Original Article

SASH1 inhibits hypoxia-induced epithelial-to-mesenchymal transition via suppression of the PI3K/Akt pathway in human pancreatic cancer cells

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Abstract: Hypoxia is a common characteristic in solid tumor development and progression and it may induce epithelial-mesenchymal transition (EMT) in cancer cells. The SASH1 (SAM- and SH3-domain containing 1) gene, a member of the SLY (SH3 domain-containing expressed in lymphocytes) family of signal adapter proteins, has been implicated in tumorigenesis of many types of cancers. However, the biological role of SASH1 remains unknown in pancreatic cancer. To unravel the function of SASH1, we explored the expression of SASH1 in pancreatic cancer tissues and investigate the effect of SASH1 on hypoxia-induced EMT in pancreatic cancer cells. Here, we found that SASH1 was lowly expressed in human pancreatic cancer cell lines, and hypoxia significantly inhibited SASH1 expression at both mRNA and protein levels in PANC-1 cells. Then, overexpression of SASH1 prevented hypoxia-induced migration/invasion and EMT process in PANC-1 cells. Moreover, overexpression of SASH1 prevented hypoxia-induced the expression of HIF-1α, phosphorylated PI3K and Akt in PANC-1 cells. Taken together, our study found that SASH1 prevented hypoxia-induced EMT partly by negatively regulating the expression of hypoxia-inducible factor (HIF)-1α via blocking PI3K/Akt signaling pathway in human pancreatic cancer cells. These results strongly suggest that SASH1 could be a promising therapeutic target for the treatment of human pancreatic cancer.

Keywords: SASH1, pancreatic cancer, hypoxia, epithelial-to-mesenchymal transition (EMT)

Introduction

Pancreatic cancer is the most aggressive cancer worldwide. Although the range of therapeutic strategies available for treatment of this disease has improved over the past decades, the 5-year survival rate for pancreatic cancer patients was less than 6% [1]. The lethality of pancreatic cancer is largely due to the aggressive local invasion, metastases, and resistance to chemotherapy [2]. The epithelial-mesenchymal transition (EMT) plays a major role in cancer progression, invasion and metastasis [3-5]. During EMT procedure, the actin cytoskeleton is reorganized and cells acquire increased cell-matrix contacts, leading to dissociation from surrounding cells and enhanced migratory and invasive capabilities [6].

Hypoxia is an essential component of the tumor microenvironment, often initiates multiple cellular responses, including proliferation, invasion and angiogenesis, resulting in the development and progression of cancer [7]. Therefore, an understanding of the phenotypic changes induced by hypoxia and the underlying molecular mechanisms is essential to develop appropriate and effective cancer treatment modalities.

The SASH1 (SAM- and SH3-domain containing 1) gene, a member of the SLY (SH3 domain-containing expressed in lymphocytes) family of signal adapter proteins, is composed of one Src homology 3 (SH3) domain, which is necessary for the protein-protein interaction and mediates the formation of signaling complexes [8]. Mounting evidence exists to suggest that SASH1 plays an important role in tumorigenesis, invasion and metastasis [9-11]. For example, Chen et al. reported that the overexpression of SASH1 may inhibit cell migration and invasion, as well as the protein expression of cyclin D1, matrix metalloproteinase-1 (MMP-1), and MMP-2 in
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However, the biological role of SASH1 remains unknown in pancreatic cancer. To unravel the function of SASH1, we explored the expression of SASH1 in human pancreatic cancer cell lines and investigate the effect of SASH1 on hypoxia-induced EMT in human pancreatic cancer cells.

Materials and methods

Cell line and culture conditions

The human pancreatic cancer cell lines (PANC-1, BxPC-3, and Capan-1) and the nonmalignant hTERT-HPNE were obtained from Centre for Cell Resources of Shanghai Institutes for Life Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a humidified incubator with 5% CO₂ and 95% air.

As the hypoxic environment, cells were exposed to 0.5% O₂ balanced with 5% CO₂/94.5% N₂ in the multi-gas incubator (MCO-5M; Sanyo, Osaka, Japan).

Quantitative real-time PCR

Total RNA from PANC-1 cells was prepared with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 5 μg of isolated RNA was subsequently reverse-transcribed using an oligo-(dT) primer and reverse transcriptase (Qiagen) according to the manufacturer's protocol. The primer sequences used for PCR amplification were: SASH1 forward, 5'-TCCCGTCACAGGAAGAAACG-3', and reverse, 5'-GATACCCATCACGTCGGTCC-3'; hypoxia-inducible factor (HIF)-1α forward, 5'-AGCCGAGGAGAACTATGAAC-3', reverse: 5'-ATTTGAGGGTGAGGAATTGG-3'. β-actin was used as an internal standard and the primers were as follows: forward, 5'-GTCCACCGCAAATGCTTCTA-3', and reverse, 5'-TGCTGTACCTTACCGTTC-3'. The cycling conditions included a holding step at 95°C for 10 minutes, and 35 cycles of 95°C for 20 seconds, 59°C for 30 seconds, and 68°C for 30 seconds. The relative fold expression was calculated using 2^ΔΔCt method. Each analysis was performed in triplicate.

Western blotting

Total protein was extracted from PANC-1 cells using RIPA lysis buffer (Beyotime, Nantong, China) according to the manufacturer's instructions. Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Whatman Schleicher & Schuell, Middlesex, UK). The membranes were blocked in 5% nonfat milk in TBST buffer (5 mM Tris-HCl, pH 7.4, 136 mM NaCl, 0.1% Tween 20) for 1 h at room temperature before hybridization with primary antibodies [anti-SASH1, anti-E-cadherin, anti-vimentin (1:2000; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-Pi3K, anti-phospho-Pi3K, anti-Akt, anti-phospho-Akt and anti-GAPDH (1:1500; Cell Signaling, Danvers, MA, USA)] overnight at 4°C. Following three washes with TBST buffer, HRP-conjugated secondary antibodies were introduced, and enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ) was used for detection. The signals were quantified by densitometry using Scion Image software (Scion Corporation, Frederick, MD, USA).

Cell migration and invasion assays

The invasive and migration behaviors of PANC-1 cells were determined by Transwell chamber (Corning Costar Corp., Cambridge, MA, USA) assay with or without coated Matrigel (BD Biosciences, Bedford, MA, USA). PANC-1 cells (5×10⁴ cells/ml) suspended in RPMI medium were added to the upper chamber. The lower chamber was filled with complete medium. The cells were incubated at 37°C in the incubator supplemented with 5% CO₂ for 24 h. Not migrating cells on the upper side of the filter were wiped off and migrating cells on the reverse side of the filter were stained with 0.1% crystal violet in 20% methanol for 30 min. Cells in the lower surface of the filter were photographed under a light microscope (100× magnification).

Construction of the pcDNA3.1-SASH1 vector and cell transfection

The full-length SASH1 open reading frame was amplified from human thyroid cancer cells by RT-PCR, and cloned into the pcDNA3.1 expression vector to construct the pcDNA3.1-SASH1 recombinant expression vector. PANC-1 cells were transfected with pcDNA3.1-SASH1 or pcDNA3.1 (empty vector) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to...
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the manufacturer’s instructions. Stable transfectants were clonally selected in complete medium containing 1 μg/ml G418 (Sigma, St. Louis, MO, USA).

Statistical analysis

Data are expressed as means ± SD. Analysis of variance (ANOVA) followed by Dunnett’s t test were performed to determine if the difference between groups was significant. Values of \( P < 0.05 \) were considered statistically significant.

Results

SASH1 was lowly expressed in human pancreatic cancer cell lines

We firstly examined levels of SASH1 in human pancreatic cancer cell lines (PANC-1, BxPC-3, and Capan-1a). As shown in Figure 1A, the expression of SASH1 mRNA was significantly decreased in human pancreatic cancer cell lines compared with the nonmalignant hTERT-HPNE cell line. Similarly, Western blot analysis showed that SASH1 protein was also obviously reduced human pancreatic cancer cell lines (Figure 1B).

Hypoxia inhibited the expression of SASH1 in PANC-1 cells

To determine whether SASH1 expression is regulated by hypoxia, we incubated PANC-1 cells under hypoxic conditions for 24 h and measured the expression of SASH1 mRNA and protein using real-time PCR and Western blot. As shown in Figure 2A, hypoxia obviously inhibited the expression of SASH1 in PANC-1 cells. Meanwhile, we confirmed hypoxia-inhibited SASH1 expression in protein levels (Figure 2B).

Figure 1. SASH1 is lowly expressed in human pancreatic cancer cell lines. A. Equal amounts of mRNA were analyzed by RT-qPCR for SASH1 expression. B. SASH1 protein expression was evaluated by Western blot. Data are shown as means ± SD (N=3). *P<0.05 vs. the hTERT-HPNE group.

Figure 2. Hypoxia inhibits SASH1 expression. A. PANC-1 cells were exposed to normoxia or hypoxia for 24 h. A. SASH1 mRNA expression was determined using RT-qPCR analysis. B. SASH1 protein expression was determined using Western blot analysis. Data are shown as means ± SD (N=3). *P<0.05 vs. the normoxia group.
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To examine the role of SASH1 in PANC-1 cell migration and invasion under hypoxic conditions, PANC-1 cells with overexpression of SASH1 were established (Figure 3A). Then, we investigated the effects of SASH1 on cell migration and invasion under hypoxia condition by the Transwell assays. As shown in Figure 3B, hypoxia treatment significantly increased migration potential of PANC-1 cells compared to normoxia-treated PANC-1 cells. However, overexpression of SASH1 prevented hypoxia-induced migration of PANC-1 cells. Similarly, overexpression of SASH1 resulted in decreased invasion and invasion under hypoxia condition by the Transwell assays. As shown in Figure 3C, hypoxia treatment significantly increased invasion potential of PANC-1 cells compared to normoxia-treated PANC-1 cells. However, overexpression of SASH1 prevented hypoxia-induced invasion of PANC-1 cells. Similarly, overexpression of SASH1 resulted in decreased invasion.
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PANC-1 cell invasion following cobalt chloride-induced hypoxia (Figure 3C).

**SASH1 prevented hypoxia-induced EMT process in PANC-1 cells**

To investigate the role of SASH1 in hypoxia-induced EMT, we transfected PANC-1 cells with overexpression of SASH1, and exposed these cells to hypoxic conditions. As shown in Figure 4, the protein level of vimentin was markedly increased at 24 h culture in hypoxia. On the other hand, the expression of E-cadherin, an epithelial marker, was significantly decreased by hypoxia. However, overexpression of SASH1 decreased the expression of vimentin, increased the expression of E-cadherin in PANC-1 cells under hypoxic condition.

**Inhibition of HIF-1α is involved in the prevention of hypoxia-induced EMT by SASH1**

Hypoxia-inducible factor (HIF)-1α has been reported to induce EMT in cancer cells [13]. Hence, we attempted to explore whether the preventing effect of SASH1 against hypoxia-induced EMT involves modulation of the HIF-1α expression. As shown in Figure 5A, hypoxia obviously inhibited the mRNA expression of HIF-1α in PANC-1 cells. Whereas, overexpression of SASH1 significantly reduced HIF-1α mRNA expression.
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expression induced by hypoxia. Similarly, overexpression of SASH1 obviously suppressed hypoxia-induced HIF-1α protein expression in PANC-1 cells (Figure 5B).

SASH1-mediated blockade of HIF-1α and EMT involved PI3K/Akt pathway

Previous study showed that activation of PI3K/Akt signaling increased the expression of HIF-1α by promoting the translation [14]. Therefore, we attempted to investigate the effect of SASH1 on PI3k/Akt signaling in PANC-1 cells under hypoxic condition for 24 h. As shown in Figure 6A, hypoxia treatment significantly increased the expression of phosphorylated PI3K and Akt but that of total Akt remained unchanged. Whereas, overexpression of SASH1 prevented hypoxia-induced the expression of phosphorylated PI3K and Akt in PANC-1 cells.

Discussion

Hypoxic regions exist in pancreatic cancer, and increased hypoxia is clearly associated with cancer cell migration and invasion. In this study, we found that SASH1 was lowly expressed in human pancreatic cancer cell lines, and hypoxia significantly inhibited SASH1 expression at both mRNA and protein levels in PANC-1 cells. Then, overexpression of SASH1 prevented hypoxia-induced migration/invasion and EMT process in PANC-1 cells. Moreover, overexpression of SASH1 prevented hypoxia-induced the expression of HIF-1α, phosphorylated PI3K and Akt in PANC-1 cells.

Previous studies have reported that SASH1 was implicated in tumorigenesis. Zeller et al. demonstrated that SASH1 expression was decreased in breast cancer cell lines [15]. Another study reported that SASH1 expression was also significantly decreased in colon cancers [9]. In line with these results, in this study, we found that SASH1 was lowly expressed in human pancreatic cancer cell lines, suggesting that SASH1 functioned as a tumor suppressor in the development of pancreatic cancer.

EMT plays an important role in pancreatic cancer metastasis. Generally, increased motility and invasion are positively correlated with EMT, which is characterized by repression of epithelial markers and induction of mesenchymal markers [16]. Furthermore, it has been report-
In conclusion, our study found that SASH1 prevented hypoxia-induced EMT partly by negatively regulating the expression of HIF-1α via blocking PI3K/Akt signaling pathway in human pancreatic cancer cells. These results strongly suggest that SASH1 could be a promising therapeutic target for the treatment of human pancreatic cancer.

Disclosure of conflict of interest
None.

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