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Original Article

PIAS3, an inhibitor of STAT3, has intensively negative association with the survival of gastric cancer

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Abstract: Objective: The present study was conducted to elucidate the prognostic prediction value of the expression of the protein inhibitor of activated signal transducer and activators of transcription 3 (PIAS3) in gastric cancer (GC). Methods: We detected the expression of PIAS3 in GC tissue, adjacent non-tumor tissue, GC cell lines, and GES-1 cell line. Besides, both clinicopathological data and follow-up records were obtained for patients' survival analyses. Results: We showed that both protein and mRNA expression of PIAS3 in GC tissue were significantly lower than those in adjacent non-tumor tissue, respectively. Besides, the relative mRNA expression value of PIAS3 in each of GC cell lines was also much lower than that in GES-1 cell line. With multivariate survival analyses, PIAS3 protein expression in GC tissues, and status of lymph node metastasis were identified to be the independently prognostic predictors of GC by using the Cox regression with bootstrapping method. Conclusions: Lower expression of PIAS3 protein, indicating the poor survival of GC, is a potential marker for prediction the prognosis of patients.

Keywords: Stomach, neoplasm, protein inhibitor of activated signal transducer and activators of transcription 3, survival, lymph node

Introduction

Although the worldwide incidence of gastric cancer (GC) was still decreasing, GC still accounts for 3% to 10% of all cancer related deaths [1]. The survival rate for GC has steadily been improving in East countries in the past decades, though it has not been showed in other nations [2]. In spite of the technical improvements in surgery, the prognostic evaluation of GC is still be considered as the critical procedure for administration the optimal treatment for improvement the survival rate for patients. Recently, researchers are still concentrating on the optimal biomarkers for precise prediction the prognosis of GC. Relatively low sensitivity and specificity in diagnosis or prognosis of GC limited most biomarkers in further use [3]. It was fully elucidated that signal transducers and activators of transcription (STAT) could be activated by upstream receptor or non-receptor tyrosine kinases in malignancies [4]. Recently, researchers reported that STAT3 activation was correlated with such diverse cellular phenotypes as differentiation, proliferation, apoptosis regulation, angiogenesis, malignant transformation, metastasis formation and drug responsiveness [5]. In addition, the activation of EGFR/STAT3 signaling pathway was demonstrated to contribute to lymph node metastasis from GC [6]. It is certain that PIAS3 was originally identified as a specific inhibitor of STAT3 [7]. However, the biological functions of PIAS3 in GC tissue have not been elucidated. Taking into account the aforementioned considerations, we designed this study to elucidate the potentially prognostic prediction of PIAS3 expression for GC patients.

Patients and methods

Data source

After the institutional review board of Tangshan People's hospital (China) approved our study, data from the cancer registry of the Tangshan

Table 1. Patient demographics

Gender	
Male	41 (74.55%)
Female	14 (25.45%)
Age at surgery	
≤ 60	32 (58.18%)
> 60	23 (41.82%)
Tumor location	
Other location	31 (56.36%)
Lower third	24 (43.64%)
Tumor size	
< 4.0	8 (14.55%)
≥ 4.0	47 (85.45%)
Tumor invasion (serosal invasion)	
Yes	47 (85.45%)
No	8 (14.55%)
Status of lymph node metastasis	
Yes	36 (65.45%)
No	19 (34.55%)
Lauren classification	
Intestinal	16 (29.09%)
Diffuse	37 (67.27%)
Mixed	2 (3.64%)
Type of gastrectomy	
Distal subtotal	25 (45.45%)
Proximal subtotal	10 (18.18%)
Total	20 (36.36%)
PIAS3 protein expression in GC tissues (IH)	
No (-, +)	38 (69.09%)
Yes (++, +++)	17 (30.91%)

Abbreviation: IH, immunohistochemical staining.

People's hospital were obtained. Data obtained from the registry were listed as follows: gender, age at surgery, tumour location, tumour size, tumour invasion (serosal invasion), status of lymph node metastasis, Lauren classification, type of gastrectomy, and follow-up vital status. Oral and written informed consents were also obtained from patients who were included in this study.

Cells

Human GC cell line AGS, KATO-III, SUN-1, SGC-7901, HGC-27, MGC-803, and BGC-823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences, (Shanghai, China). Human normal gastric mucosa cell line GES-1 lines were purchased from Biowit Technologies Corporation (Shenzhen, China). GC and GES-1 cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 (Thermo Electron Corporation, Beijing, China). Media were supplemented with 10% (v/v) FBS (Life Tech, Mulgrave Vic, Australia) and penicillin/streptomycin (10,000 IU/ml penicillin, 20 mg/ml streptomycin; Roche, Swiss). The medium was changed twice a week.

Study population and tissue samples

A total of 55 GC patients after potentially curative resection for histologically confirmed gastric adenocarcinoma from Nov 2000 to Oct 2005 were eligible for this study. Eligibility criteria for this study included: 1) histologically proven adenocarcinoma, 2) no history of gastrectomy or other malignancy, 3) no distant metastasis or peritoneal dissemination, 4) lymphadenectomy performed, 5) no gastroesophageal junction tumor or cardia tumor, 6) no patients died during the initial hospital stay or for 1 month after surgery. All patients were operated on according to the potentially curative gastrectomy. Adjuvant chemotherapy or radiotherapy was not routinely administrated in patients routinely. This study was approved by the Research Ethics Committee of Tangshan People's hospital, and written informed consent was obtained from all patients.

Surgical treatment

Curative resection was defined as a complete lack of grossly visible tumor tissue and metastatic lymph nodes remaining after resection, with pathologically negative resection margins [8]. Primary tumors were resected en bloc with limited or extended lymphadenectomy (D1 or D2-3 according to the Japanese Gastric Cancer Association (JGCA) [9]). Surgical specimens were evaluated as recommended by the seventh UICC TNM classification for GC.

Immunohistochemistry

Paraffin sections (4 μm thick) were deparaffinized and rehydrated. Antigen retrieval treatment was done at 95°C for 40 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0), and endogenous peroxidases were blocked using 3% hydrogen peroxide for 30 minutes. Purchased antibody was mouse anti-PIAS3 (Epigentek, A-8151-100, 1:150 dilution). All sections were incubated overnight with the primary antibody at 4°C. The sections were then treated with peroxidase using the labeled poly-

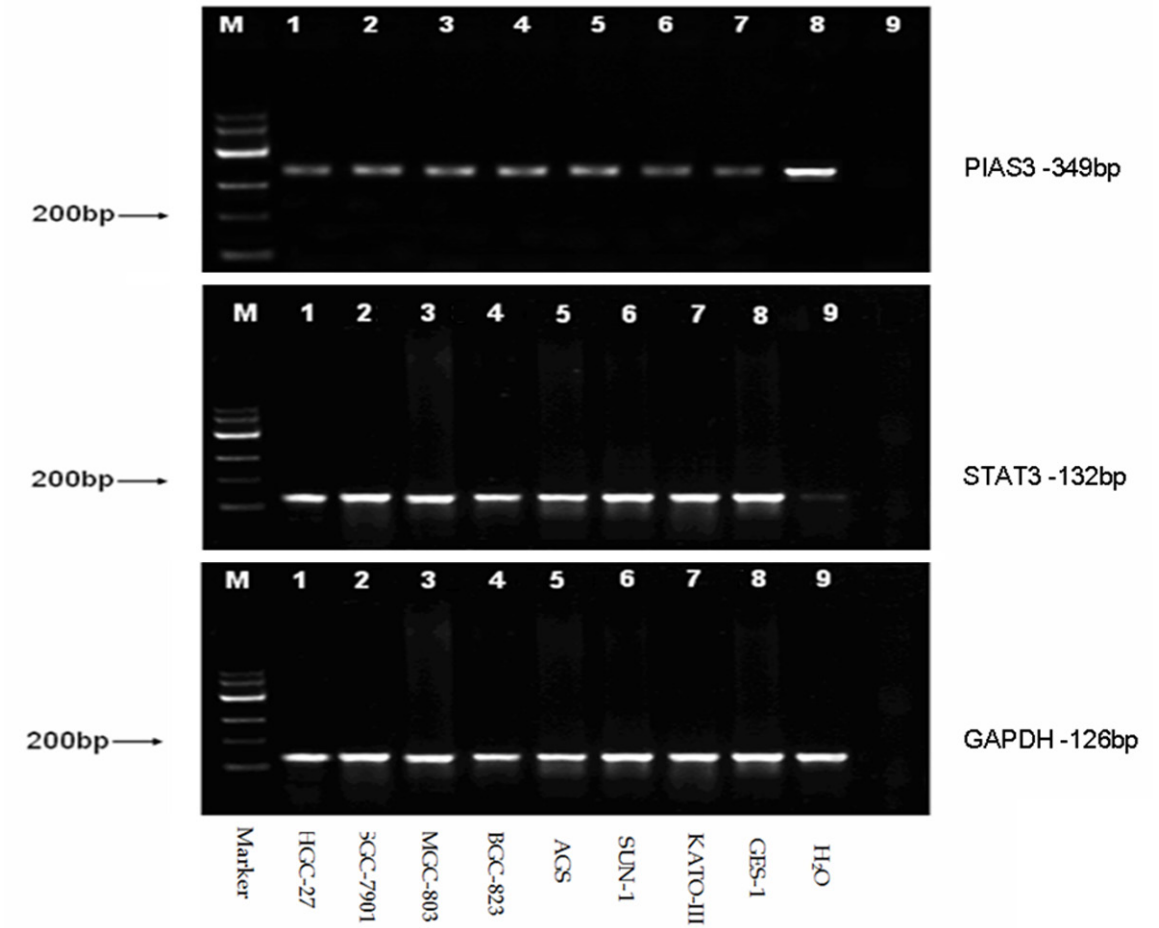

Figure 1. PIAS3 and STAT3 mRNA expression in gastric cancer cell lines and GES-1 cell line.

mer method with Zhongshan Peroxidase (Beijing, China) for 30 minutes. Antibody binding was visualized using the Avidin Biotin Complex (ABC) Elite Kit and 3,3'-diaminobenzine according to the manufacturer's instructions (City Key Laboratory of Tianjin Cancer Center, China). Sections were then counterstained in hematoxylin. For general negative controls, the primary antibody was replaced with PBS.

Microscopic assessment of PIAS3 protein expression

All sections were assessed blindly by two independent observers, and in cases of assessing disagreement a third independent assessment was performed. Staining for PIAS3 protein was considered potentially positive if there was cytoplasmic staining. The grade of staining intensity of PIAS3 protein was rated on a scale from "-" to "+++", with "-", indicating no staining; "+", weak staining; "++", moderate staining; and "+++", strong staining. The intensity scores of "++" and "+++" were considered positive staining [10]. The intensity scores of "++" and "+++" were considered positive staining, whereas the intensity scores of "-" and "+" were considered negative staining.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

For the PIAS3 and STAT3 semi-quantitative RT-PCR, RNA was extracted from all GC cell lines, GES-1 cell line, gastric adenocarcinoma tissue, and adjacent non-tumor tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA in a 20 ul volume using Reverse Transcription kit (Invitrogen, Carlsbad, CA). Primers designed and utilized for PIAS3 was as follows: Forward sequence: 5'-GAGCCGACATCCAAGGTTTAG-3', and Reverse sequence: 5'-GACAGCGAAGTTTCCATAATCC-3'. Primers designed and utilized for STAT3 was as follows: Forward sequence: 5'-GAAGGACATCAGCGGTAAGA-3', and Reverse

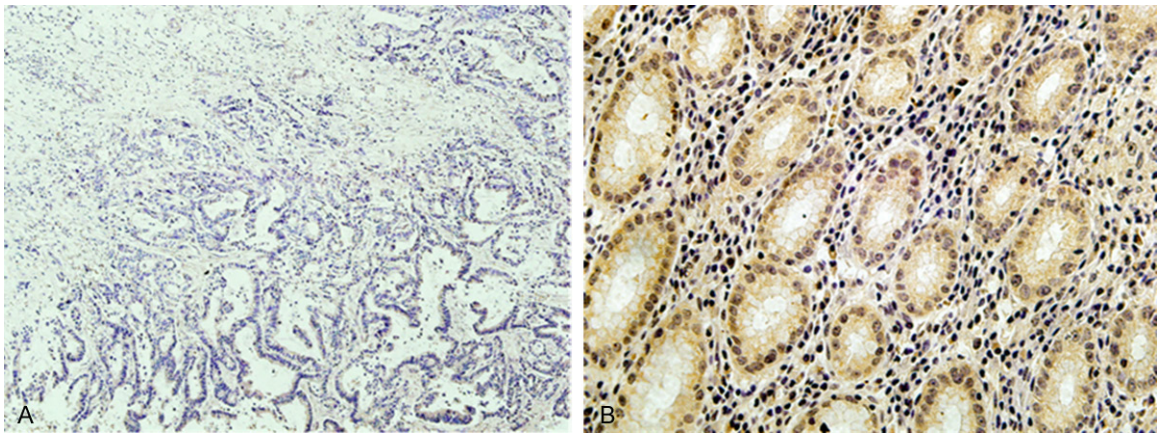

Figure 2. A. Expression of PIAS3 protein in the cytoplasm of malignant epithelium of gastric cancer tissue (× 400 magnification); B. Expression of PIAS3 in the cytoplasm of epithelium of adjacent non-tumor tissue (× 400 magnification).

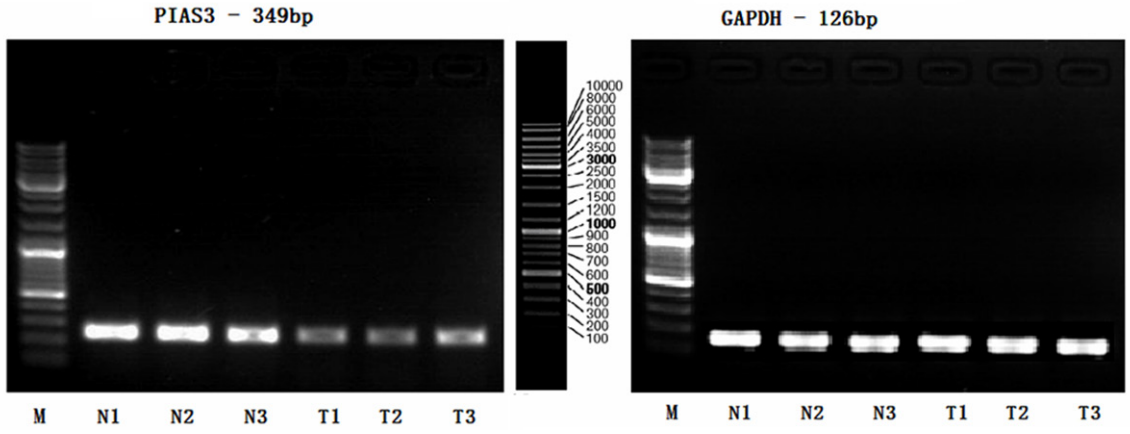

Figure 3. PIAS3 mRNA expression in gastric cancer tissue and adjacent non-tumor tissue (representation: N: adjacent non-tumor tissues; T: gastric cancer tissues).

sequence: 5'-TACTTTCCGAATGCCTCCTC-3'. The GAPDH gene was used as an endogenous control for quantitative DNA-PCR. Primers designed and utilized for GAPDH was as follows: Forward sequence: 5'-GAAGGTGAAGGTCGGAGTC-3', and Reverse sequence: 5'-GAAGATGGTGATGGGATTTC-3'. The PCR Cycling conditions for all sequences were 35 cycles of denaturation at 95°C for 3 minutes, annealing at 94°C for 30 seconds, and extension at 56°C for 30 seconds followed by a final extension at 72°C for 8 minutes. All PCR product electrophoreses were performed on a 2% agarose gel with ethidium bromide and visualized using the Gel Imager system (Asia Xingtai Mechanical and Electrical Equipment Company, Beijing, China). The relative expression value of mRNA was expressed by ratio between target mRNA gray scale value and GAPDH gray scale value. Amplifications were quantified by computerizing absorbance values with Gel Imager system.

Follow-up

After curative surgery, all patients were followed every 3-6 months for 2 year, then every year or until death. The median follow-up for the entire cohort was 38.0 months (range: 2-86). The follow-up of all patients who were included in this study was completed in December 2012. B ultrasonography, CT scans, chest X-ray, and endoscopy were obtained with every visit.

Statistical analysis

Differences in the different variables of GC patients were estimated using the X^2 test for

Table 2. Survival analysis of GC patients

Variables	Median OS (mo)	X^2 value	Univariate *P* value	HR value	Multivariate *P* value
Gender					
Male	31	0.624	0.430		
Female	34				
Age at surgery (years)					
≤ 60	34	1.474	0.225		
> 60	25				
Tumor location					
Other location	28	3.822	0.049		
Lower third	35				
Tumor size (cm)					
< 4.0	41	0.901	0.342		
≥ 4.0	31				
Type of gastrectomy					
Distal subtotal	34	3.175	0.204		
Proximal subtotal	35				
Total	21				
Lauren classification					
Intestinal	28	0.103	0.950		
Diffuse	34				
Mixed	17				
Tumor invasion (serosal invasion)					
Yes	21	1.163	0.281		
No	33				
Status of lymph node metastasis					
Yes	21	9.519	0.002	2.979 (1.349-6.576)	0.007
No	41				
PIAS3 protein expression in GC tissues (IH)					
No (-, +)	21	9.962	0.002	2.612 (1.283-5.317)	0.008
Yes (++, +++)	50				

Abbreviation: IH, immunohistochemical staining.

categorical data and independent-paired Student's t test for continuous variables. Univariate survival analysis was performed by the Kaplan-Meier method, and log-rank test was performed to determine significance. Cox regression with bootstrapping performance analysis was used to estimate the independent risk factor of overall survival (OS) of GC patients after curative surgery. *P* value less than 0.05 was considered significant. All statistical calculations were performed using PASW Statistics 18 software.

Results

Patient demographics

Patient demographics are shown in **Table 1**. The median OS of 55 GC patients was 33 months (range, 14-67 months) and 14 patients was alive when fellow-up was over.

Expression of PIAS3 and STAT3 mRNA in GC cell lines and GES-1 line

PIAS3 mRNA expression was assessed in seven GC cell lines and GES-1 line by using the RT-PCR (**Figure 1**). The relative mRNA expression value of PIAS3 in each of GC cell lines was significantly much lower than that in GES-1 cell line ($0.31 \pm 0.16_{HGC-27}$, $0.54 \pm 0.09_{SGC-7901}$, $0.42 \pm 0.14_{MGC-803}$, $0.36 \pm 0.18_{BGC-823}$, $0.66 \pm 0.21_{AGS}$, $0.57 \pm 0.16_{SUN-1}$, $0.39 \pm 0.11_{KATO-III}$, VS $2.47 \pm 0.64_{GES-1}$). In addition, STAT3 mRNA expression was also assessed in all GC cell lines and GES-1 line by using the RT-PCR (**Figure 1**). The relative mRNA expression value of STAT3 in each of GC cell lines was significantly much higher than

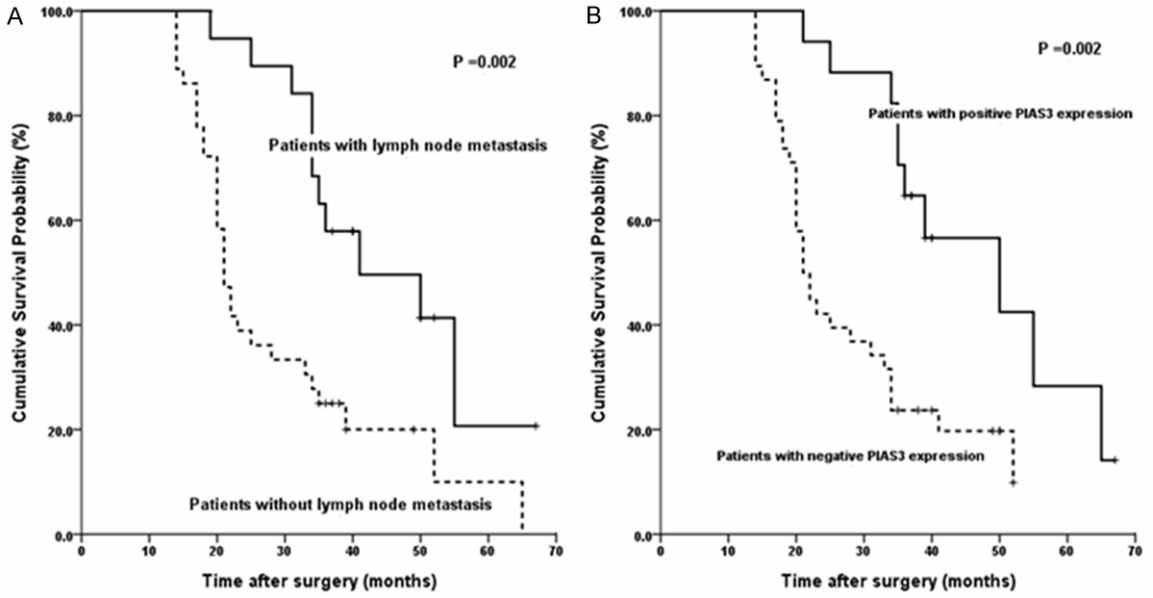

Figure 4. Survival curve for 55 GC patients after curative resection (A) according to stage subgroup of status of lymph node metastasis; (B) According to stage subgroup of PIAS3 protein expression.

that in GES-1 cell line ($2.48 \pm 1.10_{HGC-27}$, $2.67 \pm 0.93_{SGC-7901}$, $2.70 \pm 1.16_{MGC-803}$, $1.96 \pm 0.86_{BGC-823}$, $3.06 \pm 1.25_{AGS}$, $2.87 \pm 1.44_{SUN-1}$, $3.09 \pm 1.07_{KATO-III}$, VS $0.17 \pm 0.04_{GES-1}$).

Immunohistochemical staining for PIAS3 protein in GC and adjacent non-tumor tissues

PIAS3 protein expression was mainly observed in the cytoplasm (**Figure 2**). PIAS3 protein expression was detected in 33 (60.0%) (-), 13 (23.64%) (+), 6 (10.91%) (++), and 3 (5.45%) (+++) tumor samples, which represented that only 9 (16.36%) patients presented positive PIAS3 protein expression. Meanwhile, we found that 16 (29.09%) (-), 7 (12.73%) (+), 21 (38.18%) (++), and 11 (20.0%) (+++) of PIAS3 protein expression were detected in adjacent non-tumor tissues, respectively. Therefore, we demonstrated that the positive rate of PIAS3 protein expression in GC tissues was significantly higher than that in adjacent non-tumor tissues ($P < 0.001$).

Expression of PIAS3 mRNA in GC and adjacent non-tumor tissues

PIAS3 mRNA expression was detected in malignant tissues of GC and adjacent non-tumor tissues from 55 patients (**Figure 3**). The relative mRNA expression value of PIAS3 in GC tissues was significantly much lower than that in adjacent non-tumor tissues (0.59 ± 0.31 VS 2.10 ± 0.72, $P < 0.001$). PIAS3 mRNA expression in adjacent non-tumor tissues was about 3.50-fold higher than that in GC tissues.

Survival analysis

The results of survival analysis of 55 GC patients were showed in **Table 2**. Univariate analysis showed significant relationship between OS and tumor location, status of lymph node metastasis, and PIAS3 protein expression (immunohistochemical staining) in GC tissues. PIAS3 protein expression in GC tissues (HR = 2.979; $P = 0.007$), and status of lymph node metastasis (HR = 2.612; $P = 0.008$) were identified as the independent factors of OS in all enrolled GC patients following the multivariate analysis (Cox regression with bootstrapping performance) (**Figure 4**).

Discussion

More than 980 000 new GC cases are diagnosed annually, and the disease causes 730 000 deaths per year. As we know, STAT3 activity can contribute to oncogenesis and promote the metastasis of GC [6]. Under physiological conditions in normal cells, the activation of STAT proteins is rapid and transient because of negative regulation by proteins such as suppressors of cytokine signaling (SOCS) and PIAS

[11]. Although the PIAS proteins were identified to impact on the function of a number of proteins, the major process on which all these proteins act was the control of gene transcription. Thus, PIAS proteins should be considered to be transcriptional co-regulators. The other major functional part of PIAS proteins was reported to be the SP-RING domain, which was associated with the zinc-binding RING fingers and was most similar to the domains identified in a subclass of ubiquitin E3 ligases [12]. Theoretically, these somewhat functionally-redundant proteins are structurally associated with ubiquitin and are covalently attached to target proteins by a SUMO-conjugation system consisting of an E1 activating enzyme (SAE1/SAE2), an E2 ligase (Ubc9) and various E3 ligases with differing target-protein specificities [13].

Researchers demonstrated that PIAS3 was an E3 SUMO ligase, which could bind Rac1 to induce the SUMO modification within the polybasic regions of Rac1 in response to the stimulation of hepatocyte growth factor (HGF) [14]. Furthermore, PIAS3-mediated SUMO modification was also demonstrated to be essential for Rac1 activation and Rac1-mediated lamellipodium extension, cell migration, and invasion. PIAS3 expression was also identified to be correlated with STAT3 activation, and PIAS3 could control the extent and the duration of STAT3 activity in normal cells. In cancer cells, the expression of PIAS3 protein was post-transcriptionally suppressed and promoted the oncogenic effects of STAT3 activation [12]. PIAS3 was the firstly identified as a transcriptional repressor of STAT3 activation, which could inhibit transactivation of a STAT3-responsive reporter gene and could decrease the DNA-binding activity of STAT3 [12]. Besides, PIAS3 was also considered as an endogenous inhibitor of STAT3 attributing its anti-proliferative properties [15]. Owing to interference with the DNA-binding activity of STAT3, PIAS3 was identified to be a specific inhibitor of STAT3 in several human malignant diseases [16]. However, the role and the regulatory mechanisms of PIAS3 in GC are unclear.

To date, the molecular mechanism of causing the disease is still elusive. Finding reliable biomarker is important for exploring the molecular mechanism of GC. However, the levels of some important proteins cannot be accurately detected in GC tissue. PIAS3 was identified to affect the growth of cancer cells by inhibiting both the JAK/STAT and PI3-K/Akt signaling pathways or by regulating its SUMO (small-ubiquitin like modifiers) ligase activity in GC [17]. In this study, we showed that PIAS3 mRNA expression was simultaneously silence in the seven different GC cell lines, which was similar to PIAS3 mRNA expression in other human malignant disease [13]. In view of the results of STAT3 mRNA expression in the seven GC cell lines, we deduced that the silence of PIAS3 expression might be a key mechanism to incur the activation of STAT3 gene for promotion the progression of GC. With the further investigation, we demonstrated that PIAS3 mRNA expression and PIAS3 protein expression in GC tissues were significantly lower than those in adjacent non-tumor tissues, respectively. On the other hand, the immunohistochemical staining results also showed that PIAS3 protein expression was much low in GC tissues, compared to that in adjacent non-tumor tissues. Owing to the clearly negative association of STAT3 with overall survival of GC, we did think that it was obligatory to verify whether the low expression of PIAS3 in GC was associated with the poor prognosis of GC [4, 6]. With the survival analyses, we demonstrated that PIAS3 was identified to be an independent predictor of prognosis of all GC patients, as was occurrence of lymph node metastasis. The PIAS3 protein expression was positively associated with the overall survival of GC. Therefore, we thought that PIAS3 should be deemed as a promising biomarker for evaluation the prognosis of GC.

In this study, we originally elucidated that the low expression of PIAS3 in GC is specific. Although we performed this study with the small scale GC samples and the several GC cell lines, we detected the PIAS3 expression in the different stratification planes. Future research should be carried out for elucidation the clearly molecular mechanisms of low expression of PIAS3 in GC, which can provide elaborately functional information about PIAS3 in carcinogenesis and prognosis.

Disclosure of conflict of interest

None.

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References

[1] Dicken BJ, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM. Gastric adenocarcinoma: review and considerations for future directions. Ann Surg 2005; 241: 27-39.

[2] Schmidt B, Yoon SS. Varying Lymphadenec tomies for Gastric Adenocarcinoma in the East Compared with the West: Effect on Outcomes. Am Soc Clin Oncol Educ Book 2012; 32: 250-255.

[3] Li Y, Yang Y, Lu M, and Shen L. Predictive value of serum CEA, CA19-9 and CA72.4 in early diagnosis of recurrence after radical resection of gastric cancer. Hepatogastroenterolog 2011; 58: 2166-2170.

[4] Groner B. Determinants of the extent and duration of STAT3 signaling. JAK-STAT 2012; 1: 3, 211-215.

[5] Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009; 9: 798-809.

[6] Deng J, Liang H, Zhang R, Sun D, Pan Y, Liu Y, Zhang L, Hao X. STAT3 is associated with lymph node metastasis in gastric cancer. Tumour Biol 2013; 34: 2791-2800.

[7] Junicho A, Matsuda T, Yamamoto T, Kishi H, Korkmaz K, Saatcioglu F, Fuse H, Muraguchi A. Protein inhibitor of activated STAT3 regulates androgen receptor signaling in prostate carcinoma cells. Biochem Biophys Res Commun 2000; 278: 9-13.

[8] Hermanek P, Wittekind C. Residual tumor (R) classification and prognosis. Semin Surg Oncol 1994; 10: 12-20.

[9] Jaehne J, Meyer HJ, Maschek H, Geerlings H, Bruns E, Pichlmayr R. Lymphadenectomy in gastric adenocarcinoma: a prospective and prognostic study. Arch Surg 1992; 127: 290-294.

[10] Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat 3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res 2003; 9: 594-600.

[11] Shuai K, Liu B. Regulation of gene-activation pathways by PIAS proteins in the immune system. Nat Rev Immunol 2005; 5: 593-605.

[12] Rosas-Acosta G, Langereis MA, Deyrieux A and Wilson VG. Proteins of the PIAS family enhance the sumoylation of the papillomavirus E1 protein. Virology 2005; 331: 190-203.

[13] Jackson PK. A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. Genes Dev 2001; 15: 3053-3058.

[14] Castillo-Lluva S, Tatham MH, Jones RC, Jaffray EG, Edmondson RD, Hay RT, Malliri A. SUMOylation of the GTPase Rac1 is required for optimal cell migration. Nat Cell Biol 2010; 12: 1078-1085.

[15] Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD, Shuai K. Inhibition of Stat1-mediated gene activation by PIAS1. Proc Natl Acad Sci U S A 1998; 95: 10626-10631.

[16] Wang L, Banerjee S. Differential PIAS3 expression in human malignancy. Oncol Rep 2004; 11: 1319-1324.

[17] Liu LM, Yan MG, Yang DH, Sun WW, Zhang JX. PIAS3 expression in human gastric carcinoma and its adjacent non-tumor tissues. Clin Res Hepatol Gastroenterol 2011; 35: 393-398.