Protein inhibitor of activated STAT-1 is downregulated in gastric cancer tissue and involved in cell metastasis

PING CHEN1, DESHOU ZHAO2, YUNWEI SUN1, LIYA HUANG1, SHUXIAN ZHANG1 and YAOZONG YUAN1

1Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025; 2Department of Laboratory, Second Hospital Affiliated Lanzhou University, Lanzhou, Gansu 746000, P.R. China

Received April 30, 2012; Accepted July 28, 2012

DOI: 10.3892/or.2012.2030

Abstract. Protein inhibitor of activated STAT-1 (PIAS1) is a novel modulator of the JAK/STAT signaling pathway that negatively regulates the inflammatory response. It has been also reported to be downregulated in a variety of human cancer cell lines. However, the role of PIAS1 in gastric cancer remains unclear. In this study, we investigated the prognostic value of PIAS1 expression and its regulated mechanisms in gastric cancer cell metastasis. Therefore, the expression of PIAS1 was explored in gastric cancer tissues and adjacent tissues of gastric cancer with 31 cases of patients, and the prognostic value was analyzed. In addition, the growth and invasion in SGC7901 cells were investigated in the restoration of PIAS1 expression with Ad5/F35-PIAS1 or Ad5/F35-vector or PBS treatment, and the activity of P38MAPK, P-P38MAPK, JNK/SAPK, P-JNK/SAPK, ERK and P-ERK, were detected by western blotting. The tumor migratory factors MMP-9, MMP-2 and ICAM-1 were detected by western blotting. The results demonstrated that 22 of 31 (70.9%) gastric cancer specimens showed low levels of PIAS1 expression from immunohistochemistry staining using tissue microarrays. Statistical analysis suggested that the downregulation of PIAS1 was significantly correlated with tumor staging. Furthermore, we found that the restoration of PIAS1 expression mediated by Ad5/F35 virus suppressed cell proliferation and invasion accompanied by the inhibition of P38MAPK and ERK protein expression and activity, but not JNK/SAPK protein. Notably, PIAS1 restoration with the transfection of Ad5/F35-PIAS1 robustly decreased the expression of tumor migratory factors including MMP-9, MMP-2 and ICAM-1 compared to Ad5/ F35-vector. These data suggest that PIAS1 may function as a tumor suppressor to regulate gastric cancer cell metastasis by targeting the MAPK signaling pathway.

Introduction

Gastric cancer is still a major health problem and a leading cause of cancer-related death although its incidence is decreased worldwide (1). In spite of the standardization of surgical techniques and multimodal therapy, the postoperative survival of patients with advanced gastric cancer still remains very low. It was recently reported that the understanding of the molecular and genetic factors involved in gastric cancer progression may identify novel gastric biomarkers and highlight potential avenues of investigation for targeted therapies.

Recent evidence shows that PIAS1, a downstream target protein of STAT signaling pathway inhibitor, is related to anti-inflammatory response through negative regulation of NF-kB/STAT1 signaling, which mediates inflammatory cell adhesion and inhibits inflammatory injury (2). Consistently, PIAS1 null mice show increased protection against pathogenic infection and are hypersensitive to LPS-induced septic shock (3). This finding provides a theoretical basis for revealing the mechanism of reversing inflammation by PIAS1. However, recent studies demonstrated that the expression of PIAS1 in some cancer cells is significantly downregulated or lost (4). Several studies showed that PIAS1 negatively regulates Bcl2 expression and thus reverses the cancer growth. Therefore, PIAS1 not only plays functions of inflammatory inhibitor but also plays a role in the reversal of cancer cell growth (4). Therefore, in this study, the PIAS1 expression was first observed the expression of gastric cancer tissue with patient to prove that PIAS1 may be involved in the pathogenesis of cancer and to verify whether PIAS1 acts as a marker for the preclinical detection and clinical assessment of patients with gastric cancer.

The cascade reaction of mitogen-activated protein kinases (MAPKs) is one of the vital intracellular signal transduction systems, participating in many physiological progressions, such as cell growth, proliferation, differentiation and apoptosis. Three major subclasses of MAPKs, namely, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and P38MAPK have been identified (5). Related studies indicated that MAPKs are proposed to be a critical integrator of various signaling transduction systems and involved in various cellular processes including cell proliferation, differentiation, apoptosis, and transformation (6). Constitutive activation of these signaling cascades has been noted in the malignant transformation of...
various cell lines and implicated in carcinogenesis and metastatic potential of human cancers (7).

Therefore, we hypothesize that the regulation of PIAS1 is a key step in the multiple biological regulations in gastric cancer by regulating the MAPK signaling pathway. To test this hypothesis, we used gastric cancer SGC7901 cells and studied the migration response of the cells by upregulating administration of PIAS1. Additionally, to gain a better understanding of the mechanism of action of PIAS1, this study investigated further the effect of PIAS1 on the levels of the MAPK signaling pathway, tumor migratory factors including MMP-9, MMP-2, and ICAM-1, then providing a possible effective mechanism for gastric cancer treatment.

Materials and methods

Reagents

Patients and tissue samples. Tumor samples were obtained from gastric cancer patients who underwent surgery between 2002 and 2010 at RuiJin Hospital (Shanghai Jiaotong University School of Medicine, Shanghai, China). All cases were staged according the guidelines of the International Union Against Cancer (2002). There were 18 males and 13 females with ages ranging from 20 to 92 years (mean 60.9 years). All available clinicopathological data were collected (Table I). The study was approved by the ethical review board of RuiJin Hospital.

Tissue microarray construction and immunohistochemistry. Formalin-fixed paraffin-embedded samples were reviewed for tissue array construction by a pathologist. At least two core tissue biopsies were taken from morphologically representative regions of each gastric tumor. The samples of 31 primary tumor cases and 31 adjacent normal tissues samples were arranged in rows and columns to construct a tissue microarray. For staining, the sections (4 µm) were transferred to glass slides using an adhesive slide system (PSA-CS 4; Instrumedics, Hackensack, NJ, USA) to support cohesion of the array elements. The slides were de-waxed in xylene, and rehydrated in ethanol with descending concentration. Anti-PIAS1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted to 1:50) was added after blocking of endogenous peroxidase and proteins, and each section was incubated with HRP-labeled anti-goat IgG antibody. The immunostained specimens were assessed by two independent observers without prior knowledge of the clinicopathologic characteristics. PIAS1 expression was scored using two measures: staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong); the percentage of positive cells was scored as 0 (negative), 1 (<10%), 2 (11-50%), 3 (51-80%), or 4 (>80%). The two scores were multiplied and two positive cells was scored as 0 (negative), 1 (<10%), 2 (11-50%), 3 (51-80%), or 4 (>80%). The two scores were multiplied and two positive cells was scored as 0 (negative), 1 (<10%), 2 (11-50%), 3 (51-80%), or 4 (>80%).

PIAS1 expression in cells by RT-PCR. Total RNA was isolated from cells using TRIzol reagent kits (Gibco BRL, Rockville, MD, USA). The cDNA obtained from this reaction was mixed with PCR buffer, MgCl2, dNTPs, Taq DNA polymerase, and human PIAS1 gene-specific primers (the primer sequence of PIAS1, the forward primer: 5'-CCA CGC TTT CCT GCT GTA GA-3'; the reverse primer: 5'-TAT CAC ACA GGC AGT CTT GA-3') and amplified in an automated thermal cycler. The conditions of RT-PCR were as follows: 1 cycle for 5 min at 95°C; 35 cycles for 45 sec at 94°C, for 45 sec at 55°C, for 1 min at 72°C; 1 cycle for 10 min at 72°C. The PCR products were separated by electrophoresis in 1.2% agarose gels and stained with ethidium bromide. The densities of cDNA bands were analyzed by scanning densitometry using GelDoc 2000 (Bio-Rad, Baltimore, MD, USA). The band densities were normalized to GAPDH (the primer sequence of GAPDH, the forward primer: 5'-GCG TGA GAA CGG GAA GCT TGT CAG CTC CTA TGT CA-3'; the reverse primer: 5'-CAC CAA TCC TCT CTA TGG TGG TGA TGA AAG CTA-3') band densities and the results were expressed as ratio.

Scratch wound-healing assay. To measure cell motility, 4x103 cells were seeded in 6-well plates. A central linear wound was created by scraping the cell monolayer with a 200-µl sterile pipette tip. The media were carefully changed to remove any floating cells and cultured at 5% CO2 and 37°C. The migration of...
cells into the denuded areas in the scraped region was calculated at 48 h, respectively. The wound at 0 h was considered 100% of the average gap.

**Cell invasion.** For the determination of invading cells, the transwell chambers (Corning Inc., NY) were used. The transwell chamber membranes were covered with 75 µl Matrigel™ (2 mg/ml, BD Biosciences, NJ, USA) and incubated for 2 h at 37°C. The cell lines were grown in RPMI-1640 (10% FBS) medium, thus were trypsinized and suspended at a density of 1x10⁶/ml in serum-free RPMI-1640. Suspended cells (200 µl) were placed into the upper compartment of the Transwell chambers. The lower compartment of the chambers was filled with 600 µl RPMI-1640 containing 10% FBS. The transwell chamber systems were incubated in the humidified incubator (37°C, 5% CO₂) for 24 h. After incubation, the non-invaded cells and the matrigel were removed with a cotton toothpick at the top of the chamber. At the bottom of the cell swab, the invaded cells were washed with PBS and fixed with 4% paraformaldehyde and then were stained with Giemsa for 15 min. Five random fields were counted and the invasion rate was calculated to find the number of the invaded cells under the inverted microscope at magnification x200. The data representing the average cells of five fields was compared between the experimental groups and control group. Each experiment was repeated three times.

**Western blot analysis.** The levels of P38MAPK, P-P38MAPK, JNK/SAPK, P-JNK/SAPK, ERK, P-ERK, MMP-9, MMP-2, and ICAM-1 proteins were investigated in each group using western blot analysis. The cells were washed twice with PBS and then homogenized in RIPA buffer. Following centrifugation at 12,000 g at 4°C for 10 min, the supernatant was collected and stored at -80°C. Protein concentration of each sample was determined by BCA protein assay. Each sample was adjusted up to a desired protein content of 40 µg, thus denatured in loading buffer and separated by electrophoresis on 9% SDS polyacrylamide gel at 100 V for 120 min. The separated proteins were transferred to polyvinylidene difluoride membrane by using transfer buffer at 200 mA for 90 min. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)-0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS-0.1% Tween, and incubated with a primary antibody including P38MAPK, P-P38MAPK, JNK/SAPK, P-JNK/SAPK, ERK, P-ERK, MMP-9, MMP-2, and ICAM-1 with 1:1000 dilution in TBS-0.1% Tween overnight at 4°C, respectively. After three 10 min washes in TBS-0.1% Tween, the membranes were incubated with a second antibody, horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulin G (Kangcheng Inc., Shanghai, China) for 1 h at room temperature. After washing, the membranes were detected by the enhanced chemiluminescence methods (Amersham Biosciences, Piscataway, NJ, USA), and then scanned by densitometry using bio-image analysis system (Bio-Rad) for quantification. GAPDH was determined in the similar manner to anti-GAPDH antibody (diluted to 1:500, Santa Cruz) as an endogenous control for other proteins.

**Statistical analysis.** All data were expressed as the mean ± standard deviation (SD). Statistics were done by SPSS program at 10.5 versions. The χ² test was used to evaluate the correlation between PIAS1 expression and clinicopathological characteristics. The one-way analysis of variance (ANOVA) with Dunnet's multiple comparison tests was used for comparisons. A P<0.05 was considered as statistically significant.

**Results**

**Expression of PIAS1 in gastric cancer tissue.** We investigated the expression of PIAS1 in 31 cases of gastric cancer tissues and 31 cases of adjacent normal tissues of gastric cancer (Fig. 1). The results showed that PIAS1 levels were significantly lower in gastric cancer than in the adjacent normal tissues of gastric cancer in all 31 cases of patients. These results suggest that PIAS1 expression was significantly decreased in human gastric cancer.

Furthermore, the clinical relevance was confirmed by the observation that PIAS1 expression correlated with prognosis (Table I). Tumor staging was significantly associated with low expression of PIAS1 protein (P<0.05). However, a non-significant correlation was observed between PIAS1 protein expression and age with patients. No correlation was observed between PIAS1 protein expression and tumor size with patients or between PIAS1 protein expression and gender with patients (P>0.05).

**Expression of PIAS1 in gastric cancer cells.** We monitored the levels of PIAS1 transcripts in SGC7901 cells by RT-PCR assays (Fig. 2B). The data showed that at 6 h following Ad5/F35-PIAS1 treatment, PIAS1 mRNA levels were increased in SGC7901 cells but not in the cells with Ad5/F35-vector treatment. The enhanced PIAS1 gene expression returned to basal level within 72 h after stimulation. The protein expression of PIAS1 was monitored using western blot analysis (Fig. 2A). Similar to the RT-PCR data, the SGC7901 cells with Ad5/F35-PIAS1 treatment expressed a higher level of PIAS1 protein compared with SGC7901 cells with Ad5/F35-vector treatment. SGC7901 cells at 72 h of Ad5/F35-PIAS1 treatment did not show significant

<table>
<thead>
<tr>
<th>Total cases</th>
<th>Positive</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (&gt;50)</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>Low (&lt;50)</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Tumor extent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>T3, T4</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large (&gt;5)</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>Small (&lt;5)</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 1. The evaluation of PIAS1 expression investigated in gastric cancers (A1) and adjacent normal tissues of gastric cancer (B1) on tissue microarrays by hematoxylin-eosin staining and immunohistochemical method. The invasive gastric cancers are shown with negative and weak PIAS1 staining (A2-3). The adjacent normal tissues of gastric cancer showing strong PIAS1 staining of epithelial and acinar cells (B2-3). Magnification (A1-3, B1-3) x200.

Figure 2. The evaluation of PIAS1 expression was investigated in gastric cancer cell lines SGC7901 in each group. Western blot analysis showing the overexpression of PIAS1 in SGC7901 cells after they had been infected with an adenovirus containing PIAS1 construct in 24 h. The untreated cells and cells infected with adenovirus alone (vector) served as the control that showed lower expression of PIAS1 in 24 h (A1-2). These results showed similar changes to the results of RT-PCR analysis (B1-2). Note: vs. control group, △P<0.01; vs. Ad5/F35-vector treated group, ●P<0.01.
The proliferation, invasion and migration of gastric cancer cells. We investigated the effect of PIAS1 on proliferation of gastric cancer SGC7901 cells using *in vitro* MTT assay (Fig. 3). We found that the increased PIAS1 expression by transfection with Ad5/F35-PIAS1 significantly decreased the proliferative ability of SGC7901 cells.

To examine the effect of Ad5/F35-PIAS1 on cell motility, an *in vitro* scratch wound-healing assay was performed (Fig. 4). The results showed that the cells transfected with Ad5/F35-PIAS1 had a reduced migration rate compared with the control cells at 24 h (P<0.01). To further investigate the effect of Ad5/F35-PIAS1 on cell invasion, we determined the invasion ability of the three groups of cells using the trans-well chambers assay (Fig. 5). After incubation for 24 h, the number of control group and Ad5/F35-PIAS1-vector treated cells which had invaded the polycarbonate membrane of the matrigel chamber was ~3.7- and 3.3-fold greater than that of the Ad5/F35-PIAS1 treated group, respectively [(21.25±1.51) and (19.35±1.33) vs (5.70±1.56)] (P<0.05). These results provide strong evidence that PIAS1 plays a role in decreasing metastasis of gastric cancer cells.

**Expression of P38MAPK, P-P38MAPK, JNK/SAPK, P-JNK/SAPK, ERK, P-ERK, MMP-9, MMP-2, and ICAM-1 in gastric cancer cells.** The proteins of P38MAPK, P-P38MAPK, JNK/SAPK, P-JNK/SAPK, ERK, and P-ERK were expressed in each group by western blot analysis (Fig. 6A and B). The expression levels of P38MAPK, P-P38MAPK, ERK, and P-ERK proteins were decreased in gastric cancer cells SGC7901 with Ad5/F35-PIAS1 treatment compared with these cells by transfection with Ad5/F35-vector and control cells. The expression levels of JNK/SAPK, P-JNK/SAPK proteins in gastric cancer with control had no different when compared with those of Ad5/F35-PIAS1 and Ad5/F35-vector treated cells, respectively. After Ad5/F35-PIAS1 treatment, the expressions of MMP-9, MMP-2, and ICAM-1 were detected in gastric cancers cells, which were decreased compared with those of cells with Ad5/F35-vector treatment and control, respectively. The protein levels of MMP-9, MMP-2, and ICAM-1 in gastric cancer cells with control were no higher than those of Ad5/F35-vector treated cells (Fig. 6A and C).
Discussion

Gastric cancer is the most frequent malignancy of the gastrointestinal tract in Chinese populations and the second most common cause of cancer related death in the world. The prognosis of gastric cancer has been improving owing to progress in diagnostic techniques and treatment methods for gastric cancer, but the prognosis of gastric cancer which has invaded is still poor with a 5-year survival of <35% (9). Among these malignant characteristics of gastric cancer cells, metastasis is an especially complex phenomenon, which requires the involvement of many different genes in multiple steps. The adhesion molecules, metastatic chemotaxis related genes, and others have been reported to play an important role in metastasis of gastric cancers (10,11), although many aspects of gastric cancer metastasis await further clarification.

Gene activation analyses show that PIAS1 selectively regulates a subset of STAT dependent genes, with a notable preference for inflammatory cytokines (11,12). In this study, we aimed to investigated the expression of PIAS1 on gastric cancer and evaluated its relationship with development of gastric cancer. Further the study elucidated the upregulating effect of PIAS1 on gastric cancer by a series of studies including cell viability assay and cell invasion, as well as the effective mechanisms of PIAS1, in order to explore a new therapeutic agent for gastric cancer. The PIAS1 expression was significantly decreased in human gastric cancer, which was positively correlated with the development of gastric cancer, but which showed no correlation with tumor size, gender or age with patients. Our results demonstrate that the decreasing expression of PIAS1 was found in TNM stage III and IV tumors. PIAS1 might play an important role in a certain stage. The exact role of PIAS1 in gastric cancer has not been identified. Its upstream and downstream molecular targets are also unidentified. Future investigation will provide the necessary information to shed lights on these points.

Recently, the increasing evidence indicates that anticancer drugs activated many pro-inflammatory signal pathways, some of which were connected to the development of metastasis of tumor cells. The MAPK signaling is an important signaling transduction pathway activated by many different stimuli. Previous reports have shown that the modulators of MAPK signaling pathway can affect cell growth (13,14). With regard to human gastric carcinoma, the investigations of alterations in expression and activity of components of the MAPK cascade will help to understand the mechanisms of malignant behaviors of gastric cancer cells. The P38MAPK is a member of MAPK signaling pathway, mediating many cell reactions induced by stress, inflammatory cytokines or bacterial products and playing a key role in the regulation of cell cycle. For the different cell lines of gastric cell (15), the similar phenomenon can be seen when these cells are presented under P38MAPK activity. Therefore, P38MAPK pathway may be one candidate target of cancer therapy for further study. The activation of signal transduction pathways by growth factors, hormones and neurotransmitters is mediated through JNK/SAPK signaling pathway. It is the basic signal transduction pathway regulating cell proliferation and differentiation (16). Recent research data indicate that the activation of ERK cascade is involved in H. pylori-induced proliferation of gastric epithelial cells (17).
Thus, these results suggest that the MAPK signaling pathway is involved in regulation of the tumor migratory factors in cancer cells, and may contribute to cell growth and cell invasion. Furthermore, this study investigated the relationship of the MAPK signaling pathway and PIAS1 overexpression in gastric cancer. The results of the study showed that the incubation with Ad5/F35-PIAS1 for 24 h in cells could block ERK and P38MAPK proteins activity. We used Ad5/F35-vector to detect the MAPKs signaling pathway in whole gastric cancer cells, which showed that the administration of Ad5/F35-vector had no effect on the expression and activation of MAPK signaling pathway including ERK, JNK/SAPK, and P38MAPK proteins.

Local invasion and metastasis are hallmark features in cancer progression. The modulation of the adhesive and migratory properties of the disseminating tumor cells play an essential role in the process of invasion and metastasis. The ability of tumor cells to adhere and migrate across the endothelium and extracellular matrix (ECM) is considered one of the most important prerequisites for the formation of invasion and metastasis (18). ICAM-1 has been found overexpress in gastric cancer and it is correlated with cancer progression (19). The MMPs constitute a large family of endopeptidases, which are responsible for degrading almost all ECM components, with each ECM element being cleaved by a specific MMP. Consistent with their role in tumor progression, the high levels of MMP-2 and MMP-9 have been shown to correlate with poor prognosis (20). We took this experiments further by examining whether MMP-2, MMP-9 and ICAM-1 regulate the invasion of gastric cancer. We further observed the administration of Ad5/F35-PIAS1 in gastric cancer cells markedly decreased the levels of MMP-2, MMP-9 and ICAM-1 proteins. One of the mechanisms by which P38 MAPK may promote tumor cell migration and invasion is by the upregulation of MMPs. Our studies have also demonstrated that MMP-2, MMP-9 and ICAM-1 can directly stimulate cell invasion and invasion via MAPK-dependent mechanism. The low MMP-2, MMP-9 and ICAM-1 protein expression was shown in SGC7901 cells with Ad5/F35-PIAS1 treatment. Therefore, these findings provide a possible explanation how the upregulation of PIAS1 expression could inhibit the invasion of gastric cancer.

In conclusion, the downregulation of PIAS1 expression shows potential to be used as a valuable assessment marker for the progression of gastric cancer. The upregulation of PIAS1 expression inhibited the activation of P38MAPK and ERK proteins and downregulated the levels of downstream cytokines (MMP-2, MMP-9 and ICAM-1), relieving cell invasion. It might be helpful to use PIAS1 in gastric cancer for therapeutic strategy.

References