Long non-coding RNA DSCAM-AS1 promotes pancreatic cancer progression via regulating the miR-136-5p/PBX3 axis

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ABSTRACT

LncRNA down syndrome cell adhesion molecule antisense 1 (DSCAM-AS1) plays an important role in tumor progression, but its function in pancreatic cancer is unknown. DSCAM-AS1 level was evaluated by in situ hybridization (ISH) assay and qRT-PCR. DSCAM-AS1 was knocked down in pancreatic cancer cells, and its impacts on cell proliferation, invasion, and migration were detected. The binding relationship among DSCAM-AS1, miR-136-5p, and pre-B-cell leukemia homebox 3 (PBX3) was investigated by bioinformatic analysis and luciferase reporter assay. An in vivo animal model was constructed to determine the role of DSCAM-AS1 in tumor growth. Our results showed that DSCAM-AS1 was elevated in tumor tissues of pancreatic cancer patients and cell lines. DSCAM-AS1 knockdown efficiently inhibited PANC-1 cell proliferation, migration, and invasion and suppressed tumor growth. DSCAM-AS1 could promote PBX3 expression by sponging miR-136-5p, and its function in pancreatic cancer was partially mediated by the miR-136-5p/PBX3 axis. Overall, DSCAM-AS1 knockdown inhibits pancreatic cancer progression by modulating the miR-136-5p/PBX3 axis.

Introduction

Pancreatic cancer is a common invasive malignancy with approximately 227,000 deaths annually worldwide [1]. Although great advances have been achieved in pancreatic cancer diagnosis and treatment [2,3], the overall five-year survival rate is still low (about 6%) [4]. Hence, identifying novel diagnostic and therapeutic targets for pancreatic cancer is urgently needed.

LncRNAs are emerging as novel regulators in human cancer with more than 200 nt in length [5]. In pancreatic cancer, more and more LncRNAs with important roles in cancer progression have been identified, such as HOXA-AS2 [6], BX111 [7], ZEB2-AS1 [8], and HOTTIP [9]. LncRNA down syndrome cell adhesion molecule antisense 1 (DSCAM-AS1) is a newly identified LncRNA [10] with a significant impact on the development of various human cancers. For instance, DSCAM-AS1 is highly expressed in osteosarcoma tumor tissues and aggravates tumor cell proliferative rate and invasive ability [11]. Moreover, DSCAM-AS1 is involved in G (1)/S cell cycle transition of breast cancer and promotes tumor growth [10]. Although the crucial functions of DSCAM-AS1 in various cancers are known, its role in pancreatic cancer is still lacking.

MicroRNAs (miRNAs) are another group of small molecule RNAs that exert important functions in tumor progression through modulating mRNA translation and degradation [12]. MiR-136-5p is reported as a tumor suppressor in various human cancers, including bladder cancer [13], laryngeal squamous cell carcinoma [14], and retinoblastoma [15]. Moreover, previous studies have reported that pre-B-cell leukemia homebox 3 (PBX3) is a molecular target of miR-136-5p in multiple tumors, and this axis plays an essential role in cancer progression. For example, miR-136-5p prevents retinoblastoma progression by targeting PBX3 [16]. Previous studies reported that PBX3 is notably upregulated in pancreatic cancer tissues, and high PBX3 level is positively correlated
to the tumor progression of AsPC-1 and BxPC-3 cells [17].

Here, we hypothesized that lncRNA DSCAM-AS1 might play a role in pancreatic cancer progression, investigated its expression and potential function in pancreatic cancer, and revealed that miR-136-5p/PBX axis mediated the role of DSCAM-AS1 in pancreatic cancer.

Materials and methods

Tissue samples

A total of 30 patients with pancreatic cancer were recruited at West China Hospital, Sichuan University. Tumor tissues and normal pancreatic adjacent tissues were obtained by surgical resection. This study protocol was approved by the Ethics Committees of West China Hospital affiliated to Sichuan University (No. 9632). All patients signed the written informed consent. The clinical characteristics of pancreatic cancer patients are shown in Supplementary Table 1.

Cell culture

Human pancreatic cancer cell lines SW1990, BXPC-3, PANC-1, and PaCa-2, HEK-293 T, and HPDE6-C7 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in DMEM medium containing 10% FBS at 37°C with 5% additional CO2.

Cell transfection

Two specific small interfering RNA targeting DSCAM-AS1 were obtained from GenePharma (Shanghai, China). MiR mimics (5′-ACUCCAAUUGUUUGAUGAUGG-3′), NC mimics (5′-TTCCTCGAACGTGTCACGT-3′), miR-136-5p inhibitor (5′-CAACGTGCAAGAATAC-3′), and NC inhibitor (5′-UACCCCTGCATTGACCTCAAC-3′). For DSCAM-AS1 and PBX3 overexpression, the full length of DSCAM-AS1 and PBX3 was cloned into pcDNA3.1 (Gene Pharma, Shanghai, China), respectively, and the recombinant vectors were named by pDSCAM-AS1 and pPBX3. For establishing stable DSCAM-AS1 knockdown cells, short hairpin RNA against DSCAM-AS1 (sh-DSCAM-AS1, 5′-GGAGATCA CAGCCAAAGAA-3′) and negative control (sh-NC, 5′-TTCTCCGAACGTGTCACGT-3′) were transfected into PANC-1 cells, and stable DSCAM-AS1 knockdown cells were screened out as previously reported [18].

QRT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY) and reversely transcribed into cDNA using Superscript III transcriptase (Invitrogen). Gene expression was detected using PCR on an ABI 7500 qPCR system with specific primers listed in Table 1. Data were analyzed using the 2−ΔΔCt method [19].

Western blot

The expression of interest proteins was detected by Western blot as described in the previous study [20]. Total protein was isolated with RIPA lysis buffer with 1% proteinase inhibitor (PMSF, Boster, China). After separation on 8% SDS-PAGE, protein samples were transferred onto PVDF membrane and incubated with antibodies against PBX3 (47 kDa, Abcam, ab109173, 1: 500) or GAPDH (36 kDa, Abcam, ab9485, 1: 1000) overnight at 4°C. Subsequently, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibodies (1:3000, Boster, Wuhan, China) at 37°C for 1 h. The protein signals were observed with an ECL detection system.

Table 1. Sequences of primers used in qRT-PCR.

| Gene     | Forward primer (5′-3′) | Reversed primer (5′-3′) |
|----------|------------------------|------------------------|
| DSCAM-AS1| ACCACAAAAACAAACACAG    | ATGATGAGACCAACTCC      |
| MiR-136-5p| CAACCTCCAGCTGGGACTCATTTGGTTT | CCAATGCGAGTGGATTGCT    |
| U6       | GCCTGGGAGCCATATACTAAAT | CGCTCCAGGAATTTGCTGGCAT |
| PBX3     | CAATGGGAGCCAATG     | ATGTAGCTCAGGAAAAGT    |
| GAPDH    | GACCCTCTCATGGACCTCAAC | CTCTCCATGGTGGTGAAGA    |
**Luciferase reporter assay**

The wild-type (WT-) or mutant (MUT-) binding sites of DSCAM-AS1 and PBX3 were cloned into luciferase reporter vector psiCheck2. The recombinant luciferase reporter vectors and miR-136-5p mimics or mimics NC were co-transfected into HEK293T cells using Lipofectamine 2000. After two days, the luciferase activities were measured using a Dual-Glo Luciferase Assay System (Promega Corporation). The relative luciferase activity was calculated as the ratio of firefly/RENilla luciferase activity [21].

**Immunofluorescence**

Transfected PANC-1 cells were incubated with anti-PBX3 antibodies (Abcam, ab233136, 1:500) overnight at 4°C. After incubation with FITC-conjugated secondary antibodies (Abcam, ab6717, 1:1000) for 1 h and DAPI for nuclei, images were taken using a Zeiss LSM510 microscope.

**ISH assay**

The tumor tissues were fixed in 4% polyoxymethylene, embedded in paraffin, and cut into 4 μm sections. The ISH assay was performed using the Enhanced Sensitive ISH Detection Kit (POD) (Boster Biotechnology) with Digoxin-labeled DSCAM-AS1 probes from Life Technologies (Shanghai, China) [22]. The images were obtained using a Zeiss LSM510 microscope.

**In vivo model**

The animal model was conducted as previously reported [23]. 5 × 10⁶ stable sh-DSCAM-AS1-transfected PANC-1 cells were subcutaneously inoculated into BALB/c nude mice. Tumor volumes were assessed every week for five weeks based on length×width²/2. After that, mice were sacrificed, and solid tumors were weighted. To detect the proliferative rate in vivo, immunohistochemistry (IHC) assay was performed using anti-Ki67 (ab15580, 1:500, Abcam) as previously described [24]. The study was approved by the Animal Research Committee of West China Hospital, Sichuan University.

**Statistical analysis**

Data were analyzed using Graphpad Prism7 software and expressed as the mean ± standard deviation (SD). Differences between two groups and among multiple groups were compared using two-tailed unpaired Student’s t-test or ANOVA with Scheffe’s post hoc test, respectively. A p < 0.05 represents statistical significance.

**Results**

**DSCAM-AS1 was up-regulated in pancreatic cancer**

For investigating the role of DSCAM-AS1 in pancreatic cancer progression, we first detected its expression in pancreatic cancer tumor tissues and cell lines. ISH (Figure 1a) and qRT-PCR assay (Figure 1b) showed that DSCAM-AS1 was significantly upregulated in pancreatic cancer tissues (p < 0.01), and high DSCAM-AS1 level was positively correlated with tumor progression in patients (Supplementary Table 1). In addition, we found that DSCAM-AS1 levels were higher in the four cancer cell lines than in HPDE6-C7 cells, with the highest level in PANC-1 (Figure 1c). Thus, PANC-1 cells were used for the subsequent studies. Overall, these results suggested that DSCAM-AS1 was highly expressed in pancreatic cancer tumor tissues and cell lines.

**DSCAM-AS1 knockdown inhibited pancreatic cancer progression in vitro**

After detecting DSCAM-AS1 expression, we knocked DSCAM-AS1 down in PANC-1 cells by transfecting two DSCAM-AS1 siRNAs to investigate its function (both p < 0.01, Figure 2a). DSCAM-AS1 knockdown significantly decreased viability (p < 0.01, Figure 2b), colony numbers (p < 0.01, Figure 2c), migration (p < 0.01, Figure 2d), and invasion (p < 0.01, Figure 2e) of PANC-1 cells, suggesting that DSCAM-AS1 knockdown exerted an inhibitory role in pancreatic cancer cell proliferation, migration, and invasion.
DSCAM-AS1 knockdown prevented tumor growth

Subsequently, we established an in vivo animal model by subcutaneously inoculating mice with stable sh-DSCAM-AS1-transfected PANC-1 cells to examine the function of DSCAM-AS1 in pancreatic cancer growth. We found that DSCAM-AS1 knockdown significantly reduced DSCAM-AS1 level in tumor tissues (p < 0.01, Figure 3a) and notably inhibited tumor growth (p < 0.01) (Figure 3b-d). qRT-PCR analysis showed that DSCAM-AS1 knockdown also significantly upregulated miR-136-5p (p < 0.01) and downregulated PBX3 (p < 0.01) in tumor tissues (Figure 3e). Western blot assay confirmed the reduction of PBX3 in tumor tissues by DSCAM-AS1 knockdown (Figure 3f). Furthermore, IHC assay showed that DSCAM-AS1 knockdown obviously decreased Ki-67 positive cells in mice (p < 0.01, Figure 3g). Overall, DSCAM-AS1 knockdown slowed pancreatic tumor growth in vivo.

Figure 1. DSCAM-AS1 was upregulated in pancreatic cancer. (a) IHC assay of DSCAM-AS1 in tumor tissues. ×400, scale bar = 50 μm. (b and c) DSCAM-AS1 level in tumor tissues (n = 30) (b) and cancer cell lines (c).
**DSCAM-AS1 directly bound to miR-136-5p**

Current studies revealed multiple action mechanisms of DSCAM-AS1 in tumor progression, including regulating gene expression by sponging miRNAs to disrupt their functions. Thus, we first confirmed the effects of miR-136-5p mimics and miR-136-5p inhibitor on miR-136-5p levels (both $p < 0.01$, Figure 4a) and predicted the potential binding between DSCAM-AS1 and miR-136-5p via bioinformatics analysis using Starbase software (Figure 4b). To confirm the prediction, we performed luciferase reporter assays in PNAC-1 cells co-transfected with DSCAM-AS1 3′-UTR WT/Mut with miR-136-5p mimics/NC mimics. The results showed that miR136-5p overexpression notably reduced the luciferase activity of DSCAM-AS1 3′-UTR WT ($p < 0.01$) but had no effect on DSCAM-AS1 3′-UTR MUT (Figure 4c). Furthermore, DSCAM-AS1 knockdown significantly elevated miR-136-5p level in PNAC-1 cells (both $p < 0.01$, Figure 4d). Moreover, miR-136-5p mimics significantly reduced DSCAM-AS1 level ($p < 0.01$) while miR-136-5p inhibitor enhanced DSCAM-AS1 level ($p < 0.01$) in PNAC-1 cells (Figure 4e). Furthermore, miR-136-5p levels were significantly reduced in tumor tissues ($p < 0.01$, figure 4f) and had a significantly negative correlation with DSCAM-AS1 level ($p < 0.01$, Figure 4g). We further investigated the correlation of miR-136-5p expression with patients’ clinicopathological parameters. The results showed that high miR-136-5p levels were negatively correlated with tumor progression in patients...
Taken together, these results suggested that DSCAM-AS1 directly targeted miR-136-5p in pancreatic cancer, and miR-136-5p was downregulated in pancreatic tumor tissues.

**MiR-136-5p mimics inhibited pancreatic cancer behaviors, possibly via regulating PBX3 in vitro**

To investigate the function of miR-136-5p in pancreatic cancer, miR-136-5p mimics were transfected into tumor cells. The results showed that miR-136-5p mimics significantly inhibited viability (p < 0.05), colony formation (p < 0.01), migration (p < 0.01), and invasion (p < 0.01) of PANC-1 cells compared with NC mimics (Figure 5a-d). We further investigated the downstream pathway of miR-136-5p using Starbase and found that PBX3 might be a downstream gene of miR-136-5p (Figure 5e). Luciferase reporter assay showed that miR-136-5p overexpression significantly reduced the relative luciferase activity of PBX3 3′-UTR WT (p < 0.01) but had no impact on PBX3 3′-UTR MUT (Figure 5f). PBX3 expression was significantly increased in pancreatic cancer tissues compared with normal controls (Figure 5g). Furthermore, high PBX3 were positively correlated with tumor progression in patients (Supplementary Table 3). Next, we performed rescue experiments by transfecting DSCAM-AS1
overexpression vector into PANC-1 cells (p < 0.01, Figure 5h) and found that miR-136-5p mimics reduced PBX3 expression (p < 0.01) and co-transfection of DSCAM-AS1 with miR-136-5p mimics attenuated the inhibitory impact of miR-136-5p mimics on PBX3 expression (p < 0.01, Figure 5i). Immunofluorescence staining also confirmed that DSCAM-AS1 overexpression relieved the inhibitory effect of miR-136-5p mimics on PBX3 in PANC-1 cells (Figure 5j). Taken together, these results suggested that miR-136-5p played a tumor suppressor role in pancreatic cancer via targeting PBX3.

**DSCAM-AS1 regulated pancreatic cancer progression via modulating miR-136-5p/PBX3**

We further performed rescue experiments to investigate the role of miR-136-5p/PBX3 axis in the function of DSCAM-AS1. PANC-1 cells were transfected in vitro with miR-136-5p mimics, or co-transfected with miR-136-5p mimics and DSCAM-AS1 overexpressing vector (both p < 0.01, Figure 6a). DSCAM-AS1 overexpression notably reversed the inhibitory impacts of miR-136-5p mimics on pancreatic cancer cell proliferation, invasion, and migration (all p < 0.01, Figure 6b-e), suggesting that miR-136-5p mediated the pro-tumor function of DSCAM-AS1.

To investigate the regulatory axis of DSCAM-AS1 and PBX3, we constructed a PBX3 overexpressing vector and confirmed that its transfection significantly increased PBX3 at mRNA and protein levels (p < 0.01, Figure 7a and b). Then we transfected PANC-1 cells with si-DSCAM-AS1-1 or co-transfected with si-DSCAM-AS1-1 and PBX3 overexpressing vector and found that si-DSCAM-AS1-1 significantly reduced PBX3 level (p < 0.01) and co-transfection of PBX3 overexpressing vector obviously abolished the effect of si-DSCAM-AS1-1 on PBX3 level (p < 0.01) (Figure 7c and d). The function assays showed that PBX3 overexpression efficiently reversed the inhibitory impacts of si-DSCAM-AS1-1 on the phenotype of PANC-1 cells (Figure 7e-h). Taken together, these results suggested that miR-136-5p/PBX3 axis mediated the pro-tumor function of DSCAM-AS1-1 in pancreatic cancer.
The study investigated the expression and function of lncRNA DSCAM-AS1 in pancreatic cancer. We found that lncRNA DSCAM-AS1 was downregulated in pancreatic cancer tissues and cell lines and directly targeted MiR-136-5p to mediate its oncogenic role in the tumorigenesis and progression of pancreatic cancer. Moreover, miR-136-5p bound to PBX3 to downregulate its expression to further promote tumor proliferation, migration, and invasion. Our findings extended our knowledge of lncRNA mechanism in pancreatic cancer progression and provided a potential therapeutic target.

LncRNAs play fundamental roles in carcinogenesis. As a typical lncRNA, lncRNA DSCAM-AS1 is first identified in screening differentially expressed transcripts between breast cancer cells and benign breast samples [25]. Subsequent studies have demonstrated that lncRNA DSCAM-AS1 is upregulated and plays a pro-tumor role in lung cancer, colorectal cancer, osteosarcoma, hepatocellular carcinoma, melanoma, and cervical cancer [26]. For example, DSCAM-AS1 is upregulated in many human cancers and functions via miR-384/AKT3 in colorectal cancer [27], miR-877-5p/ATXN7L3 in cervical cancer [28], and miR577/HMGB1 in non-small cell lung cancer [29]. This study investigated its role in pancreatic cancer and found that DSCAM-AS1 was also notably upregulated in pancreatic cancer. In addition, its high expression in tumor tissues was associated with malignant tumor progression in patients, implying that DSCAM-AS1 might be a prognostic biomarker for pancreatic cancer. Other studies also showed that serum DSCAM-AS1 level was increased in patients with hepatocellular carcinoma than in healthy controls [30]. In the future,
we will investigate the potential of serum DSCAM-AS1 as a prognostic marker for patients with pancreatic cancer. DSCAM-AS1 knockdown efficiently inhibited tumor progression, including proliferation, migration, invasion, and in vivo growth, consistent with previous reports. Recent studies have shown that non-coding RNAs (ncRNAs), including lncRNAs, are enriched in exosomes, and exosome treatment has become a promising therapeutic strategy [31]. Hence, identifying DSCAM-AS1 inhibitor-related drugs and how to deliver them to the human body is the next stage of our work.

MiRNAs represent a category of non-coding RNAs with ~22 bp in length. Tumor molecular studies have demonstrated that multiple miRNAs play important roles in tumor progression [32–36]. For example, microRNA-409 is demonstrated to act as a tumor suppressor in pancreatic carcinoma and is associated with a good prognosis in tumor patients [37]. LncRNAs have been identified as specific sponges for miRNAs and inhibit their expression [38]. DSCAM-AS1 was reported to participate in tumor progression by sponging several miRNA targets, such as miR-101-3p in gastric cancer [39], miR-216b in colorectal adenocarcinoma [40], and miR-338-3p in hepatocellular carcinoma [30]. Our study revealed a negative correlation between DSCAM-AS1 and miR-136-5p levels in tumor tissues and confirmed their binding relationship. Moreover, the rescue experiments further confirmed the regulatory network of DSCAM-AS1 and miR-136-5p in pancreatic cancer. MiR-136-5p is a well-known tumor suppressor miRNA in multiple tumor types. For example, miR-136-5p is downregulated in hepatocellular
carcinoma tissues compared with para-non-tumor tissues, and its high expression is associated with a good prognosis [41]. Exosomal miR-136-5p derived from anlotinib-resistant NSCLC cells confers anlotinib resistance in non-small cell lung cancer through targeting PPP2R2A [42]. However, as far as we know, there is no study exploring the role of miR-136-5p in the progression of pancreatic cancer. In addition, whether one or more downstream miRNA targets mediate the role of DSCAM-AS1 in pancreatic cancer needs to be investigated in the future, which will contribute to our understanding of the functions of DSCAM-AS1 in pancreatic cancer.

Previous studies have indicated that miRNAs could regulate gene expression by binding to the 3' untranslated region (3'-UTR) of their targeted genes in cancer [43]. Here, we predicted the downstream targets of miR-136-5p and found that PBX3 might be a target of miR-136-5p. DSCAM-AS1 could positively regulate PBX3 by modulating miR-136-5p. Low PBX3 level due to si-DSCAM-AS1-1 transfection suppressed tumor cell progression, and PBX3 overexpression reversed the inhibitory effect of si-DSCAM-AS1-1. This phenotype was consistent with a previous finding that high PBX3 levels were closely related to the aggravated progression of pancreatic cancer in vitro [17]. A series of rescue experiments revealed the regulatory axis of DSCAM-AS1/miR-136-5p/PBX3 in pancreatic cancer. However, a single miRNA might play its essential role in human cancers by targeting one or more downstream genes [44,45]. For example, miR-136-5p could target several downstream genes, including CBX4 in cervical cancer [46], BCL2 in non-small cell lung cancer [47], ROCK1 in head and neck

**Figure 7. DSCAM-AS1 regulated pancreatic cancer progression via modulating PBX3.** (a and b) The transfection efficiency by qRT-PCR (a) and Western blot (b). (c and d) PBX3 level by qRT-PCR (c) and Western blot (d). (e) CCK-8 assay. (f) Colony formation assay. (g) Migration assay. (h) Invasion assay. ×200, scale bar = 100 μm.
cancer [48], MMP2 in hepatocellular carcinoma [49], and XIAP in ovarian tissues [50]. Hence, other potential regulatory axes for miR-136-5p in pancreatic cancer should be explored.

Although our study revealed a regulatory axis in pancreatic cancer, whether manipulating miR-136-5p and PBX3 in vivo could affect the function of DSCAM-AS1 should be demonstrated in subsequent experiments.

Conclusion

DSCAM-AS1 promoted pancreatic cancer progression by regulating the miR-136-5p/PBX3 axis, suggesting that this axis could be a novel target for pancreatic cancer treatment.

Research Highlights

DSCAM-AS1 plays a pro-tumor role in pancreatic cancer. DSCAM-AS1 promotes PBX3 expression via sponging miR-136-5p. DSCAM-AS1/miR-136-5p/PBX3 participates in pancreatic cancer progression.

Ethical Approval and Consent to participate

All patients signed the written informed consent. All procedures were approved by West China Hospital, Sichuan University Ethics Committee and Animal Ethics Committee. Procedures operated in this research were completed in keeping with the standards set out in the Announcement of Helsinki and laboratory guidelines of research in China and the principles on ethical animal research outlined in the Basel Declaration.

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Availability of supporting data

The data that support the findings of this study are available on request from the corresponding author.

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