The effects and mechanisms of blockage of STAT3 signaling pathway on IL-6 inducing EMT in human pancreatic cancer cells in vitro

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Aberrant Signal transducers and activators of transcription-3 (STAT3) signaling pathway is a major cause of tumor invasion and metastasis; the underlying mechanisms, however, are not well understood. Epithelial-mesenchymal transition (EMT) is an early event that occurs during invasion of cancers of an epithelial origin. It remains elusive whether STAT3 signaling pathway is involved in EMT. The objective of this study was to evaluate the effect of blockage of STAT3 signaling pathway on IL-6 inducing EMT in human pancreatic cancer cells. We used SW1990 cells and induced them to undergo EMT by exposing these cells to soluble factor interleukin-6 (IL-6). The expression of Snail, E-cadherin, and Twist was detected by reverse transcription-PCR, real-time PCR, and Western blotting. Cell morphology was observed under invert phase-contrast microscope. The invasion ability was determined by cell invasion assay in vitro. Our results demonstrated that STAT3 signaling pathway was involved in pancreatic cancer cell invasion and EMT, and that EMT induced by IL-6 was associated with the activation of STAT3 signaling pathway. Inhibition of STAT3 signaling pathway by silencing of the STAT3 gene with RNAi blocked STAT3 signaling pathway activation and suppressed EMT in pancreatic cancer cells. Collectively, the STAT3 signaling pathway plays an important role in the process of EMT of pancreatic cancer by regulating Snail gene expression. Better understanding of STAT3 signaling pathways in EMT may contribute to development of novel therapeutic strategies in invasion and metastasis of pancreatic cancer.

Key words: STAT3, IL-6, EMT, Pancreatic cancer

Pancreatic cancer is a highly lethal disease, which is usually diagnosed in an advanced stage for which there is little or no effective therapies. It remains the fourth most common cause of cancer-related death in the western world. The annual incidence rate of pancreatic cancer is almost identical to the mortality rate, approximately 42,470 new cases are diagnosed each year in the United States, and approximately 35,240 patients die from this disease[1]. Because of the aggressive natural history of this disease, less than 10% of these cases constitute candidates for surgical resection at the time of diagnosis. Although an adjuvant treatment regimen after surgical resection seems to prolong survival, the precise treatment protocol including drug-of-choice is still debated and the focus of several ongoing clinical trials[2]. Effective systemic therapy capable of reversing the aggressive biology of this disease is currently not available. Thus, Attempts to better understand the molecular characteristics of pancreatic cancer is one of the most important issues for treatment.

Signal transducer and activator of transcription 3 (STAT3) is an oncogene that is activated by phosphorylation of a conserved tyrosine residue in response to extracellular signals and oncogenes. Once tyrosine is phosphorylated, two STAT3 monomers form dimers through reciprocal phospho-tyrosine–SH2 interactions. The dimers are phosphorylated STAT3 (p-STAT3), which translocate to the nucleus and bind to cognate DNA sequences, regulate the transcription of target genes and modulate fundamental cellular processes, such as proliferation and differentiation. Inappropriate and constitutive activation of STAT3 may be responsible for pancreatic cancer progression through regulating the expressions of target genes, such as Bcl-xL, Mcl-1, Bcl-2, Fas, cyclin D1, survivin, c-Myc, VEGF, MMP-2 and MMP-9[3]. Specifically, the level of activated STAT3 protein has been found to be associated with invasion in various types of tumors. For example, activation of STAT3 in thymic epithelial tumors[4], colorectal adenocarcinoma[5], and cutaneous squamous cell
carcinoma[6] correlates with local invasion and lymph node metastasis. Inhibition of STAT3 signaling pathway suppresses cancer cell growth, invasion and induces apoptosis in various cancers [7]. STAT3 signaling pathway may be one of the common pathways involved in regulating cancer invasion and metastasis.

Epithelial tight junctions form a functional and morphologic boundary on the cell surface and participate in the maintenance of epithelial integrity that protects multicellular organisms from the external environment. During Epithelial to mesenchymal transition (EMT), cells undergo a developmental switch from a polarized, epithelial phenotype to a highly motile fibroblastoid or mesenchymal phenotype[8], which is often accompanied by the dissolution of epithelial tight junctions, the loss of cell adhesion, down-regulated expression of some epithelial markers, as well as the acquisition of migratory and invasive properties. EMT is associated with the loss of expression and/or mis-localization of proteins involved in the formation of cell-cell junctions, such as E-cadherin, zonula occludens (ZO-1) and claudin, and the gain of expression of mesenchymal proteins such as fibroblast specific protein 1 (FSP-1), smooth muscle actin and fibronectin, as well as certain integrins [9]. EMT is actively involved not only in tissue homeostasis, wound healing, fibrosis, and embryonic development but also in tumor invasion, metastasis[10-13]. In recent years, EMT has been received much attention. EMT has been associated with poor prognosis in several cancers, including prostate, breast, non-small-cell lung, pancreatic cancers and colorectal cancers[14-18]. However, its regulatory mechanism has not been well understood. Previous studies of other solid tumors support the association of increased EMT with the ability of cancer cells to migrate, invade and metastasize[8, 19-21]. EMT is a key step during embryonic morphogenesis and is involved in the progression of primary tumors toward metastasis[22].

RNA interference (RNAi) is the process by which double-stranded RNA induces poten and specific inhibition of eukaryotic gene expression and has been widely used by researchers to silence the expression of many target genes because of their high specificity and apparent non-toxicity[23]. Furthermore, systems based on lentiviral vectors have provided new solutions to achieving stable shRNA-mediated knockdown[24]. In our previous studies, we successfully constructed the LV (lentivirus)-STAT3siRNA (STAT3 small interfering RNA) and proved that it can suppress expression of STAT3 gene in SW1990 cells[25].

The pleiotropic cytokine interleukin-6 (IL-6) is a major activator of STAT3; IL-6 stimulates the formation of tyrosine-phosphorylated STAT3 (p-STAT3) in cancer cells[26]. In our previous studies, we used IL-6 to activate STAT3 signal in pancreatic cancer cells and proved that IL-6 can increase expression of STAT3 gene in pancreatic cancer cells [27].

Our present study was to investigate the roles STAT3 signaling pathway plays in EMT of pancreatic cancer. We constructed lentivirus vector mediating RNA interference targeting STAT3 in SW1990, and then used IL-6 to stimulate STAT3-siRNA SW1990 cells, observed the changes of morphology, adhesion and invasion capability, the activation of STAT3 signaling pathway and expression of EMT related genes including E-cadherin, Snail, TWIST. Our results demonstrate that activation of the STAT3 signaling pathway is critical for EMT and invasive behavior of pancreatic cancer cells and silencing of the STAT3 gene with RNAi may provide a novel strategy for investigation of the role of STAT3 gene in the invasion of human pancreatic cancers.

Materials and methods

Cells and reagents. Human pancreatic cancer cell lines SW1990 was obtained from American Type Culture Collection (ATCC). Tumor cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator with an atmosphere of 5% CO2, 95% air at 37°C. In our previous studies, we constructed three lentivirus vector mediating RNAi targeting of STAT3 (LV-STAT3siRNA-1, 2, 3) and proved LV-STAT3siRNA-2 had the most obvious gene silencing effect. We also constructed a negative control scrambled siRNA expression vector (LV-Con). LV-Con and LV-STAT3siRNA-2 expression vector were carried out in SW1990 cells, and SW1990 LV-Con transfectants (SW1990-Con) and SW1990 LV-STAT3siRNA transfectants (SW1990-RNAi) were established as recently described in details [25]. Recombinant IL-6 (Peprotech, Princeton, USA) was dissolved in 5-10mM Acetic Acid to a concentration of 0.1-0.5mg/mL and then diluted with the culture medium for experiments. SW1990, SW1990-Con and SW1990-RNAi cells were treated with 100ng/mL IL-6 for 24 hours.

Invasion assay. Invasion assay was performed using a specialized invasion chamber that included a 24-well tissue culture plate with 12 cell-culture inserts (Chemicon, Temecula, CA, USA). The inserts contained an 8 µm pore size polycarbonate membrane with a precoated thin layer of basement membrane matrix (ECMatrix). Briefly, medium supplemented with 10% fetal bovine serum was poured into the lower chamber as a hemo-attractant. After reaching 60–70% subconfluence, cells were removed from the upper surface of the membrane compartment. After 48 h incubation at 37°C, non-invasive cells were removed from the upper surface of the membrane using a moist cotton-tipped swab. Invasive cells on the lower surface of the membrane, which had invaded the ECMatrix and had migrated through the polycarbonate membrane, were stained with the staining solution for 20 min and rinsed with distilled water several times. Invasiveness was quantitated by selected ten different views (400 times) and counted the number of invasion cells.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction from pancreatic cancer cells was
performed with Trizol Reagent (Life Technologies, Rockville, MD, USA). Then, 2 µg of total RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (Promega, Madison, WI, USA) to synthesize cDNA samples. Subsequently, 2 µL of cDNA product was then subjected to PCR amplification with Taq DNA polymerase (Sangon, Shanghai, China) on a thermal cycler using the following primers. The PCR primers used to detect each factor were as follows: STAT3, sense strand 5'-CCACGAAGGAGACTGAGCAT-3', antisense strand 5'-GCCAGACCAAGAGAGGACGC-3', with a product length of 149bp; Snail, sense strand 5'-CAGACCCACTCAGATGTCAC-3', antisense strand 5'-CATAGTTAGTCACCACTC-3', with a product length of 557bp; Twist, sense strand 5'-GGAGTCCCGAGTCTAC-3', antisense strand 5'-CCTGTCTCGCTTCTCTTT-3', with a product length of 527bp; E-cadherin, sense strand 5'-ATTCTGATTCTGCTGCTCTTG-3', antisense strand 5'-AGTATGCTAATGCTGTG-3', with a product length of 420bp; and β-actin, sense strand 5'-ATCTGGCGACCAACCTCTCAATGACTGGG-3', antisense strand 5'-CTGATACCTCCTGCTGTGATACCACATCGC-3', with a product length of 138bp. The PCR conditions were as follows: one cycle of denaturing at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 40 s for 40 cycles. A standard calibration curve for expression of each mRNA was generated using a spectrophotometer. The expression of STAT3 was determined using an assay kit (Bio-Rad). Lysates containing 100 µg of protein were mixed with loading buffer with 5% β-mercaptoethanol, and heated for 5 min at 100°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes by semidyrid blotting. Membranes were incubated in blocking buffer (tris buffered saline TBS), 0.1% Tween 20, and 5% non-fat dry milk for 1 h at room temperature, followed by hybridization with anti-p-STAT3 (tyr-705) antibody (Cell Signaling Technology; 1:1000 dilution), anti-STAT3 antibody (Cell Signaling Technology; 1:1000 dilution), anti-Snail antibody (Cell Signaling Technology; 1:1000 dilution), anti-E-cadherin antibody (Cell Signaling Technology; 1:1000 dilution), anti-TWIST antibody (Cell Signaling Technology; 1:1000 dilution), anti-β-actin antibody (Lab Vision, Fremont, USA; 1:100 dilution), at 4°C overnight. After three washes in TBS/0.1% Tween 20, the membranes underwent hybridization with a horseradish peroxidase-conjugated secondary antibody rabbit IgG (Santa Cruz Biotechnology; 1:5000 dilution) for 1 h at room temperature. After three washes in TBS/0.1% Tween 20, signals were detected by chemiluminescence using the western blotting luminol reagent (Santa Cruz Biotechnology).

**Statistical analysis.** All assays were conducted three times and found to be reproducible. Data were expressed as mean ± SD. Statistical correlation of data between groups was checked for significance by Student’s t test. Differences with P<0.05 were considered significant. These analyses were performed using SPSS 11.0 software.

**Results**

**Effects of LV-STAT3siRNA-2 and IL-6 on STAT3 expression of pancreatic cancer cells.** The expression of STAT3 was analyzed at the mRNA level by semiquantitative RT-PCR (Fig. 1A) and real-time PCR (Fig. 1B). Protein expression of STAT3 and p-STAT3 was evaluated by Western blotting (Fig. 1C) in the human pancreatic cancer cell lines. RT-PCR and real-time PCR indicated that the levels of STAT3 mRNA expression in the transfectants containing LV-STAT3siRNA-2 (SW1990-RNAi) were significantly decreased as compared to that of parental SW1990 or SW1990-Con cells. Moreover, enforced expression of LV-STAT3siRNA-2 in SW1990 cells significantly suppressed STAT3 and p-STAT3 protein expression as determined by Western blotting analysis. SW1990, SW1990-Con and SW1990-RNAi cells were treated with 100ng/mL IL-6 for 24 hours. After treatment, in SW1990, SW1990-Con and SW1990-RNAi cells, STAT3 protein expression had no significant change, but p-STAT3 protein expression increased significantly; in SW1990-RNAi cells, both STAT3 and p-STAT3 protein expression had no significant change.

**Change of STAT3 signaling pathway activation influence morphology of SW1990 cells.** Cell morphology was observed under invert phase-contrast microscope. Pancreatic cancer cells SW1990 exhibit a typical polygonal cobblestone epithelial
morphology when grown in culture. Both LV-Con and LV-STAT3siRNA-2 had no significant effects on cell morphology of SW1990 cells. After treatment with 100ng/mL IL-6 on SW1990 and SW1990-Con cells for 24 hours, all cells underwent typical EMT morphological and cellular changes [28-30]: cancer cells started to loose cell contacts, scattered from cell clusters and acquired a spindle-shaped and fibroblast-like phenotype. However, after treatment with 100ng/mL IL-6 on SW1990-RNAi cells for 24 hours, only some SW1990-RNAi cells underwent typical EMT morphological and cellular changes. The results of cell morphology were shown in Figures 2.

**Figure 1.** Effects of LV-STAT3siRNA-2 and IL-6 on STAT3 expression of pancreatic cancer cells
The expression of STAT3 were analyzed at the mRNA level by RT-PCR and real-time PCR. STAT3 and p-STAT3 protein expression was evaluated by Western blotting in the human pancreatic cancer cell lines. A, RT-PCR. B, real-time PCR. C, Western blotting. 1, SW1990 group; 2, SW1990-Con group; 3, SW1990-RNAi group; 4 IL-6+SW1990 group; 5, IL-6+SW1990-Con group; 6, IL-6+SW1990-RNAi group. Western blotting results showed that stable transfection of LV-STAT3siRNA-2 suppressed STAT3 protein (P=0.000) and p-STAT3 protein level (P=0.001). Then, SW1990, SW1990-Con, SW1990-RNAi group cells were treated with 100ng/mL IL-6 for 24 hours. In SW1990 and SW1990-Con group, STAT3 protein expression had no change, but p-STAT3 protein expression increased(P=0.042;P=0.003); in SW1990-RNAi group, both STAT3 and p-STAT3 protein expression had no significant change.

**Change of STAT3 signaling pathway activation influence invasion ability of SW1990 cells.** We performed an in vitro invasion assay. The invasion system was set up as described in Materials and methods. LV-Con had no effect on invasion of SW1990 cells. When compared with parental cells or cells transfected with LV-Con, LV-STAT3siRNA-2 transfected cells showed a substantial reduction in invasive capacity. After treatment with 100ng/mL IL-6 on SW1990, SW1990-Con and
SW1990-RNAi group cells for 24 hours, cell invasion ability significantly increased compared with their corresponding group. However, the invasion ability of IL-6+SW1990-RNAi group was significantly lower compared with IL-6+SW1990 group or IL-6+ SW1990-Con group. The results of cell invasion were shown in Figures 3.

**Change of STAT3 signaling pathway activation influence Expression of Snail, Twist and E-cadherin.** The expression of Snail, Twist, and E-cadherin were analyzed at the mRNA level by semiquantitative RT-PCR (Fig. 4A) and real-time PCR (Fig. 4B). Protein expression was evaluated by Western blotting (Fig. 5) in the human pancreatic cancer cells. Twist showed no expression in SW1990 pancreatic cancer cell lines. Silencing of STAT3 expression by stable transfection of LV-STAT3siRNA-2 can significantly suppressed the expression of Snail and markedly increased the expression of E-cadherin at mRNA and protein level. After treatment with 100ng/mL IL-6 on SW1990, SW1990-Con cells for 24 hours, the mRNA and protein expression of Snail significantly increased and the mRNA and protein expression of E-cadherin markedly decreased. After treatment with 100ng/mL IL-6 on SW1990-RNAi cells for 24 hours, the expression of E-cadherin markedly decreased; the expression of snail protein and mRNA level increased, but have no statistical significance.

**Discussion**

STAT3 is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncoproteins. There is much evidence that implicates the important roles for STAT3 in cell proliferation and survival in diverse human cancers, including pancreatic cancer. JAK is responsible for the tyrosine phosphorylation of STAT3 in response to extracellular signals and oncogenes. The pleiotropic cytokine IL-6 is a major activator of STAT3, IL-6 stimulates the formation of tyrosine-phosphorylated STAT3 (p-STAT3) in cancer cells [31,32]. Through the JAK/STAT3 signaling pathway, IL-6 plays an important role in cell proliferation, apoptosis, angiogenesis, metastasis, and other biological activities[33].

In our study, through analyzing expressing of STAT3, p-STAT3, we found IL-6 can activate STAT3 signaling pathway by upregulating the expressing of p-STAT3. Through examining the invasive ability of pancreatic cancer cells with a cell invasion assay kit, we found that IL-6 could increase invasion ability of SW1990 cells. However, STAT3 silence with RNAi in SW1990 cells could decrease the invasion ability of pancreatic cancer cells. Therefore, there is a strong relationship between IL-6-STAT3 signal pathway and the invasive ability of human pancreatic cancer cells.
EMT is a crucial process in tumor progression providing tumor cells with the ability to escape from the primary tumor, to invade tissues and to migrate to distant regions. EMT requires a loss of cell-cell adhesion and apical-basal polarity, as well as the acquisition of a fibroblastoid motile phenotype and the adhesion ability to extracellular matrix. EMT is considered to promote the early stages of invasion and metastasis that allow primary tumor cells to invade the basement membrane and disseminate into the circulation. Several genes have emerged in recent years that induce EMT, with important implications for...
tumor progression. Loss of E-cadherin expression is a hallmark of EMT. Many solid cancers such as prostate cancer, pancreatic cancer, renal cancer, gastric cancers, and certain types of breast cancers are associated with a loss or down-regulation of E-cadherin expression[11,34]. Reduced E-cadherin expression contributes to the transition of adenoma to carcinoma in animal models and is inversely correlated with tumor stage[21]. Loss of E-cadherin can be attributed to gene mutations, or to inhibition of gene transcription stimulated by activation of certain cellular signaling pathways that result in the activation of repressors of E-cadherin gene transcription, such as Snail and Twist[34,35]. Twist is frequently overexpressed in many human cancers[36]. The elevated Twist protein levels are associated with advance tumor stage and poor prognosis in several types of cancer[37-39]. Further, increased Twist in cancer cells has been shown to promote metastatic ability in vivo and induce EMT in vitro[40,41]. The transcriptional factor Snail has been described to be direct repressors of E-cadherin in vitro and in vivo[42,43]. Snail binds to E-cadherin and leads to the initiation of the invasive process through the down-regulation of E-cadherin[44].

EMT is regulated by complex signaling networks, including the Ras–Raf–Erk signal transduction cascade, EGF/EGFR pathway, TGF-β and Raf/MAPK/Erk signaling etc. The potential roles of STAT3 signaling pathway during EMT remain completely unknown. Recent studies have reported that EGF/EGFR signaling pathways induce cancer cell EMT via STAT3-mediated
TWIST gene expression[45]. Oncostatin M has been shown to induce EMT in tubular epithelial cells through activation of JAK/STAT3 pathway[46]. Recently, gastrin has been reported to induce EMT in colon cancer through the JAK/STAT3 pathway [47], while gastrin-releasing peptides have been shown to activate EGFR in pancreatic cancer[48]. In addition, STAT3 signaling pathway may be critical for EMT biology in cancer. In this study, Twist showed no expression in SW1990 pancreatic cancer cell lines, when analyzed the changes of cell morphology, invasion ability and expression of Snail and E-cadherin.

In our study, all cells underwent typical EMT morphological changes and cell invasion ability significantly increased after treatment with IL-6 on SW1990 and SW1990-Con cells. However, after treatment with IL-6 on SW1990-RNAi cells, only some SW1990-RNAi cells underwent typical EMT morphological changes and the invasion ability of IL-6+SW1990-RNAi group was significantly lower compared with IL-6+SW1990 group or IL-6+ SW1990-Con group. These findings demonstrated that IL-6 could promote EMT process and invasion ability and that STAT3-siRNA could inhibit EMT process and invasion ability induced by IL-6 in SW1990 cell. Therefore, we now showed that the activation of STAT3 signaling pathway may be a pre-requisite for IL-6-induced EMT in SW1990 cell lines, as STAT3-siRNA in SW1990 cells not only led to an inhibition of STAT3 signaling pathway activation but also inhibition of EMT process and invasion ability.

In our study, after treatment with IL-6 on SW1990-RNAi group cells, we found IL-6 can decreased expression of E-cadherin, but IL-6 didn't markedly increased Snail expression in SW1990-RNAi group. These findings highlighted the potential significance of STAT3 signaling pathway in EMT of pancreatic cells. We suggested that IL-6 can promote invasion and EMT via activation of many signaling pathways in SW1990 cells, STAT3 signaling pathway is one of these pathways. The molecular mechanism that STAT3 signaling pathway inducing EMT of pancreatic cancer cells may be mainly via up-regulated Snail gene, down-regulated E-cadherin might have certain role, but needs further verification.

On the whole, the present study indicates that siRNA targeting of STAT3 via a lentivirus vector system effectively sustains knock-down of the STAT3 gene expression in SW1990 cells. The lentivirus RNAi vector targeting STAT3 has been successfully constructed, which will provide a tool for the further study on function of STAT3 gene in pancreatic cancer cells. Many signaling pathways were involved in the process of EMT, STAT3 signaling pathway is one of them. Our study found that STAT3 signaling pathway plays an important role by regulating expression of Snail and E-cadherin in EMT and invasion of pancreatic cancer. Hence, STAT3 signaling pathway may represent novel therapeutic targets for pharmacological intervention in the management of pancreatic cancer EMT and invasion in the future.

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