

SATB1 Promotes Pancreatic Cancer Growth and Invasion Depending on MYC Activation

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Received: 12 March 2015 / Accepted: 10 June 2015 / Published online: 25 June 2015
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Abstract

Background SATB1 plays an important role in human malignant progression, inducing cancer cell proliferation and metastasis by regulating downstream gene expressions. However, little is known about the underlying mechanisms in which SATB1 promotes pancreatic cancer tumorigenesis.

Aims To investigate SATB1 expression levels and its biological functions in promoting pancreatic cancer growth and invasion.

Methods SATB1 expression levels were detected in seven human pancreatic cancer cell lines and 16 pairs of normal pancreatic/pancreatic cancer tissues using RT-PCR and western blot. SW1990 or Capan-1 cells stably

knockdown (shRNA) or transiently knockdown (siRNA) SATB1 cells, and PANC-1 stably overexpressing SATB1 cells were investigated with MTT, EdU assay, flow cytometry, and transwell invasion assay for cell proliferation and invasion activity. The binding of SATB1 to MYC promoter region was examined using reporter assay. Expression of SATB1 in 68 pancreatic cancer samples was studied by immunohistochemical staining and scoring.

Results SATB1 was overexpressed in pancreatic cancer tissues samples, showing strong correlation with pancreatic cancer invasion depth and tumor staging. SATB1 induced MYC mRNA and protein expression; promoted pancreatic cancer cell growth; increased cell population in S phase; and enhanced pancreatic cancer cell invasion in vitro. On the other hand, SATB1 knockdown showed opposite effects. Furthermore, MYC blocking in SATB1-

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overexpressing cells attenuated the promotion of pancreatic cancer cell growth and invasion. Our data also indicated that SATB1 bound to specific promoter region of MYC.

Conclusions SATB1 is overexpressed in pancreatic cancer, promoting cancer cell proliferation and invasion through the activation of MYC.

Keywords Special AT-rich binding protein 1 · Pancreatic carcinoma · Cell cycle · Myelocytomatosis viral oncogene

Abbreviations

SATB1	Special AT-rich binding protein 1
MYC	Myelocytomatosis viral oncogene
MARs	Matrix attachment regions
DMSO	Dimethyl sulphoxide
PBS	Phosphate-buffered saline
DMEM	Dulbecco's minimum essential medium
FBS	Fetal bovine serum
ELISA	Enzyme-linked immunosorbent assay
PI	Propidium iodide
FACS	Fluorescence-activated cell sorter
PVDF	Polyvinylidene fluoride
ECL	Chemiluminescent
EdU	5-Ethynyl-2'-deoxyuridine
BURs	Base-unpairing regions

Background

Pancreatic cancer is the fifth leading cause of cancer death in Japan [1], the sixth leading cause of cancer death in China, and the fourth leading cause of cancer death in the USA [2]. Due to a lack of obvious symptoms during the early stages of pancreatic cancer, very few patients receive an early diagnosis, and most are not diagnosed until they reach the late stages of this disease. To further exacerbate the issue, advanced local disease or distant metastases prevent the majority of pancreatic cancer patients with a definitive diagnosis from responding to nearly all of the present treatments [3]. The limitations associated with its diagnosis and treatment make the mortality rate of pancreatic adenocarcinoma the highest among all cancers [4]. However, in patients who are suitable for resection, 5-year survival rates of approximately 5 % are observed, providing evidence that surgery and subsequent chemical therapy offer the only chance of a potential cure and long-term survival [5, 6]. Better understanding of the changes that occur at the gene and protein levels during pancreatic cancer progression will bring better prognosis to patients.

Chromatin architecture plays an important role in the regulation of nuclear function. Matrix attachment regions

(MARs) are specialized genomic sequences that show a high affinity for the nuclear matrix and are able to tether chromatin loops in vivo [7, 8]. MARs form specific binding sites on the minor groove of AT-rich DNA for proteins that recognize DNA structure, rather than specific DNA sequences [9]. At present, several proteins have been identified that bind to MARs, including the special AT-rich binding protein 1 (SATB1) [10]. SATB1 is found predominantly in thymocytes, and it regulates gene expression by folding chromatin into loop domains, tethering specialized DNA elements to an SATB1 network structure [11, 12]. The overexpression of SATB1 has recently been found in many different kinds of human tumors including breast cancer, gastric cancer, and rectal cancer [13–15], promoting cancer cell invasion and metastasis [16, 17]. SATB1 expression level also shows strong correlation with the progression of rectal cancer [18], and it is an independent prognostic marker for gastric cancer [19]. However, the role of SATB1 in pancreatic cancer development and progression is still unknown.

The proto-oncogene MYC (*v-myc* avian myelocytomatosis viral oncogene homolog) encodes a nuclear phosphoprotein transcription factor that regulates a large number of genes involved in many critical cellular functions including proliferation, cell cycle progression, differentiation, and apoptosis [20–22]. Amplification of MYC has been reported in different kinds of human cancers [23–26]. The overexpression of MYC is observed in more aggressive breast cancer and correlates with poor prognosis and distant metastasis [27, 28], but the biological function of MYC in pancreatic cancer progression has yet to be fully understood.

Matrix metalloproteinase (MMP) family is involved in the breakdown of extracellular matrix in normal physiological processes as well as in pathological processes, such as arthritis and tumor metastasis [29–31]. MMP2 and MMP9 are important members of MMP family, promoting cancer cell angiogenesis, invasion, and metastasis [32, 33]. Recently, a study has shown that both MMP2 and MMP9 were induced after the overexpression SATB1 in breast cancer [16], but the exact mechanism is unknown.

The primary objective of this study was to investigate the role of SATB1 in promoting pancreatic cancer proliferation and invasion and identifying the underlying molecular mechanism. We have uncovered the overexpression of SATB1 in pancreatic cancer tissues compared with adjacent normal samples. We have demonstrated that SATB1 regulates pancreatic cancer proliferation, cell cycle progression, and invasion through the activation of MYC. We also have shown strong correlation between SATB1 expression and pancreatic cancer tumor invasion depth and staging. These novel findings underscore the important role of SATB1 in promoting pancreatic cancer tumorigenesis

and the potential therapeutic target of SATB1 in pancreatic cancer.

Methods

Cell Culture and Reagents

Human pancreatic cancer cell lines, including PANC-1, SW1990, CFPAC-1, BxPC-3, HPAC, MIAPaCa-2, and Capan-1, were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin (Gibco). The cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO₂. Cells were passaged every 2–3 days to support exponential growth. SATB1, MMP2, MMP9, MYC and β -actin antibodies, SATB1 siRNA, and MYC siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated mouse and goat secondary antibodies were purchased from Genscript (Nanjing, China).

Specimen Collection and Ethics Statement

Sixteen pairs of human primary pancreatic carcinomas and adjacent normal pancreatic tissue samples and 68 formalin-fixed paraffin-embedded pancreatic cancer tissue samples were surgically removed and snap-frozen in liquid nitrogen or fixed in 10 % formalin and embedded in paraffin; these tissues samples were obtained from fresh pancreaticoduodenectomy (Whipple resection) or from the body and tail of pancreas resections, along with pathology summaries at the Department of General Surgery of the First Affiliated Hospital of Nanjing Medical University between 2006 and 2009. Pancreatic cancer staging was followed the guideline as we described before [34]. All human tissue samples were obtained according to the ethical guidelines of the Declaration of Helsinki. This study was approved by Ethics Committee of Nanjing Medical University. All patients participated after having provided informed consent. Tumor stage was evaluated in accordance with the tumor-node metastasis (TNM) classification system UICC/AJCC 2002.

SATB1 Knockdown or Overexpression and MYC Knockdown

SATB1 shRNA was designed based on the SATB1 mRNA sequence [GenBank: NM_002971] with BLOCK-iTTM RNAi Designer (Invitrogen Life Technologies, Carlsbad, CA, USA): SATB1-shRNA oligoduplexes (5'-

GGATTTGGAAGAGAGTGTC-3') were synthesized and cloned into pGCsi-H1/Neo/GFP (Genechem, Shanghai, China). Pancreatic cancer cell line SW1990, which expresses high level of endogenous SATB1, was transfected with pGCsi-H1/Neo/GFP-SATB1-shRNA or empty vector using Lipofectamine 2000 (Invitrogen Life Technologies) following the manufacturer's instruction. Forty-eight hours later, the transfected cells were selected and maintained in media containing 800 μ g/ml G418 (Invitrogen Life Technologies). The full-length SATB1 coding sequence (CDS) was cloned into pcDNA3.1 mammalian expression construct (Invitrogen Life Technologies) defining as pcDNA3.1-SATB1. Pancreatic cancer cell line PANC-1 expressing low level of endogenous SATB1 was transfected with pcDNA3.1-SATB1 or empty vector using Lipofectamine 2000 (Invitrogen Life Technologies) following the manufacturer's instruction. PANC-1 cells stably expressing SATB1 or empty vector was selected and maintained in media containing 600 μ g/ml G418. SATB1 siRNA was transfected using RNAiFectinTM transfection reagent (Applied Biological Materials Inc, Richmond, BC, Canada) in SW1990 or Capan-1 cells with relative high levels of SATB1 following manufacturer's protocol. MYC siRNA was transfected with RNAiFectinTM transfection reagent (Applied Biological Materials Inc) in PANC-1 cells that stably overexpressing SATB1 and in control cells following the manufacturer's protocol to knock down MYC.

Cell Growth Curves

Cellular growth was evaluated by MTT assays. SW1990 stably knockdown, PANC-1 stably overexpressing SATB1, or control cells were seeded at a density of 1.0×10^3 cells/well in 96-well plates. After cells were allowed to adhere for 2 h, and at every 12 h until 96 h, MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well at a final concentration of 0.5 mg/ml, followed by incubation at 37 °C for 4 h. The medium was then removed, and 200 μ l of DMSO was added to each well. The absorbance of the mixture was measured at 490 nm using a microplate ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). The relative cell number was calculated using the following equation: relative cell number = mean experimental absorbance/mean 2-h absorbance.

Cell Cycle Analysis

SW1990 stably expressing SATB1 shRNA or empty vector cells were incubated in six-well plates (5×10^4 cells/well). Forty-eight hours later, cells were fixed with 80 % chilled ethanol and then incubated with 0.5 % Triton X-100 solution containing 1 mg/ml RNase A (Qiagen,

Germantown, MD, USA) at 37 °C for 30 min. Propidium iodide (PI) (Sigma) was added to each well at a final concentration of 50 µg/ml later, followed by a 30-min incubation in the dark. Cellular DNA content was analyzed using a FACS machine (Becton–Dickinson, Franklin Lakes, NJ, USA). Data were processed using WinMDI29 software (Becton–Dickinson).

Cell Migration and Invasion Assays

Cell migration and invasion assays were performed using 24-well transwell plates (8-µm pore size) from Sigma. In the invasion assays, Matrigel (BD Biosciences, Morrisville, NC, USA) was diluted to 1 mg/ml in serum-free, cold DMEM, and 100 µl of the diluted Matrigel was added to each insert chamber. The plates were incubated at 37 °C for 4 h to allow gelling. SW1990 stably knockdown, PANC-1 stably overexpressing SATB1, or control cells were harvested from culture plates and re-suspended in serum-free culture medium at a density of 10⁵ cells/ml. After collecting the cell samples, 100 µl of the cell suspension was added into each insert chamber. The lower chamber was filled with 600 µl of culture media containing 10 % FBS. After 24 h of incubation, non-invading cells on the top of the membrane were removed using cotton swabs. Invaded cells on the bottom of the membrane were fixed with 4 % paraformaldehyde for 15 min, followed by staining with 0.05 % crystal violet for 2 h. Photographs were taken, and all the cells on the entire membrane were counted. The relative invasion activity was calculated after normalization to cell migration.

RT-PCR and Quantitative Real-Time PCR

Total RNA was extracted from cancer cell lines using Trizol reagent (Invitrogen Life Technologies). Total RNA (1 µg) was reverse-transcribed by A3500 AMV RT-PCR (reverse transcription polymerase chain reaction) system (Promega, Madison, WI, USA). The products of the PCR were electrophoresed on 1 % agarose gels, visualized by ethidium bromide staining and quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). The results were analyzed using SDS 2.3 software (Invitrogen Life Technologies). β-Actin was used as the internal positive control and reference gene for the normalization of PCR cycle numbers to ensure a linear amplification of templates in each experiment. The quantitative real-time PCR was performed and analyzed in a 7900HT (Invitrogen Life Technologies) using SYBR Green Real-time PCR Master Mix (TOYOBO, Shanghai, China).

Western Blotting

Total protein was extracted using a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 % Triton

X-100, 0.1 % SDS, and 1 mM EDTA supplemented with protease inhibitors and phosphatase inhibitors provided by Sigma. The protein extract was loaded, size-fractionated by SDS–polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Bio-Rad). After blocking, the membranes were incubated with primary antibodies at 4 °C overnight. Membranes were probed with specific antibodies, and proteins were visualized by using HRP-conjugated secondary antibodies and Immobilon Western Chemiluminescent HRP Substrate Detection Reagent (Millipore, Billerica, MA, USA). Gel loading was normalized for equal β-actin.

EdU Cell Proliferation Assay

Cell proliferation was evaluated using the ClickiT[®] EdU (5-ethynyl-2'-deoxyuridine) assay (Invitrogen Life Technologies), which measures actively proliferating cells. EdU is incorporated as thymidine analog in the DNA of newly dividing cells and is detected by a copper-catalyzed reaction with Alexa Fluor 594 dye (red fluorescence). SW1990 stably knockdown, PANC-1 stably overexpressing SATB1, or control cells (1.5 × 10⁴) were cultured in 8-well culture slides for 48 h. EdU labeling was done by incubating cells with 10 µM EdU solution prepared in pre-warmed complete medium at 37 °C in an atmosphere containing 5 % CO₂ for 1 h. Next, cells were fixed in 3.7 % paraformaldehyde solution prepared in 1× PBS for 15 min at room temperature followed by two washes with 3 % BSA in PBS. Then, cells were permeabilized by treating with permeabilization buffer (0.5 % Triton X-100 in PBS) for 20 min. After rinsing the cells with wash solution, cells were incubated with 1× ClickiT[®] reaction cocktail containing ClickiT[®] reaction buffer, CuSO₄ solution, 1× ClickiT[®] reaction buffer additive, and Alexa Fluor 594 dye for 30 min at room temperature in a dark humidified chamber. Before visualizing under a fluorescence microscope (Olympus Co., Tokyo, Japan) at 40× magnification, cells were washed twice with 3 % BSA in PBS and the nuclei are counterstained with blue fluorescent Hoechst 33342. All experiments were performed in triplicate, and 500 cells were counted from each experiment. The percentage of cells with nuclear EdU staining was calculated and graphed.

Evaluation of Immunohistochemical Staining and Scoring

Patterns of staining, staining intensities, and percentages of SATB1-expressing cells in 68 pancreatic cancer tissue samples were recorded. The staining patterns were evaluated using the immunoreactive score (IRS) recommended by Stegner [35]. In this scoring system, IRS = SI (staining

intensity) \times PP (percentage of positive cells). SI was defined as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was determined as 1, 0–9 % positive cells; 2, 10–50 % positive cells; and 3, >50 % positive cells. One hundred cells were counted in each of ten 40 \times visual fields from different areas of each random section chosen for IRS evaluation. Then, the average IRS was calculated for each sample. The intensity of SATB1 staining was defined as “negative” or “positive,” corresponding to IRS values below 1 or above 1, respectively. Sample sections were reviewed and scored by two researchers including one pathologist.

Dual-Luciferase Reporter Assay

Thirteen different lengths of MYC 5' promoter regions (–3090 to +100) were cloned into pGL3 luciferase reporter vector (Promega, Madison, WI, USA) and defined as pGL3-R1 to R13. SW1990 stably knockdown SATB1 or control cells in 24-well plates were transfected with 100 ng of pGL3-R1 to R13 and 50 ng of normalization plasmid pRL-TK Renilla. The dual-luciferase reporter assay was performed 48 h after transfection using a luciferase assay system (Promega). Activities were normalized to pRL-TK activity and further normalized to pGL3-basic group.

Statistical Analyses

Each experiment was performed at least in triplicate. PCR results were analyzed using Quality One software (BIORAD). Statistical analyses were performed using SPSS 13.0. MTT, migration/invasion, EdU, promoter reporter, qRT-PCR assays, and growth curve analysis in cells were assessed using the two-tailed Student's *t* test, Mann–Whitney *U* test, or one-way ANOVA. The Chi-squared test was used to compare categorical groups. A *P* value less than 0.05 was considered significant.

Results

SATB1 Is Overexpressed in Human Pancreatic Cancer Cell Lines and Tissue Samples

The PCR and western blot data demonstrated that SATB1 mRNA and protein were expressed in six human pancreatic cancer cell lines: MIAPaCa-2, BxPC-3, HPAC, CFPAC-1, Capan-1, and SW1990 (Fig. 1a, b). At the same time, our results indicated that SW1990 expressed highest level of endogenous SATB1 in seven pancreatic cancer cell lines, while PANC-1 with relative low level of SATB1 mRNA or protein (Fig. 1b). We next examined SATB1 mRNA and protein expression level in 16 pairs of human pancreatic

cancer and adjacent normal pancreatic tissue samples using RT-PCR or western blot. The results demonstrated that SATB1 mRNA was overexpressed in pancreatic cancer samples compared with normal tissue (Fig. 1c, *P* < 0.05), whereas 87.5 % (14 out of 16) cancer samples expressed higher SATB1 protein level than adjacent normal tissue samples (Fig. 1d, left panel). The quantification data showed significantly higher levels of SATB1 expressed in pancreatic cancer tissue samples compared with normal pancreas (Fig. 1d, right panel, *P* < 0.001). These results suggested that SATB1 is frequently overexpressed in human pancreatic cancer.

SATB1 Promotes Pancreatic Cancer Cell Proliferation and Invasion

Previous studies have shown that the overexpression of SATB1 increased breast cancer cell proliferation and metastasis [16]. Therefore, to investigate whether the overexpression of SATB1 in pancreatic cancer promotes cell proliferation and invasion, we evaluated cell growth and invasion activity using PANC-1 stably overexpressing SATB1 cells and SW1990 stably knockdown SATB1 cells. The SATB1 knockdown in SW1990 cells and overexpression in PANC-1 cells were confirmed by RT-PCR (Fig. 2a, b; left panels) and western blot (Fig. 2a, b; right panels). Cell growth curve analysis results indicated that knockdown of SATB1 in SW1990 cells (SATB1 shRNA) significantly inhibited cell proliferation compared with control cells (Control; Fig. 2c). On the other hand, SATB1 overexpression in PANC-1 (pcDNA SATB1) promoted cell growth compared with control cells (pcDNA; Fig. 2d). In line with these results, cell cycle distribution analysis demonstrated that knockdown of SATB1 in SW1990 obviously decreased the percentage of cells in S phase compared with control cells (Fig. 2e, *P* < 0.01) indicating inhibition of cell growth by knockdown of SATB1. We further confirmed these results using EdU cell proliferation assay. Our data demonstrated that only 14 % of SW1990 SATB1 knockdown cells were positive for nuclear EdU staining (red fluorescence), whereas 31 % of control cells showed a similar staining (Fig. 3a, *P* < 0.001). By contrast, 43 % of SATB1-overexpressing PANC-1 cells displayed positive nuclear EdU staining, while only 24 % of control cells were positive (Fig. 3b, *P* < 0.001). These data strongly indicated that SATB1 promotes pancreatic cancer proliferation through regulating cell cycle progression. We next examined the role of SATB1 in regulating pancreatic cancer cell invasion ability. Transwell cell migration/invasion assay results indicated that knockdown of SATB1 in SW1990 cells significantly decreased invasive activity compared with control cells (Fig. 3c, *P* < 0.001). On the other side, the overexpression of SATB1 in PANC-1 cells

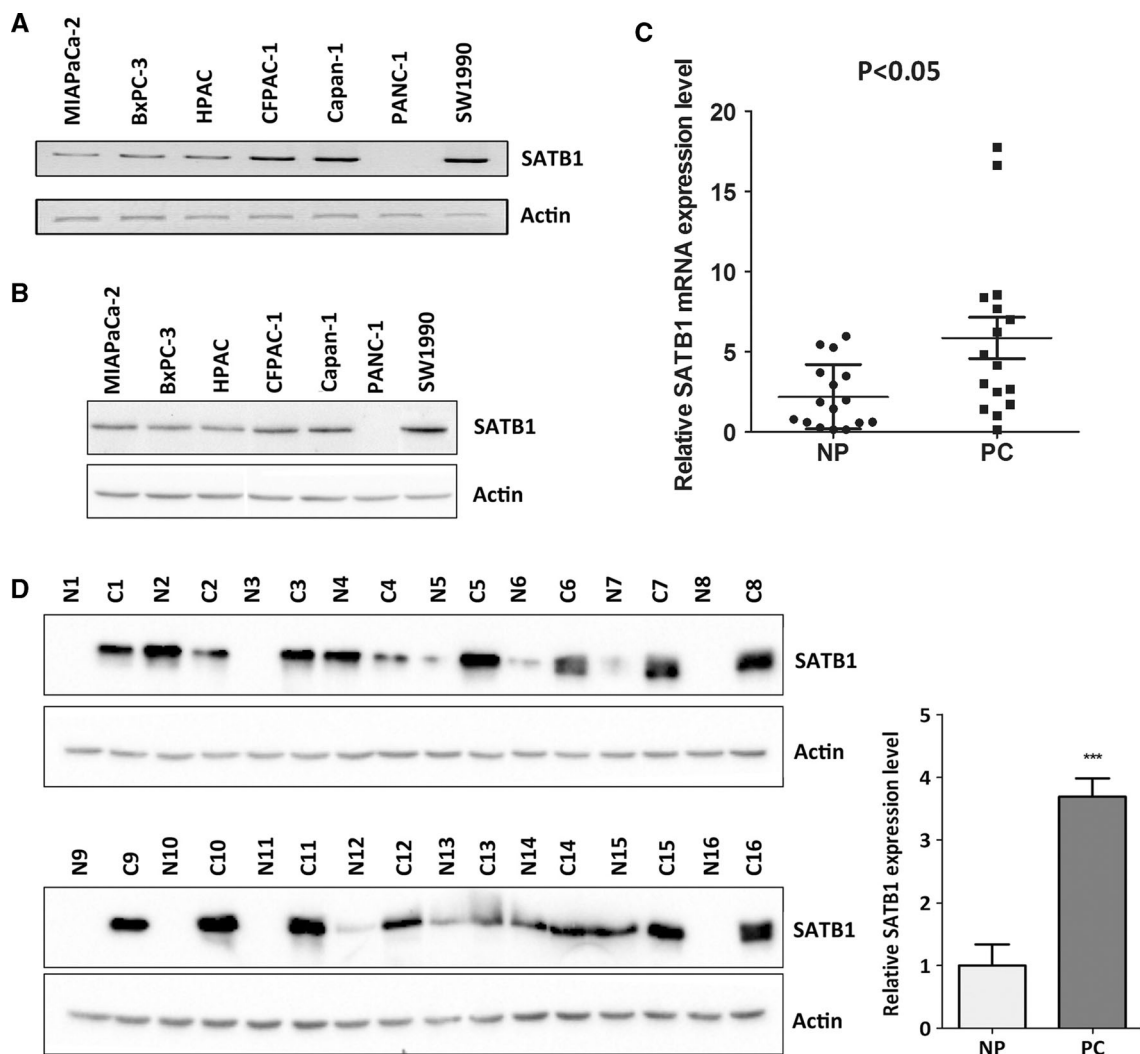


Fig. 1 Overexpression of SATB1 in pancreatic cancer cell lines and tissue samples. **a** PCR analysis of SATB1 mRNA expression level in seven human pancreatic cancer cell lines. **b** SATB1 protein expression level in pancreatic cancer cell lines. **c** Real-time PCR showing SATB1 mRNA expression level in 16 pairs of human pancreatic cancer tissue samples. *Horizontal bars* indicate the arithmetic mean.

Error bars indicate standard deviation (SD). $P < 0.05$, Mann-Whitney U test. **d** Western blot analysis of SATB1 protein expression level in 16 pairs of human pancreatic cancer (C) or normal pancreas tissue (N) samples. **e** Quantification data of panel (d) $***P < 0.001$, t test

strongly increased invasive activity than control cells (Fig. 3d, $P < 0.001$). To further confirm our results, we transiently knocked down SATB1 in SW1990 and Capan-1 cells using SATB1 siRNA transfection. Our results demonstrated that SATB1 siRNA knockdown significantly decreased SATB1 protein level in SW1990 and Capan-1 cells (Fig. 4a). As expected, the transiently knockdown of SATB1 (SATB1 siRNA cells) decreased both nuclear EdU staining-positive cell number and invasion activity in SW1990 (Fig. 4b, d) or Capan-1 (Fig. 4c, e) cells compared with control. These results clearly indicated that SATB1 promotes pancreatic cancer cell proliferation and invasion.

SATB1 Regulates MYC and MMP9 in Pancreatic Cancer Cells

To investigate the underlying mechanisms by which SATB1 promotes pancreatic cancer proliferation and invasion, we examined MYC and MMP9 mRNA and protein expression levels in SATB1 overexpression or knockdown cells. Our data indicated that the stably knockdown of SATB1 in SW1990 cells significantly decreased the mRNA and protein levels of MYC and MMP9 compared with control cells (Fig. 5a). On the other hand, PANC-1 SATB1-overexpressing cells induced more MYC and MMP9 mRNA or protein levels than control cells (Fig. 5b). These data clearly

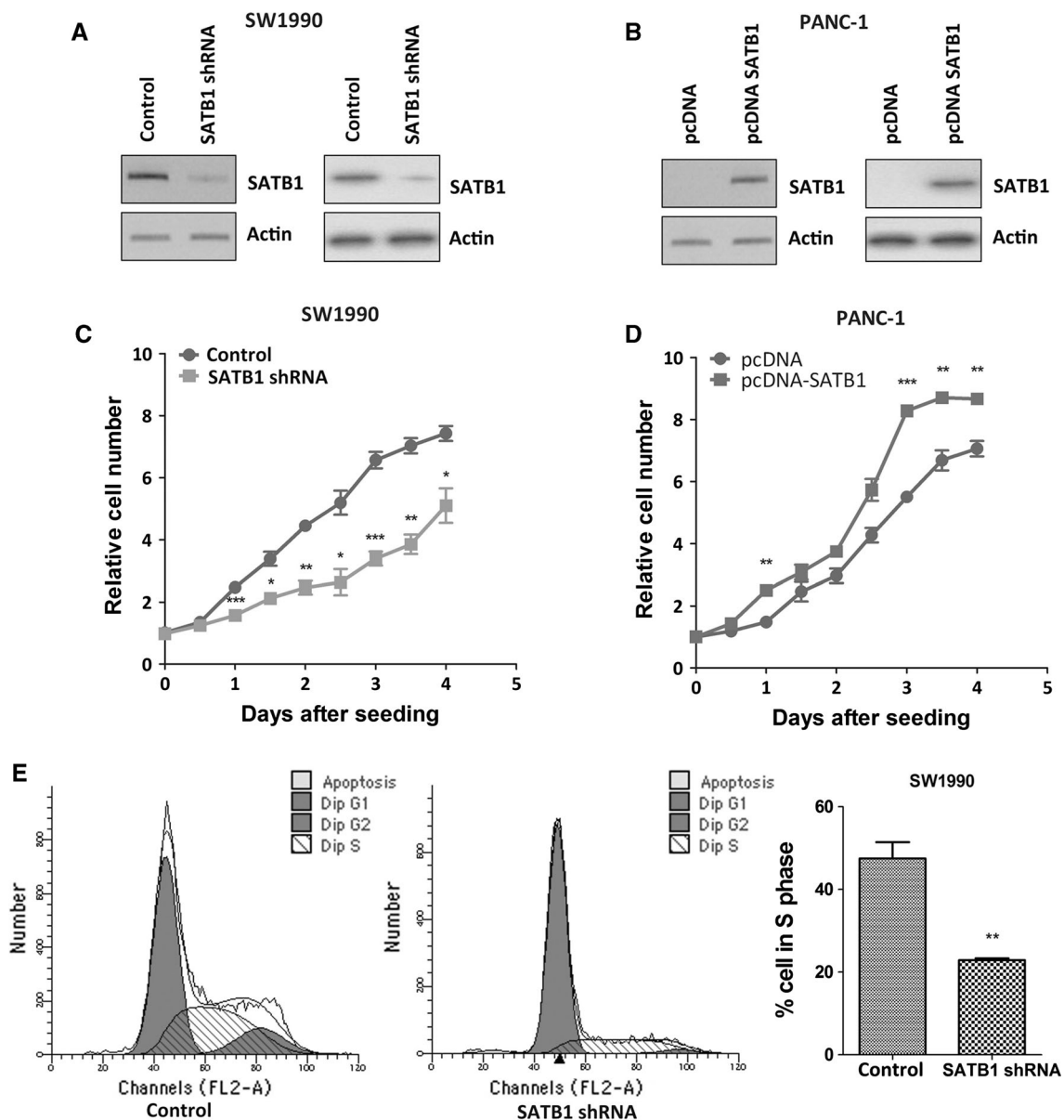


Fig. 2 Stably overexpression or knockdown of SATB1 regulates pancreatic cancer cell proliferation. **a** SATB1 expression level detected by PCR (*left panel*) and western blot (*right panel*) in SW1990 stably knockdown SATB1 (SATB1 shRNA) and control (control) cells. **b** SATB1 expression level detected by PCR (*left panel*) and western blot (*right panel*) in PANC-1 stably overexpressing SATB1 (pcDNA SATB1) or control (pcDNA) cells. **c** MTT cell

growth curve analysis of SW1990 stably knockdown SATB1 or control cells. **d** MTT cell growth curve analysis of PANC-1 stably overexpressing SATB1 or control cells. **e** Cell cycle distribution analysis of control (*left panel*) or SW1990 stably knockdown SATB1 cells (*middle panel*) and quantification data (*right panel*). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, t test

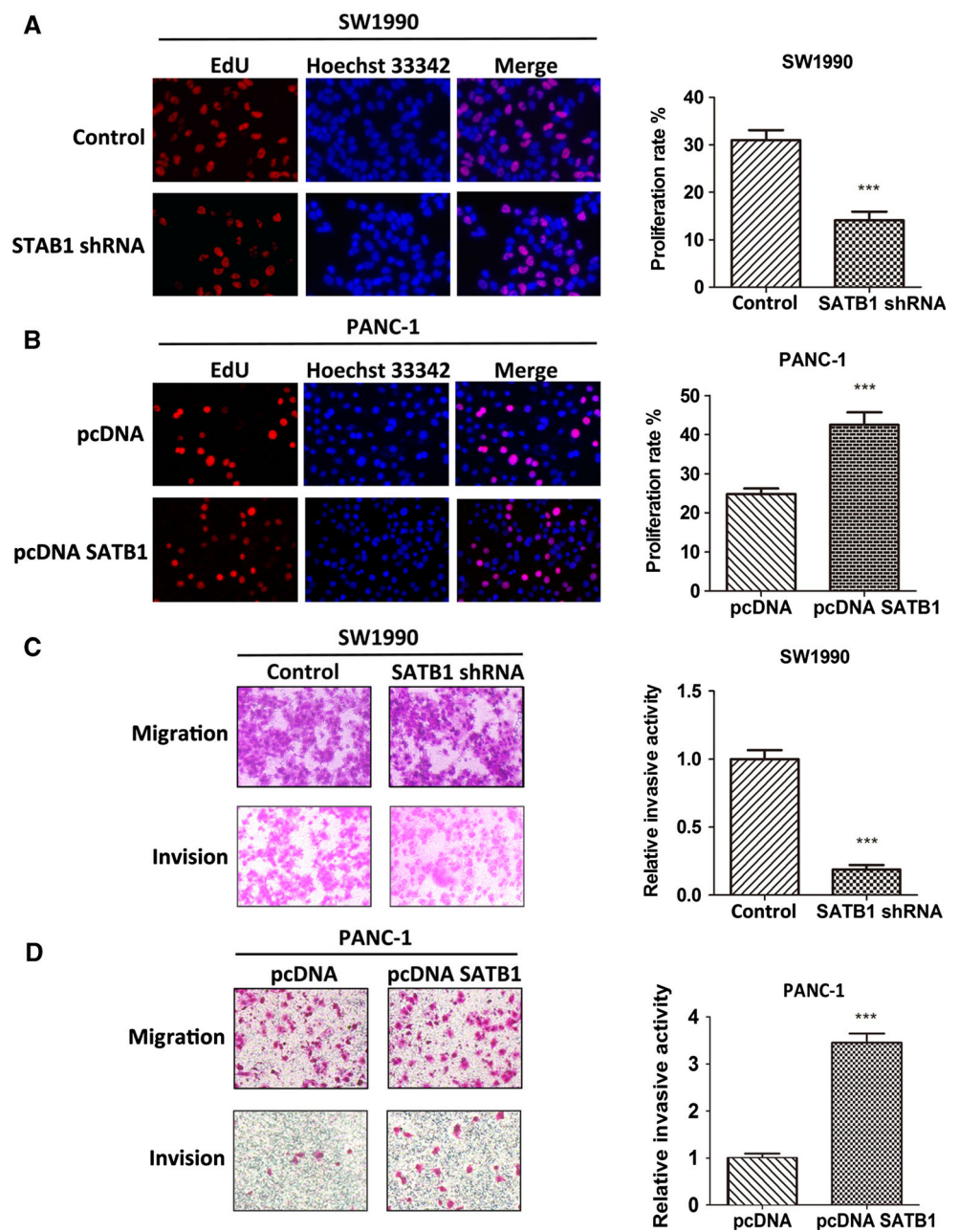
indicated that SATB1 regulates the expression level of MYC and MMP9 in pancreatic cancer cells.

Blocking MYC Attenuates SATB1-Mediated Proliferation and Invasion in Pancreatic Cancer Cells

To further study whether SATB1 promotes pancreatic cancer proliferation and invasion through the induction of

MYC, we knocked down MYC with MYC siRNA in PANC-1 stably overexpressing SATB1 or control cells. Western blot results demonstrated that the inhibition of MYC by siRNA-attenuated SATB1 induced the expression of MMP9 protein level (Fig. 5c) in PANC-1 stably overexpressing SATB1 cells. Cell growth curve and invasion assay analysis demonstrated that the knockdown of MYC expression using siRNA eliminated the stimulation of cell growth and invasion by SATB1 in PANC-1 stably

Fig. 3 Stably overexpression or knockdown of SATB1 regulates pancreatic cancer cells growth and invasion. **a** EdU assay analysis of SW1990 stably knockdown SATB1 (SATB1 shRNA) or control (control) cells (*left panel*) and quantification data (*right panel*). **b** EdU assay analysis of PANC-1 stably overexpressing SATB1 (pcDNA SATB1) or control (pcDNA) cells (*left panel*) and quantification data (*right panel*). **c** Transwell migration/invasion analysis of SW1990 stably knockdown SATB1 or control cells (*left panel*) and quantification data (*right panel*). **d** Transwell migration/invasion analysis of PANC-1 stably overexpressing SATB1 or control cells (*left panel*) and quantification data (*right panel*), *** $P < 0.001$, t test



overexpressing SATB1 cells compared with control siRNA (Fig. 5d, e). Therefore, our results demonstrated: (1) SATB1 promoting pancreatic cancer proliferation and invasion through the induction of MYC; (2) the induction of MMP9 by SATB1 in pancreatic cancer was dependent on MYC induction; (3) SATB1-MYC-MMP9 axis played an important role in pancreatic cancer progression.

SATB1 Binds to a Specific MYC Promoter Region Inducing MYC mRNA Expression

To investigate whether SATB1 binds to MYC promoter regions, we analyzed MYC promoter activities in SW1990

stably knockdown SATB1 or control cells. Firstly, seven different lengths of MYC promoter regions (R1 to R7, from -3090 to +100) were investigated using dual-luciferase reporter assay. The result showed that SATB1 knockdown significantly decreased luciferase activity in MYC promoter region R1 to R3 compared with control cells (Fig. 6a, b; $P < 0.001$) but not in R4 to R7 (Fig. 6a). This indicated that MYC promoter region -2100 to -1567 contains SATB1 binding sites. Secondly, another six different lengths of MYC promoter regions (R8 to R13, from -2100 to -1560) were selected and examined to further identify the specific SATB1 binding sites in MYC promoter region. Our data indicated that the knockdown of

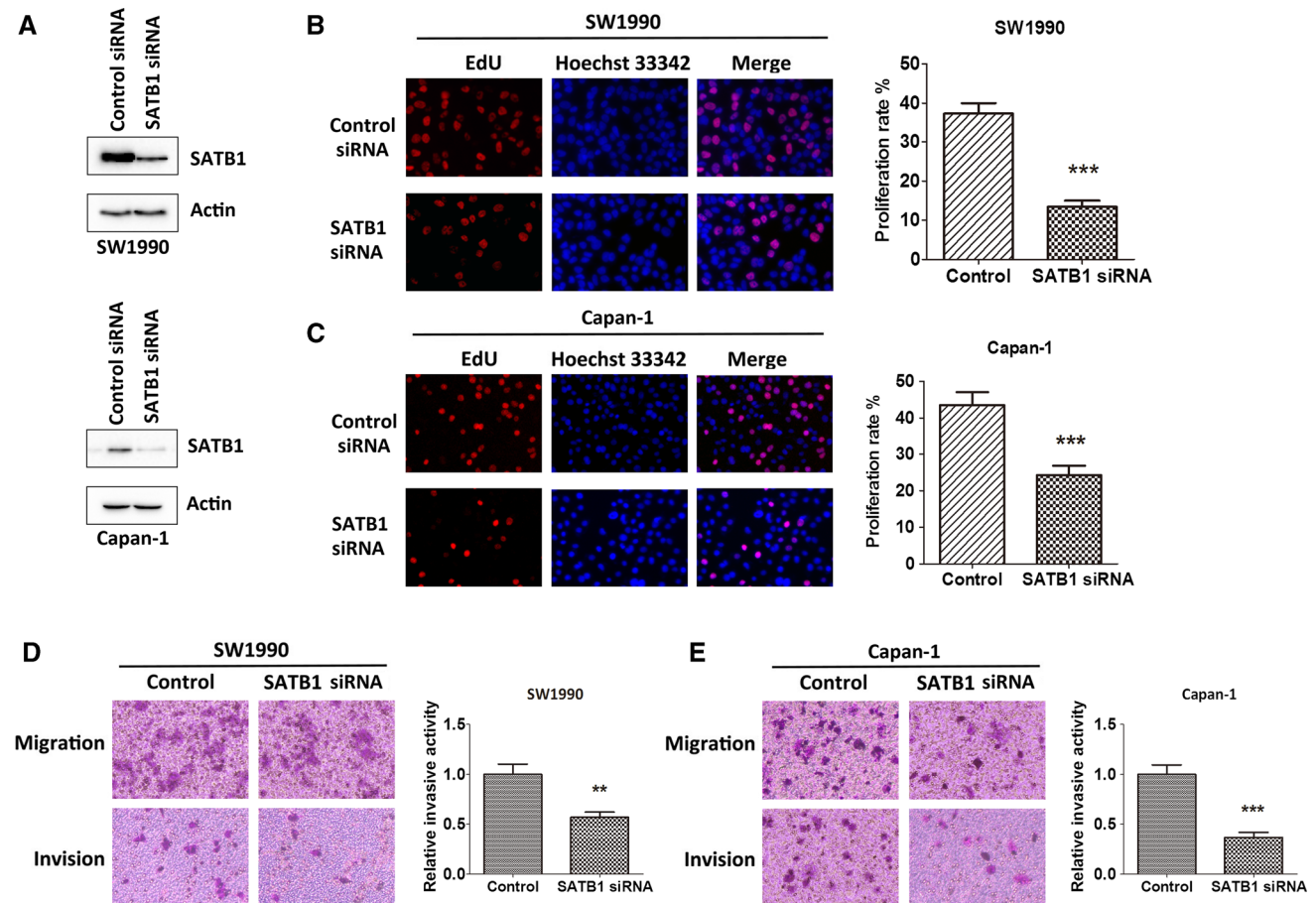


Fig. 4 Transiently knockdown SATB1 with siRNA decreases pancreatic cancer cells proliferation and invasion. **a** Western blot results showing SATB1 protein levels in PANC-1 (upper panel) or Capan-1 (lower panel) transiently knockdown SATB1 by siRNA or control cells. **b, c** EdU cell proliferation analysis of SW1990 or Capan-1

transiently knocking down SATB1 or control cells (left panels) with quantification data (right panels), $***P < 0.001$, *t* test. **d, e** Transwell migration/invasion analysis of SW1990 or Capan-1 knocking down SATB1 or control cells (left panels) with quantification data (right panels), $**P < 0.01$, $***P < 0.001$, *t* test

SATB1 decreased luciferase activity in MYC promoter region R8 to R11 compared with control cells (Fig. 6a, c; $P < 0.001$) but with no difference in R12 and R13. These results demonstrated that SATB1 binds to the specific MYC promoter region between -1774 and -1741 inducing MYC mRNA expression.

Overexpression of SATB1 in Human Pancreatic Cancer Samples Correlates with Tumor Progression

To examine whether SATB1 overexpression promotes human pancreatic cancer invasion and metastasis, we investigated the correlation between SATB1 expression and cancer clinic-pathologic features in 68 formalin-fixed paraffin-embedded pancreatic cancer tissue samples. Our analysis showed that SATB1 expression level was significantly higher in T3 + T4 pancreatic cancer samples compared with T1 + T2 samples in cancer invasion depth (Table 1, $P = 0.028$). In tumor staging, advanced

pancreatic cancer samples (stage II–III + stage III) expressed higher level of SATB1 than low staging samples (stage I + stage II) (Table 1, $P < 0.001$). These data clearly demonstrated that SATB1 expression level correlates with pancreatic cancer invasion and metastasis.

Discussion

Pancreatic cancer is the leading cause of cancer-related death in Western countries [36]. Although much work has been done to improve the diagnosis and treatment of this malignancy, it has proved difficult to inhibit its progression [37]. Adenocarcinoma cancer cells share common malignant biological properties. Same oncogenes or tumor suppressor genes and their associated signaling pathways may be involved in tumor formation from different organs, such as the TP53 gene or PI3K/AKT signaling pathways

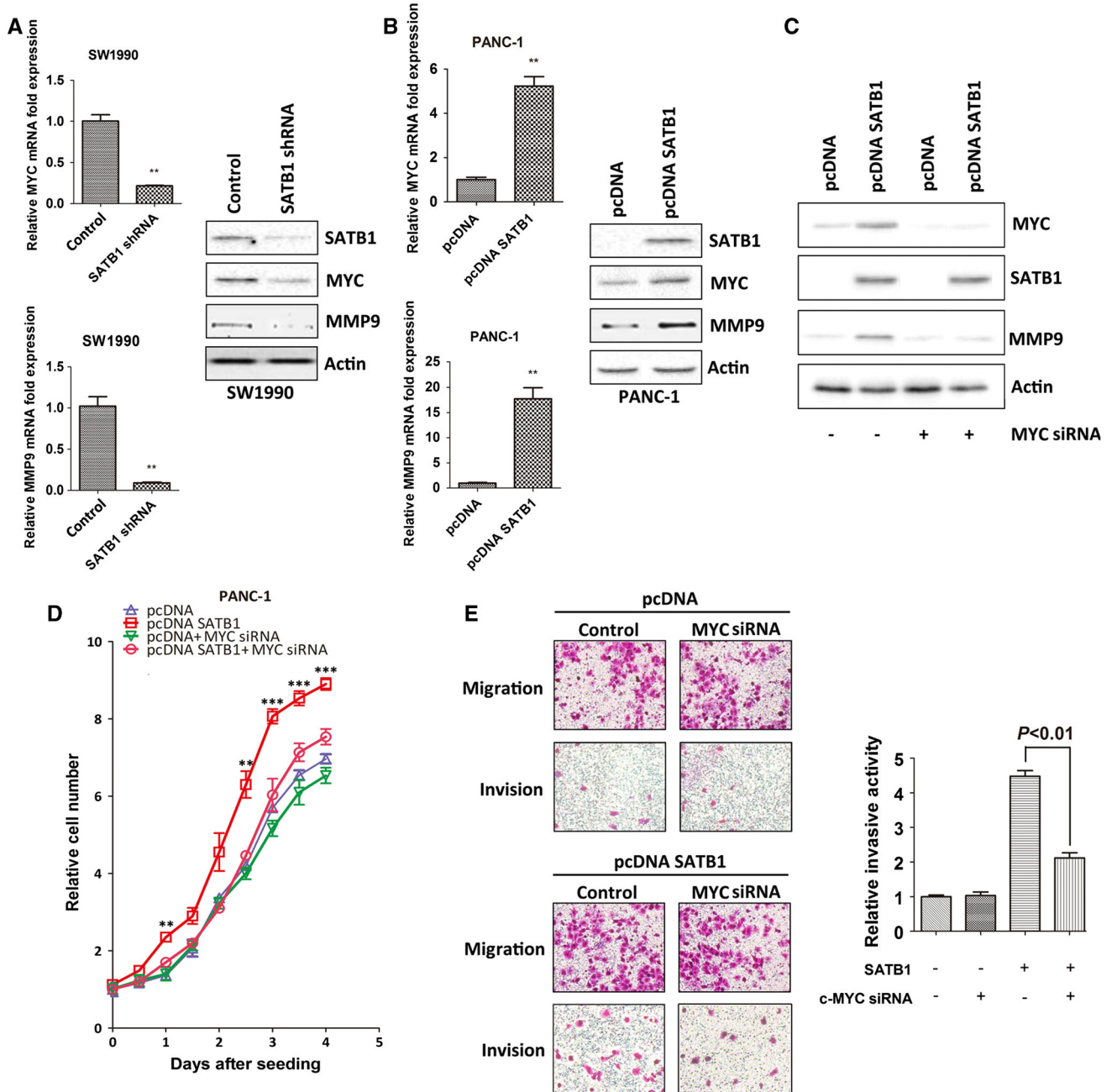


Fig. 5 SATB1 promotes pancreatic cancer proliferation and invasion dependent on MYC and MMP9 induction. **a** MYC and MMP9 mRNA or protein expression levels detected by qRT-PCR (left panels) and western blot (right panel) in SW1990 stably knockdown SATB1 (SATB1 shRNA) or control (Control) cells. **b** MYC and MMP9 mRNA or protein expression levels detected by qRT-PCR (left panels) and western blot (right panel) in PANC-1 stably overexpressing SATB1 (pcDNA SATB1) or control (pcDNA) cells. **c** Western blot analysis of MYC or MMP9 protein expression levels

in PANC-1 stably overexpressing SATB1 or control cells with or without MYC siRNA knockdown. **d** MTT cell growth curve analysis of PANC-1 stably overexpressing SATB1 or control cells with or without MYC siRNA knockdown, $**P < 0.01$, $***P < 0.001$, compared with control cells, *t* test. **e** Transwell migration/invasion analysis of PANC-1 stably overexpressing SATB1 or control cells with or without MYC siRNA knockdown (left panel) and quantification data (right panel), $P < 0.01$, one-way ANOVA

[38–40]. Although SATB1 was first reported in breast cancer [16], we have identified it as a strong potential treatment target for pancreatic cancer.

In this study, we investigated SATB1 expression in pancreatic cancer cell lines and tissue samples. Our results demonstrated that SATB1 promotes pancreatic cancer

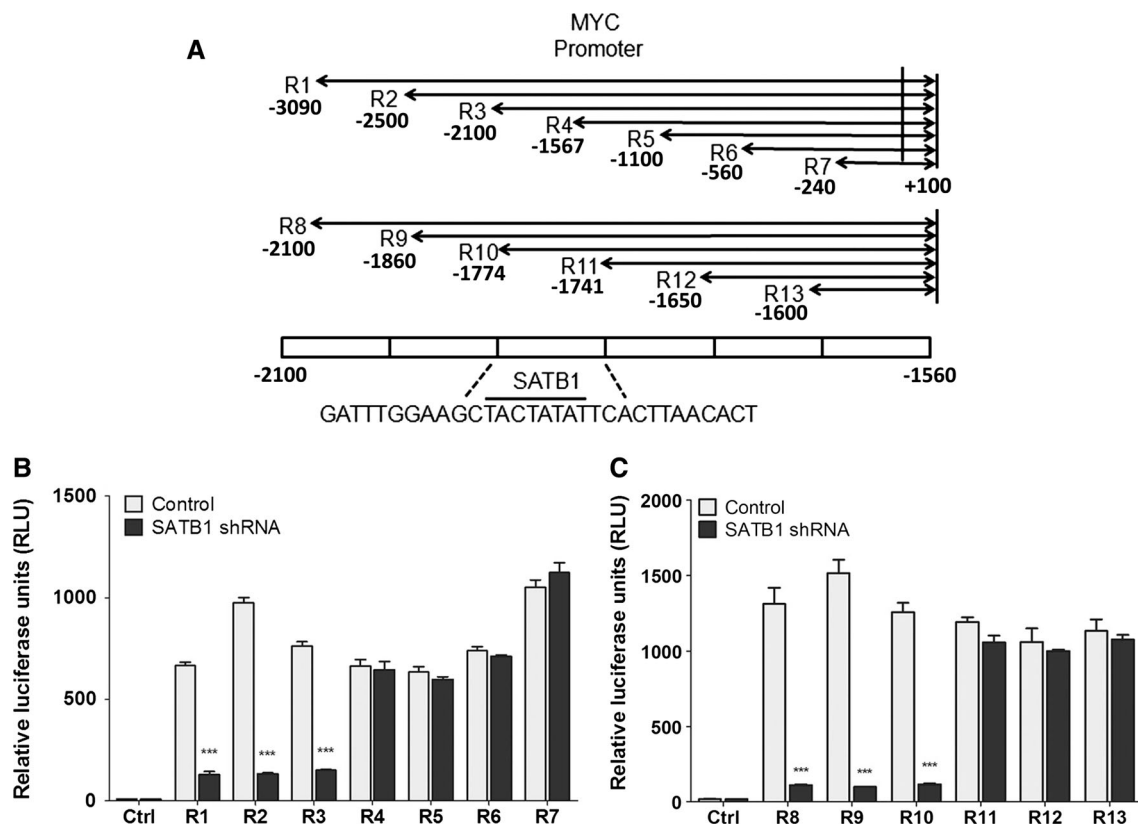


Fig. 6 Specific SATB1 binding sites localize in MYC promoter region. **a** Thirteen different lengths of MYC promoter regions were cloned into PGL3-basic reporter plasmid (*upper panel*). Localization of specific SATB1 binding site in MYC promoter region in pancreatic cancer cells (*lower panel*). **b** First round of luciferase MYC promoter reporter assay analysis in R1 to R7 MYC promoter regions in

SW1990 stably knockdown SATB1 (SATB1 shRNA) or control (Control) cells. **c** Second round of MYC promoter reporter assay analysis in R8 to R13 MYC promoter regions in SW1990 stably knockdown SATB1 (SATB1 shRNA) or control (Control) cells. *** $P < 0.001$, t test

proliferation and invasion through the regulation of MYC. This is supported by several lines of evidence: (1) We showed that SATB1 was overexpressed in pancreatic cancer cells and tissue samples; (2) SATB1 promoted pancreatic cancer cells proliferation and invasion depending on the induction of MYC; (3) using luciferase reporter assay, we found that SATB1 induced MYC expression by binding to a specific region of the MYC promoter; and (4) clinical data from human primary pancreatic cancer samples indicated that SATB1 was strongly correlated with tumor invasion depth and staging.

SATB1 was originally identified as a protein that binds to base-unpairing regions (BURs) of DNA [41], folding chromatin to regulate gene expression [12, 42]. SATB1 overexpression was observed in many kinds of human tumors, promoting cancer cells growth and metastasis by changing the gene expression profile [16, 43, 44]. Herein, we examined that the role of SATB1 plays in pancreatic cancer. Our data demonstrated that SATB1 was overexpressed in pancreatic cancer cell lines and tissue samples. And we further discovered SATB1

significantly promoted pancreatic cancer cell proliferation and invasion. These results clearly indicated that SATB1 could be a promising target for inhibiting pancreatic cancer progression.

MYC overexpression and continued presence are associated with many human malignancies including pancreatic cancer, promoting cancer cells proliferation, invasion, angiogenesis, and metastasis [45–49]. MYC helps cancer cells to pass through the restriction point and progress from the G1 to the S phase [50]. And recently, a study has shown that SATB1 bound to -1531 to -1507 promoter sequence from the transcription start site of MYC gene [42]. MMP9 is a target of MYC, promoting cancer cells invasion and metastasis [51]. In this study, we investigated SATB1-MYC-MMP9 axis in pancreatic cancer. Our results demonstrated that SATB1 regulates MYC and MMP9 expression in mRNA and protein levels, indicating that MYC or MMP9 could be direct downstream targets of SATB1. This is further confirmed by the results that knockdown of MYC significantly attenuated SATB1's oncogenic function and the induction of MMP9. Previous

Table 1 Correlation of clinic-pathological features of the patients with pancreatic cancer and SATB1 expression

Clinic-pathologic features	No. of cases	SATB1 protein expression		<i>P</i> value
		Positive	Negative	
Age (year)				
<60	26	17	9	0.927
≥60	42	27	15	
Gender				
Male	41	28	13	0.446
Female	27	16	11	
Depth of invasion				
T1 + T2	13	5	8	0.028*
T3 + T4	55	39	16	
TNM staging				
I + I-II	23	7	16	< 0.001*
II	9	5	4	
II-III + III	36	32	4	
Lymph node metastasis				
Present	26	19	7	0.256
Absent	42	25	17	
Perineural invasion				
Yes	34	24	10	0.310
No	34	20	14	

P value represents the probability from a Chi-square test for SATB1 expression in pancreatic cancer tissues

studies suggested that SATB1 was identified as a protein that binds to gene promoters, regulating gene and protein expression levels [41, 52]. Our data indicated that SATB1 directly binds to a specific MYC promoter region, which identifying MYC as a downstream target of SATB1. A similar SATB1 binding sequence is reported in the human and mouse MYC promoter region [42]. Since SATB1 regulates gene expression in different ways by binding to the 5' BURs [52], future studies could explore the further various molecular mechanisms of MYC regulation by SATB1 in pancreatic cancer.

A recent study indicated that the expression of SATB1 was an independent predictor of a significantly shorter recurrence-free survival and overall survival in pancreaticobiliary-type periampullary adenocarcinomas [53]. Our similar findings between SATB1 expression level and pancreatic cancer pathogenic-clinical features indicated that SATB1 was highly involved in cancer invasion and metastasis. Altogether, this is strong evidence confirming our *in vitro* results, showing that SATB1 promotes pancreatic cancer proliferation and invasion and plays a key role in tumor progression. Due to the difficulty in diagnosing pancreatic cancer in the early stage and the lack of effective treatment in the advanced stages of the disease [54, 55], our novel findings make SATB1 a potential therapeutic target of pancreatic cancer.

Conclusions

To our knowledge, these are the first results clearly demonstrating that SATB1 promotes pancreatic cancer cell proliferation and invasion through activation of MYC. The results elucidate the importance of SATB1 in understanding pancreatic cancer tumorigenesis, making SATB1 a promising therapeutic target for pancreatic cancer.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No. 81072031 [BA10]) and Natural Science Foundation of Jiangsu Province (No. BK2010585).

Conflict of interest The authors declare that they have no conflict of interest.

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