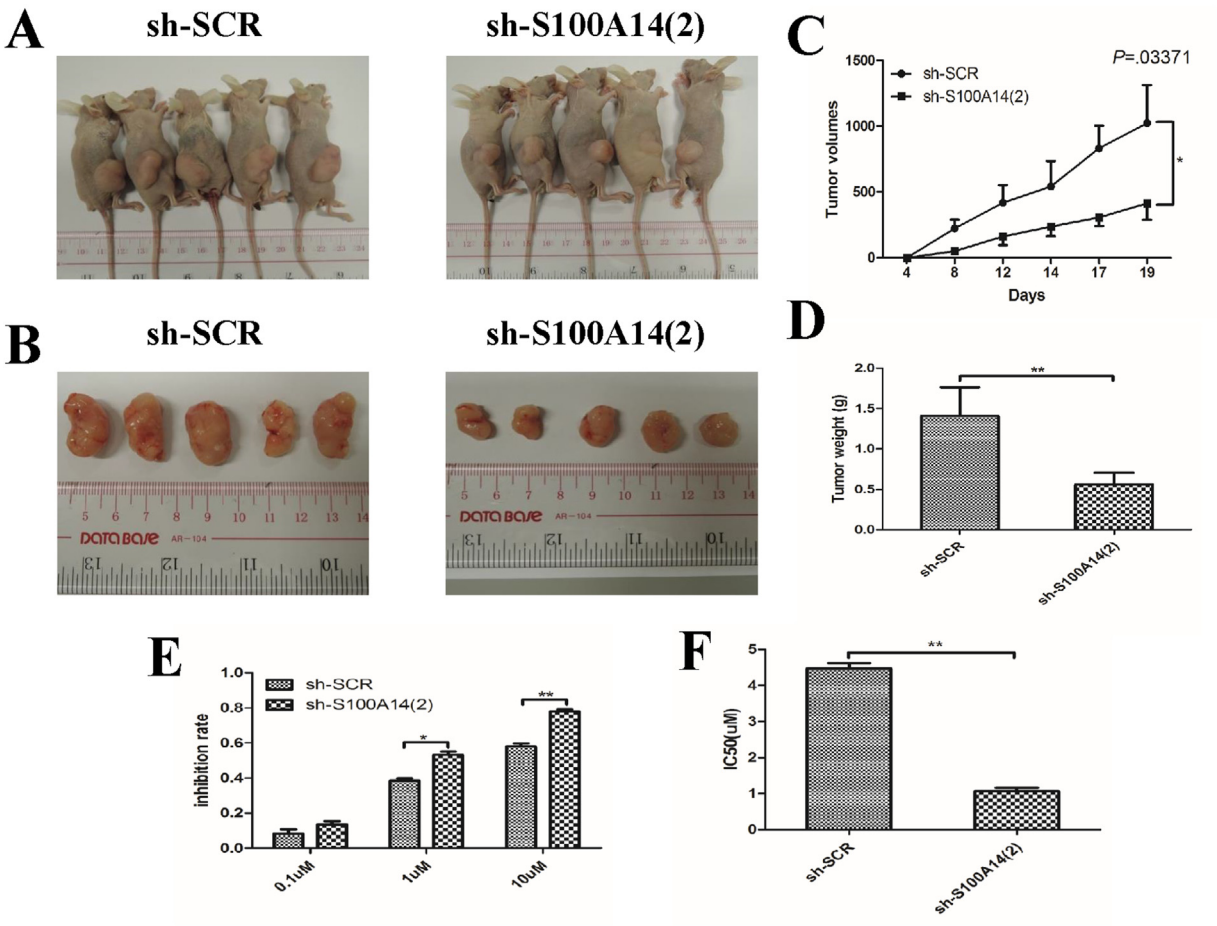

Fig. 7. S100A14 knockdown in SW1990 cells decreased tumor growth in nude mice xenografts and inhibited gemcitabine chemoresistance. **A**: For the xenograft, a total of 1.0 × 106 cells were injected subcutaneously into the nude mice (n = 5). **B**: Gross images of tumor masses from representative mice from each group (n = 5). **C**: SW1990 cells infection with sh-S100A14(2) showed reduced subcutaneous tumor growth in nude mice (n = 5). **D**: SW1990 cells infection with sh-S100A14(2) showed reduced subcutaneous tumor weight in nude mice (n = 5). *P < .05; **P < .01. **E**: Compared with CAPAN2 control cells, CAPAN2-shS100A14 knockdown cells exhibited much slower growth. **F**: Compared with CAPAN2 control cells, CAPAN2-shS100A14 knockdown cells exhibited a lower IC50 for gemcitabine. *P < .05; **P < .01.

The clinical importance of S100A14 in pancreatic cancer was also suggested, as there was a report showing S100A14 was one of the biomarkers in a six-gene panel to offer helpful prognostic stratification information, and was suitable to transfer to clinical use in pancreatic cancer patients [30]. However, there remains a lack of investigation into the functional roles of S100A14 in PDAC. Our study demonstrated that S100A14 overexpression resulted in significant enhancements in oncogenic properties including cell proliferation, clonal expansion, cell transformation, migration and invasion. Conversely, inhibiting S100A14 led to the suppression of these phenotypes. More importantly, the depletion of S100A14 in SW1990 cell decreased xenografted tumor growth in nude mice. Our study employed the subcutaneous xenograft models to study the tumorigenesis of PDAC cells in vivo and showed that inhibition of S100A14 effectively attenuated the growth of PDAC tumor. Subcutaneous xenograft models could allow the measurement of tumor growth and volume but failed to provide a relevant tumor microenvironment. Orthotopic xenograft represents a better tumor growing model that provides a more relevant tumor microenvironment but is difficult for monitoring tumor growth quantitatively. Despite this limitation, our findings indicate that S100A14 is one of the important proteins promoting PDAC carcinogenesis and progression.

Innate or acquired resistance to chemotherapy is a hallmark of PDAC. We also showed that down-regulation of S100A14 impaired the resistance of PDAC cells to gemcitabine. The present study indicated that the knockdown of S100A14 in CAPAN2 cells exhibited less cell survival upon gemcitabine treatment and achieved a lower IC50 for gemcitabine in vitro. It indicated that knockdown of S100A14 could enhance the chemosensitivity of PDAC cells to gemcitabine. However, the specific mechanism of S100A14 involvement in the resistance of chemotherapy requires further investigation. Nonetheless, given the role of S100A14 in regulating gemcitabine response, the S100A14 protein may be recognized as a novel biomarker predicting the response of chemotherapy for patient suffering from pancreatic cancer.

In summary, we reported that S100A14 expression was frequently overexpressed in pancreatic cancer clinical samples and PDAC cells. Moreover, the abundance of S100A14 was positively correlated to the increase of cancer stages, which supported the development of S100A14 as a biomarker for diagnosis and prognosis in pancreatic cancer. S100A14 promoted cell growth, transformation, progression and gemcitabine resistance of PDAC. Future studies are warranted to investigate the underlying mechanisms contributed to the oncogenic properties of S100A14 in PDAC.

Declaration of competing interest

The author reports no conflicts of interest in this work.

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