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Original Article

Reverse correlation of Jab1 and Smad4 in PANC-1 cells involved in the pathogenesis of pancreatic cancer

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Abstract: Objective: Steps in the genetic basis of pancreatic cancer (PC) have been recently identified, however, Studies focusing on the relationship between Jab1 and Smad4 in PC are rarely reported. This study was performed to examine the expression patterns and association of Jab1 and Smad4 in PC cells for gaining a further understanding of PC pathogenesis. Methods: Human pancreatic cancer cell line PANC-1 cells were infected with retrovirus vector containing GFP, HA-Jab1, siGFP, and siJab1 respectively. The expression of Jab1 and Smad4 in PANC-1 cells was analyzed by Western blot and immunocytochemistry. Subsequently, the effect of overexpression of Jab1 on cell proliferation inhibition mediated by TGF-β was examined with MTT colorimetry. Results: The expression of Smad4 in PANC-1 cells was inhibited after the overexpression of Jab1. Inversely, the expression of Smad4 was increased after the down-regulation of Jab1 silenced by SiRNA. Smad4 expression in PANC-1 cells was negatively correlated with Jab1 expression. In addition, the cell proliferation inhibitory effect induced by TGF-β in PANC-1 cells was attenuated after the overexpression of Jab1. Conclusions: The reverse correlation of Jab1 and Smad4 in PANC-1 cells may be involved in the Pathogenesis of PC. Jab1 can cause degradation of Smad4 via TGF-β signal pathway, consequently contributing to the proliferation of PC cells.

Keywords: Jab1, Smad4, TGF-β, pancreatic cancer

Introduction

Pancreatic ductal adenocarcinoma (PC), the fourth leading cause of cancer mortality in the world [1, 2], is characterized by advanced clinical stages at diagnosis and extremely poor prognosis [3]. In 2010, the estimated incidence of PC in China was 40,394 males, and an estimated 34,509 cases died from this kind of disease [4]. The incidence and mortality rates are almost equal, clearly reflecting the malignant degree of PC.

Progress of the genetic study of PC have been made recently, followed by the identification of the activation of oncogene K-ras and dysfunction of the tumor suppressor genes p16INK4a, p53, and DPC4 (deleted in pancreatic carcinoma locus 4) as characteristic features of invasive PC [5, 6]. The protein instability of DPC4, which is also known as Smad4, is a common phenomenon in pancreatic carcinoma cells [7]. Recently, some mutations in Smad4 have been shown to target the protein for rapid degradation via the ubiquitin-proteasome pathway [8, 9], indicating the important role of the protein instability of Smad4 in tumors. Studies of the mechanisms by which Smad4 is degraded in pancreatic carcinoma should be instructive for further understanding the role of Smad4 in human neoplasia, and may also be helpful to the development of effective therapeutic intervention strategies for patients with PC.

Previously, studies demonstrated that Jab1 (Jun activation domain binding protein 1) plays a key role in inducing protein degradation of DPC4/Smad4 [10]. Jab1 is also known as CSN5 as it is the fifth component of the COP9 signalosome complex (CSN). Jab1-integrated CSN also induces degradation of the tumor suppressor p53 and the cell cycle inhibitors p27kip1 and p21cip1 [11, 12], all of which are actively involved in suppressing the development of PC. Therefore, Jab1 in the CSN acts as a negative regulator of important pancreatic tumor suppressors by targeting them for degradation.

However, Studies focusing on the associations between Jab1 and Smad4 in PC are rarely reported. And the involvement of their relationship in the pathogenesis of PC remains undetermined. In this study, we overexpressed and suppressed Jab1 expression, and detected the expression of Smad4 in human pancreatic cancer cells, the purpose of which is to gain a further understanding of the relationship between Jab1 and Smad4 in PC cells, and reveal their roles in PC pathogenesis.

Materials and methods

Cell culture and virus infection

PANC-1 human pancreatic cancer cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% ampicillin/streptomycin (all from Gibco, San Diego, CA, USA) in a 37°C humidified incubator with 5% CO_2 and 95% air. Then PANC-1 was infected with retrovirus vector containing pMSCVneo/GFP, pMSCVneo/HA-Jab1, pMSCVneo/U6-GFP (siGFP), and pMSCVneo/U6-Jab1 (siJab1).

Western blot analysis

Cell lysates in radioimmunoprecipitation assay buffer were prepared and all the samples were quantified using the standard BCA method. Equal amounts of protein samples were loaded onto 10% SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The blots were blocked in 5% skim milk for 2 h and then incubated overnight at 4°C with primary antibody. The next day, blots were washed and incubated with secondary antibodies. Jab1 and Smad4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HA antibody was purchased from CRP Inc. (Denver, PA, USA). The secondary antibodies were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Immunoreactive protein bands were detected by enhanced chemiluminescence (ECL, Invitrogen, USA) and exposure to X-ray film. The intensity of the resulting bands was quantified by using the Elec-trophoresis System Tannon-2500R and Gel Image System Ver.4.00.

Immunocytochemistry analysis

Cells were grown on glass coverslips in six-well plates to 50% confluence. After overnight growth, the cells were infected with retrovirus vector. At the end of the experimental period, the cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS, and treated with 0.5% Triton-X 100 for 10 min. Next, the cells were immunostained with antibody for 2 h at room temperature, followed by incubation with secondary antibodies for 30 min. Coverslips were then stained with DAB and hematoxylin and then reviewed.

MTT assays

Cellular proliferation was assayed by CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA). In brief, after transfection, 50 μl of MTT solution (5 mg/ml) was added to the culture medium. After 4 h at 37°C, the medium was removed, and 50 μl of acidified isopropanol was added to each well. The color was allowed to develop for 5 min, and optical density at 570 nm was determined with amicroplate reader. Then the mean value converted to a percentage relative to control.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 and GraphPad Prism 5.01. All data are presented as mean ± SD. Comparison of two samples was carried out using the Student's t-test, *P* value of less than 0.05 was considered statistically significant.

Results

The overexpression of Jab1 inhibits the expression of Smad4 in PANC-1 cells

In this study, we overexpressed Jab1 by infection of PANC-1 cells with a retrovirus containing pMSCVneo-HA-Jab1 and pMSCVneo-GFP (control). Two stable cell lines (PANC-1-Jab1 and PANC-1-GFP) have been generated by infecting PANC-1 cells with these two viruses individually. The infection efficiency was determined to be approximately 90% (**Figure 1A**). We then assessed the levels of Jab1 and Smad4 in the cells by Western blot analysis. We found that Jab1 was elevated in PANC-1 cells infected with virus containing HA-Jab1 compared with cells infected with virus containing GFP, however, Smad4 was correspondingly reduced in PANC-1

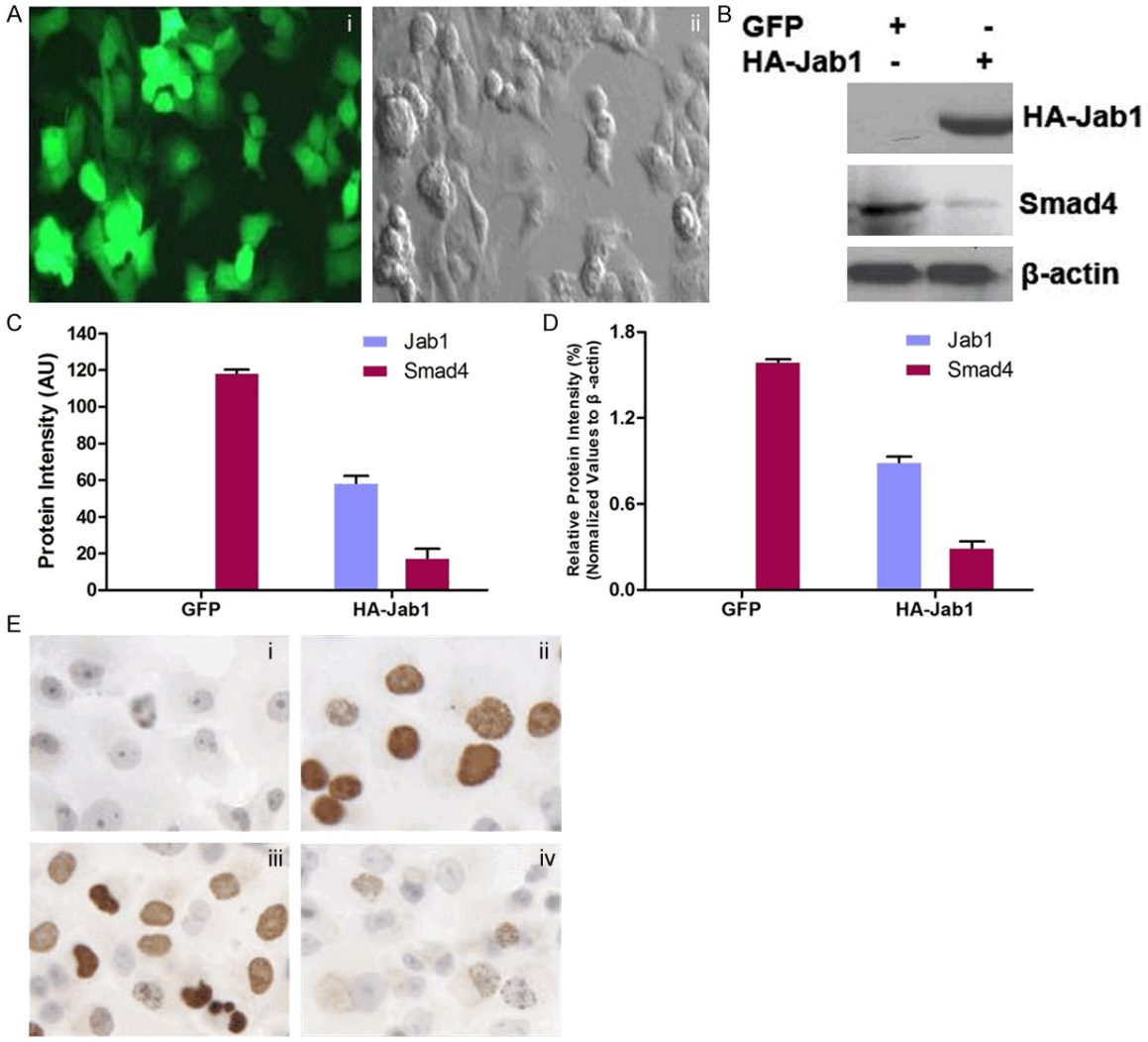

Figure 1. The overexpression of Jab1 inhibits the expression of Smad4. (A) GFP is efficiently overexpressed in PANC-1 cells. PANC-1 cells were infected with a retrovirus containing pMSCVneo-GFP. Green light representing GFP expression (i). Cell density (ii). (B) Jab1 overexpression decreases endogenous Smad4 expression. PANC-1 cells were infected with virus containing GFP or HA-Jab1. Cells were harvested and expression levels of Jab1, Smad4, β-actin were measured by Western blot analysis with antibodies against HA, Smad4, β-actin. (C and D) The intensity of the bands in B was quantified (C), and the protein ratio of Jab1 and Smad4 to β-actin was calculated (D). (E) Expression levels of Jab1 and Smad4 were measured by immunocytochemistry analysis. Jab1 was examined in PANC-1 cells infected with virus containing HA-Jab1 (ii) and in PANC-1 cells infected with virus containing GFP (i). Smad4 was examined in PANC-1 cells infected with virus containing HA-Jab1 (iv) and in PANC-1 cells infected with virus containing GFP (iii). Original magnifications: × 400 (A, E).

cells infected with virus containing HA-Jab1, suggesting that overexpression of Jab1 resulted in a significant reduction in the levels of Smad4 (**Figure 1B**). The intensity of Jab1 and Smad4 quantified demonstrated the same trend (**Figure 1C** and **1D**). We also examined the levels of Jab1 and Smad4 via immunocytochemistry analysis in PANC-1 cells infected with virus containing pMSCVneo-HA-Jab1 (**Figure 1Eii** and **1Eiv**) and pMSCVneo-GFP (**Figure 1Ei** and **1Eiii**). Likewise, we found that Jab1 was elevated in PANC-1 cells infected with virus containing HA-Jab1 compared with cells infected with virus containing GFP, however, Smad4 was reduced in PANC-1 cells infected with virus containing HA-Jab1 compared with cells infected with virus containing GFP. Immunocytochemistry showed the same results that overexpression of Jab1 resulted in a significant reduction in the levels of Smad4.

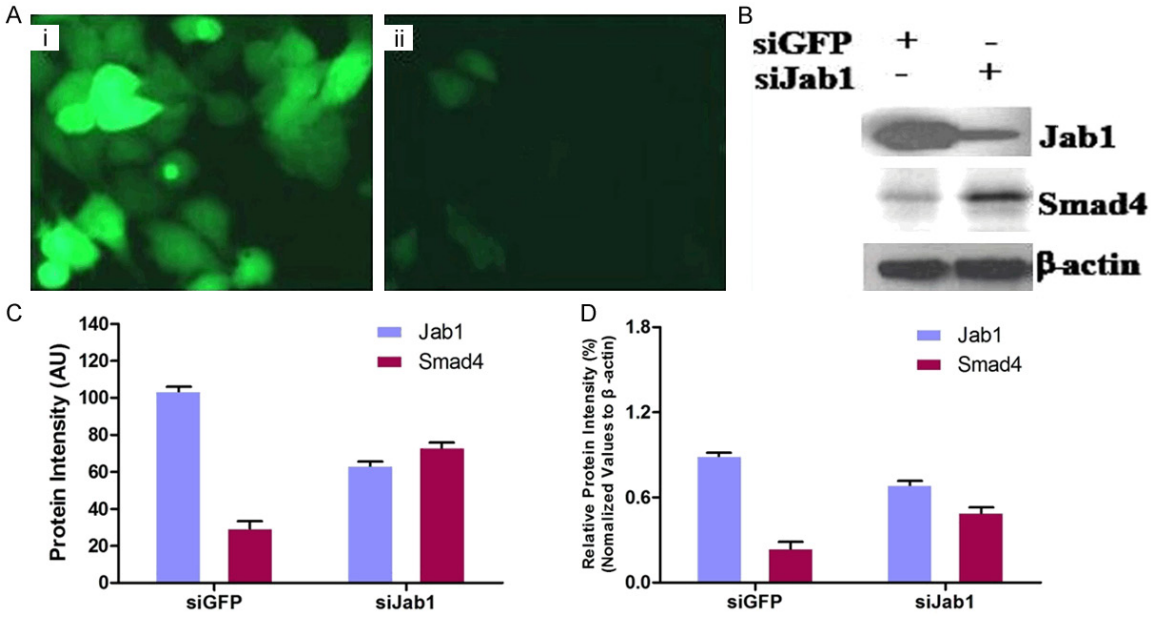

Figure 2. The down-regulation of Jab1 increases the expression of Smad4. (A) GFP is normally expressed in PANC-1 cells infected with virus containing blank plasmid pMSCVneo/U6 (i). GFP is efficiently suppressed in PANC-1 cells infected with virus containing pMSCVneo/U6-GFP (ii). Green light representing GFP expression. (B) suppression of Jab1 by retroviral delivery of SiRNA increases endogenous Smad4 expression. PANC-1 cells were infected with virus containing siGFP or siJab1. Cells were harvested and expression levels of Jab1, Smad4, β-actin were measured by Western blot analysis with antibodies against HA, Smad4, β-actin. (C and D) The intensity of the bands in B was quantified (C), and the protein ratio of Jab1 and Smad4 to β-actin was calculated (D). Original magnifications: × 400 (A).

The down-regulation of Jab1 silenced by SiRNA increases the expression of Smad4 in PANC-1 cells

Therefore, we infer that if Jab1 is down-regulated in pancreatic cancer cells, the expression of Smad4 should be elevated. To verify this hypothesis, PANC-1 cells were firstly infected with retrovirus containing used pMSCVneo-GFP, we found that GFP is efficiently suppressed in cells infected with virus containing pMSCVneo/U6-GFP (**Figure 2Aii**) compared with cells infected with virus containing blank plasmid pMSCVneo/U6 (**Figure 2Ai**), indicating that siGFP construction can significantly decrease the expression of GFP and work normally. Then we developed retroviral siRNA delivery vector pMSCVneo/U6-GFP (siGFP, irrelevant siRNA control) and pMSCVneo/U6-Jab1 (siJab1) to determine the levels of Smad4 after a reduction in the levels of Jab1 in PANC-1 cells. The levels of Jab1 and Smad4 in the cells were assessed by Western blot analysis. We found that Jab1 was reduced in PANC-1 cells infected with virus containing siJab1 compared with cells infected with virus containing siGFP, however, Smad4 was correspondingly elevated in PANC-1 cells infected with virus containing siJab1, suggesting that down-regulation of Jab1 resulted in a significant elevation in the levels of Smad4 (**Figure 2B**). The intensity of Jab1 and Smad4 quantified demonstrated the same trend (**Figure 2C** and **2D**).

The overexpression of Jab1 impairs the cell proliferation inhibitory effect induced by TGF-β

To further study the effect of overexpression of Jab1 on cells, We overexpressed Jab1 by infection of PANC-1 cells with a retrovirus containing pMSCVneo-HA-Jab1 and used pMSCVneo-GFP (control). After stimulation of TGF-β1 (5 ng/ml) to the cells for 48 hours, the effect of overexpression of Jab1 on cell proliferation inhibition mediated by TGF-β was examined with MTT assays. We found that the TGF-β-induced cell proliferation inhibitory effect was significantly reduced in cells infected with virus containing HA-Jab1, compared with cells infected with virus containing GFP, indicating that Jab1 can promote pancreatic cancer cells proliferation

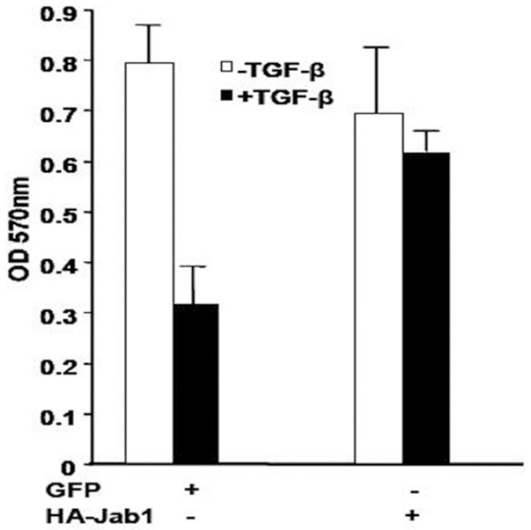

Figure 3. Overexpression of Jab1 reverses TGF-β-induced cell proliferation inhibitory effect.

via inhibition of TGF-β signaling pathway (**Figure 3**). Our findings also revealed that Jab1 may induce Smad4 protein instability through TGF-β signaling pathway, so as to contribute to the occurrence of pancreatic cancer.

Discussion

Previously, study has also identified inactivation of tumor suppressor genes p16INK4a, p53, and DPC4 [13], in which only Smad4 is characteristic in pancreatic carcinoma cells. Mutations in Smad4/DPC4 have been identified in 50% of pancreatic adenocarcinomas but only in 10% or less of other cancers, and other tumor suppressor genes have no strong specificity in PC [14, 15], suggesting a possible pivotal role of Smad4 in pancreatic tumorigenesis. However, our previous result indicates that Smad4 mRNA levels have no statistical difference compared with control group. So the Smad4 protein expression may be irrelevant to its mRNA levels. Meanwhile, our previous immunohistochemistry data demonstrated that of 35 patients, 85.7% of the samples were considered to be Smad4 negative, much higher than 30% of Smad4 homozygous deletion reported [14]. These results indicate that Smad4 protein instability is a common phenomenon in PC cells even though Smad4 has no mutations. Therefore, compared with Smad4 gene mutation, the instability of Smad4 protein may play a significant role in Smad4 functional loss in PC cells.

The ubiquitin proteasome pathway controls degradation of the majority of regulatory proteins in mammalian cells [16-18]. Our previous studies have showed that SCFβ-TrCP1, a ubiquitin (E3) ligase, is a critical determinant for Smad4 protein degradation in PC cells [19]. We also found that Jab1 is a critical factor regulating the stabilization or degradation of Smad4 by ubiquitin-proteasome pathway [10]. Jab1 is known as CSN5 as it is the fifth component of the COP9 signalosome complex [20, 21], the current work demonstrated that Overexpression or suppression of Jab1 can correspondingly decrease or increase the levels of Smad4. That is to say, Smad4 expression in PANC-1 cells was negatively correlated with Jab1 expression. It is generally known that transforming growth factor β (TGF-β) superfamily plays important roles in exerting cellular growth inhibition functions [22]. Our study reveals that overexpression of Jab1 can impair TGF-β-induced cell proliferation inhibitory effect in PANC-1 cells, providing possible research prospects for effective therapeutic intervention strategies for patients with PC. The overexpression of Jab1 has been reported in many tumors [23-25] including PC [26], but the exact mechanism is still unclear. Our findings suggested that the overexpression of Jab1 may decrease Smad4 stability via TGF-β signaling pathway, consequently causing further degradation of Smad4 and possibly providing additional evidence for the pathogenesis of PC.

In summary, we found Jab1 to be inversely correlated with Smad4 levels in PANC-1 cells, and the cell proliferation inhibitory effect induced by TGF-β can be attenuated after the overexpression of Jab1. Despite all of these promising findings, this study is considered preliminarily and more work needs to be done before Jab1 and Smad4 can be regulated into clinical practice to treat PC patients. In future studies, we plan to test expressions of them in vitro model system, such as nude mouse xenograft assay, and then determine effects of Jab1 on Smad4 in clinical trial. We will also investigate the defined molecular mechanism by which Jab1 regulates other gene beyond Smad4. Regardless, this study demonstrated modulation of the Jab1 gene product may also provide a novel target for experimental therapies in pancreatic cancer patients.

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Disclosure of conflict of interest

None.

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