

Constitutive activation of STAT3 is predictive of poor prognosis in human gastric cancer

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Abstract Abnormalities in signal transducer and activator of transcription (STAT) signaling, especially STAT3 and STAT5, are involved in the oncogenesis of several human cancers, including gastric cancer (GC). However, the downstream targets of STAT3 and STAT5 are not fully identified, and the precise roles and the prognostic value of STAT3 and STAT5 in GC have not been fully characterized. In this study, we used ChIP-on-chip to identify STAT3 and STAT5 target genes on a whole genome scale in AGS cells, a human GC cell line. A total of 2,514 and 1,314 genes were identified as STAT3 and STAT5 target genes, which were mainly related to cell growth, metabolism, differentiation, adhesion, immune response, and stress response. Furthermore, we depleted STAT3 and STAT5 with a small interfering RNA,

respectively. Our results demonstrate that STAT3, but not STAT5, is involved in GC cell growth and cell cycle progression through regulation of gene expression, such as Bcl-2, p16^{ink4a} and p21^{waf1/cip1}. Moreover, expression of pSTAT3^{Tyr705} correlates with TNM stage, differentiation and survival, and is a significant prognostic factor in GC. Therefore, our findings provide novel evidence that STAT3 may be a potential therapeutic target for GC treatment and pSTAT3^{Tyr705} expression can predict prognosis in GC.

Keywords Gastric cancer · STAT3 · STAT5 · Prognosis

Introduction

Gastric cancer (GC) is the second most common cause of cancer-related deaths worldwide, accounting for more than 1 million deaths/year [1]. Despite advances in early diagnosis and therapeutic treatments for GC in recent years, overall patient survival has not significantly improved. The development of additional effective prevention and treatment strategies for GC is limited by an incomplete understanding of the critical signaling pathways involved in GC tumorigenesis.

Signal transducers and activators of transcription (STAT) is a family of transcription factors, including STAT1, 2, 3, 4, 5a, 5b, and 6 [2]. STATs play important roles in numerous physiological processes, including immune function, inflammatory response, cell growth, and differentiation. Recently, increasing evidence has suggested that abnormalities in STATs signaling, especially STAT3 and STAT5, are involved in the oncogenesis of several cancers [3–5]. For example, constitutive activation of STAT3 correlates with cell proliferation in breast [6] and non-small-cell lung carcinoma [5]. STAT5 expression correlates with aggressiveness in prostate

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cancer [1, 7]. Generally, the molecular mechanisms that control GC progression are related to altered expression of different proto-oncogenes, tumor suppressor genes, and cytokines and their receptors, including Ras, Src, and the epidermal growth factor receptor [5, 8]. Notably, these abnormalities involve the STATs signaling pathways; however, there is little knowledge of the relationship between GC and STATs. Therefore, increased understanding of the molecular mechanisms of STATs signaling in GC may allow the identification of novel therapeutic targets.

Although STATs are transcription factors which modulate transcription of numerous genes, no comprehensive identification of the endogenous genes which STATs bind to has been performed. This study was undertaken to assess the biological significance of STAT3 and STAT5 in GC, and the relationship with GC prognosis. We performed ChIP-on-chip to identify STAT3 and STAT5 target genes on a whole genome scale in the human GC cell line AGS. Furthermore, gene ontology (GO) analysis and pathway analysis were used to analyze the major functions of the target genes. We also silenced STAT3 and STAT5 using small interfering RNA (siRNA) in two human GC cell lines (AGS and MKN-28), and investigated in the effect on cell viability, apoptosis and cell cycle progression. In addition, STAT3 and STAT5 expression and activation were quantified using immunohistochemistry in human GC samples to determine the relationship with prognosis and clinical outcome. The overall aim of this study was to determine the precise roles of STAT3 and STAT5 in human GC progression, and to test the hypothesis that STAT3 and STAT5 could serve as potential therapeutic targets for the treatment of GC.

Materials and methods

Cell culture and transient transfection of STAT3 and STAT5 siRNA

Two human GC cell lines were used in this study. AGS, a poorly differentiated GC line, and MKN-28, a well-differentiated GC line. The cells were cultured in RPMI 1640 media (Gibco, Shanghai, China), supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified 5% CO₂ atmosphere.

Commercial siRNAs were obtained from Dharmacon Inc. (Lafayette, CO) and used to target human STAT3 (GenBank accession number NM_003150) and STAT5 (NM_003152 STAT5a and NM_012448 STAT5b). Cells were transfected with siRNA (50 nM) by using the DharmaFECT™ 1 siRNA transfection reagent according to the manufacturer's instructions. Nonspecific siRNA (Dharmacon Inc.) was used as a negative control and the selective silencing of STAT3 and STAT5 was confirmed by Western blotting.

ChIP-on-chip

AGS cells were fixed in crosslinking solution containing 1% formaldehyde (Sigma, St Louis, MO) for 10 min at 37°C, and then cross-linking was stopped by the addition of glycine solution. After washing with cold phosphate-buffered saline, the cells were scraped, collected by centrifugation, the cell pellet was lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0), and the extracts were sonicated until DNA fragments of 500–1,000 bp were formed. The protein-DNA complexes were precipitated using antibodies against STAT3, STAT5 or polyclonal rabbit anti-IgG as a negative control and mixed with agarose. After washing, the agarose was incubated with elution buffer at 65°C for 15 min, and the eluted samples were incubated at 65°C for 4 h to reverse the crosslinking. The samples were then amplified, labeled and hybridized to a ChIP-GLAS microarray (Aviva Systems Biology, San Diego, CA). The GLAS-chip is an oligo-based human promoter array, containing 20,000 40-mer-oligonucleotides representing unique sequences in the promoter region of 20,000 genes. The arrays were scanned and the data were statistically analyzed according to the manufacturer's instructions. The ratio value of every gene was calculated by cutting off the value in control antibody group, and the STAT3 and STAT5 target genes were defined as genes with a ratio value ≥ 2.0 .

GO category and pathway analysis

GO analysis was applied to analyze the major functions of the STAT3 and STAT5 target genes according to the Gene Ontology key functional classification of the NCBI [9]. Pathway analysis [10, 11] was used to identify the significant pathways for the target genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the BioCarta and Reactome bioinformatics databases. Fisher's exact test and the χ^2 test were used to analyze the GO and pathway categories, respectively, and the false discovery rate (FDR) [12] was used to correct the *P* values. The FDR was defined as $FDR = 1 - (N_k/T)$, where N_k is the number of Fisher's test *P* values less than χ^2 test *P* values. We computed *P* values for the GO of all target genes. Enrichment analysis was used to provide a measure of the significance of the function. As the enrichment value of a gene increases, the gene function is more specific, which helps to identify the GOs with the most concrete functional description. Within the significant categories, enrichment (Re) was calculated as: $Re = (n_f/n) / (N_f/N)$ where n_f is the number of target genes within the particular category, n is the total number of genes within the same category, N_f is the number of target genes in the entire microarray, and N is the total number of genes in the microarray [13].

Western blot and antibodies

Whole cell lysates were prepared from the cancer cell lines, and Western blot analysis was performed using standard protocols [8]. Briefly, equal amounts of protein (50–200 µg/lane) were separated by sodium-dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose (Amersham, UK), probed with primary antibodies specific to the protein of interest (1:1,000), incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) and detected using the SuperSignal West Femto Substrate enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as a loading control. All antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA), except for GAPDH (Kangchen, Shanghai, China) and phospho-STAT5 (pSTAT5^{Tyr694/Tyr699}; Santa Cruz Biotechnology, Santa Cruz, CA).

Cell viability assay

Cell viability was assessed using the Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) tetrazolium salt (WST-8)-based colorimetric assay [5, 7]. Briefly, control and treated GC cells were seeded into 96 well plates at 5×10^3 cells/well. At the specified time points, 10 µl CCK-8 solution was added to each well, the plate was incubated for 1 h and cell viability was determined using a microplate reader at 450 nm. Data were expressed as the percentage of viable cells as: relative viability (%) = $[A_{450}(\text{treated}) - A_{450}(\text{blank})] / [A_{450}(\text{control}) - A_{450}(\text{blank})] \times 100\%$.

Flow cytometry cell cycle analysis

Approximately 1×10^6 cells were removed at specified time points, washed twice with PBS, fixed in cold ethanol for 30 min, incubated with propidium iodide for 30 min, and analyzed using a flow cytometer (BD, San Diego, CA).

Quantification of apoptosis

Apoptosis was determined using flow cytometry analysis and Annexin-V FITC/propidium iodide (PI) double staining was performed in accordance with the manufacturer's protocol (Biovision, Mountain View, CA). Briefly, both suspended and trypsinized adherent cells (5×10^5) were collected, resuspended in 500 µl binding buffer containing 5 µl Annexin-V FITC and 5 µl PI, incubated for 5 min in the dark at room temperature, and immediately analyzed by flow cytometry (BD, San Diego, CA).

Gastric cancer patients and follow-up

Archival specimens were obtained with informed consent from 305 GC patients at Ren-Ji Hospital (Shanghai, People's Republic of China) between December 2002 and December 2004. Histopathology was performed according to the WHO classification. Tumor staging was defined according to the 6th edition of the Tumor–Node–Metastasis (TNM) classification of the International Union against Cancer. Patients were followed up until 31 December 2009, with a mean follow-up of 39.7 months (range, 4–84 months).

Immunohistochemical staining

Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. A three-step streptavidin-biotin-horseradish peroxidase immunohistochemical method was performed using the LSAB+ kit (DakoCytomation, Copenhagen, Denmark) according to the manufacturer's instructions with STAT3 (1:200), pSTAT3^{Tyr705} (1:50), STAT5 (1:100), and pSTAT5^{Tyr694/Tyr699} (1:50) primary antibodies.

The slides were examined independently by two investigators blinded to both the clinical and pathological data. Protein expression was quantified based on the extent of staining (percentage of positive tumor cells) and classified into two categories: negative (0–15% tumor cells positive) and positive (more than 15% tumor cells positive) [8, 14, 15].

Ethics statement

All patients provided written informed consent before enrollment, and the study protocol was approved by the Ethic Committee of Shanghai Jiao-Tong University School of Medicine Renji Hospital, and the study was carried out according to the provisions of the Helsinki Declaration of 1983.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 (SPSS, Chicago, IL). Results were expressed as the mean ± standard deviation (SD). The Student's *t* test was used for comparisons between groups. The associations between two categorical variables were evaluated using Fisher's exact test or the Chi-square test. Overall survival (OS) was defined as the interval between surgery and death or the last follow-up. Cumulative survival time was calculated using the Kaplan–Meier method and analyzed using the log-rank test. Cox's proportional hazards regression model was used for analysis of independent prognostic factors. Results were considered significant if the *P* value was less than 0.05.

Results

GO enrichment and pathway analysis of STAT3 and STAT5 target genes

By setting the filter for ratio values OF ≥ 2.0 , 2,514, and 1,314 genes were identified as STAT3 and STAT5 target genes, respectively, in AGS cells ([Electronic supplementary material](#)). These genes were sorted by enrichment of GO categories and pathway analysis. Our data showed that the highly enriched GOs of the STAT3 target genes were linked to cytokine production, carrier proteins/membrane transport, cell signaling/communication, metabolism, cellular transducers/signal transduction (Fig. 1a). In contrast, the significant GOs corresponding to the STAT5 target genes included immunology/immune response, cell proliferation, cytokine production, cell differentiation and metabolism (Fig. 1b).

Pathway analysis was mainly based on the KEGG, BioCarta and Reactome bioinformatics databases. The identified

Fig. 2 The functional role of STAT3 and STAT5 in gastric cancer (GC) cell growth and cell cycle progression. **a** GC cells were transfected with *STAT3* and *STAT5* siRNAs and cell viability (percentage of viable cells) was determined using the CCK-8 assay. *STAT3* siRNA inhibited GC cell growth up to 72-h post-transfection, after which time the cells recovered. Values are mean \pm SD of three experiments. **b** GC cells were transfected with *STAT3* and *STAT5* siRNA and cell cycle analysis was performed. At 72 h after transfection, *STAT3*-silenced cells were blocked in the G1 phase. **c** RNAi induces downregulation of STAT signaling in gastric cancer cells. Western blot analysis of AGS and MKN-28 cells demonstrating that *STAT3* and *STAT5* siRNA induced downregulation of STAT3 and STAT5 at 72-h post-transfection. Additionally, Bcl-2 was downregulated and p16^{ink4a} and p21^{waf1/cip1} were upregulated. Data are representative of three independent experiments. GAPDH was used as the loading control

target genes were sorted by enrichment of signaling pathway categories. Our results demonstrate that the significant pathways for the STAT3 target genes included the insulin signaling pathway, Wnt signaling pathway, cell cycle, apoptosis, and MAPK signaling pathway (Fig. 1c). In contrast, the significant pathways corresponding to the STAT5

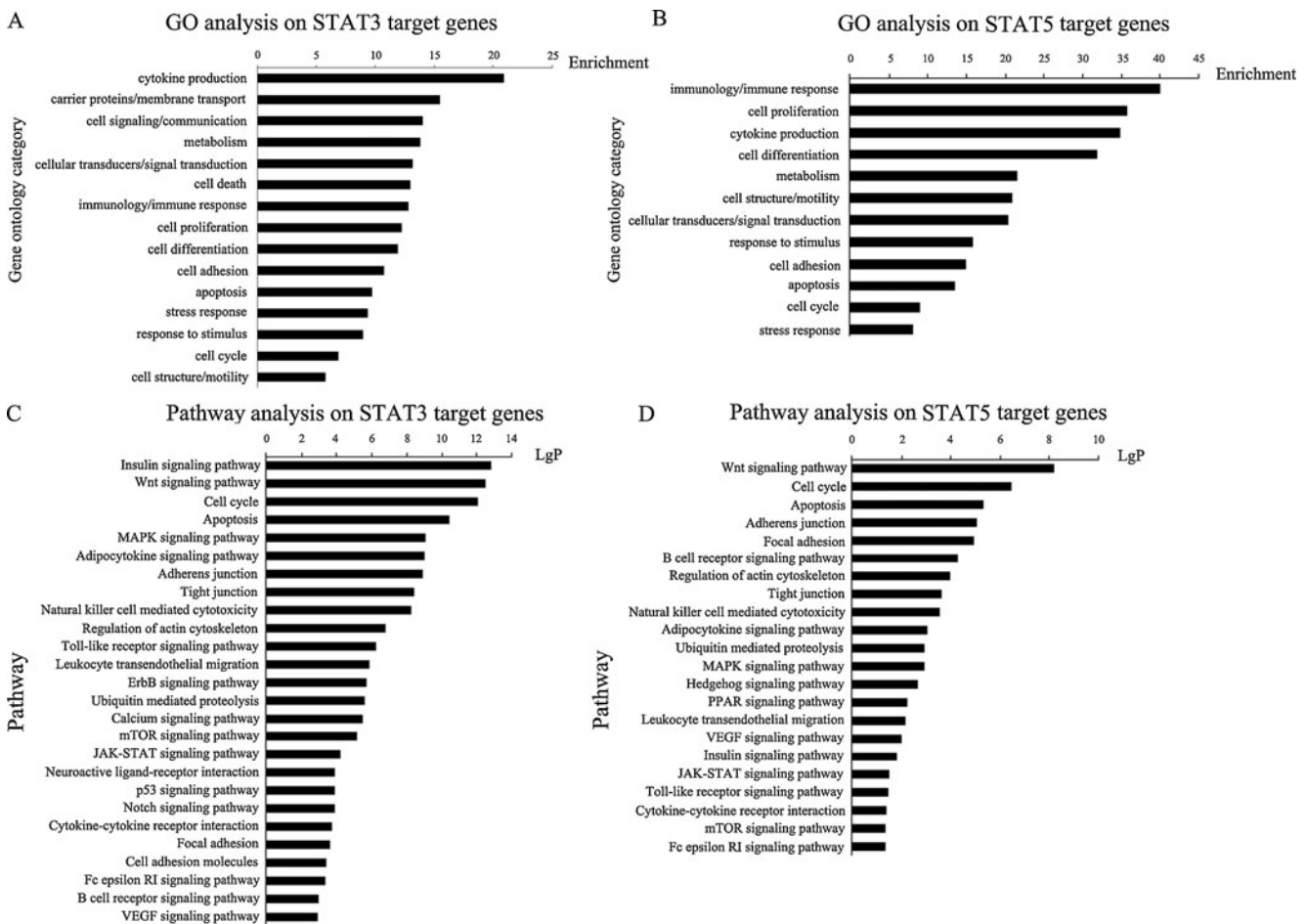
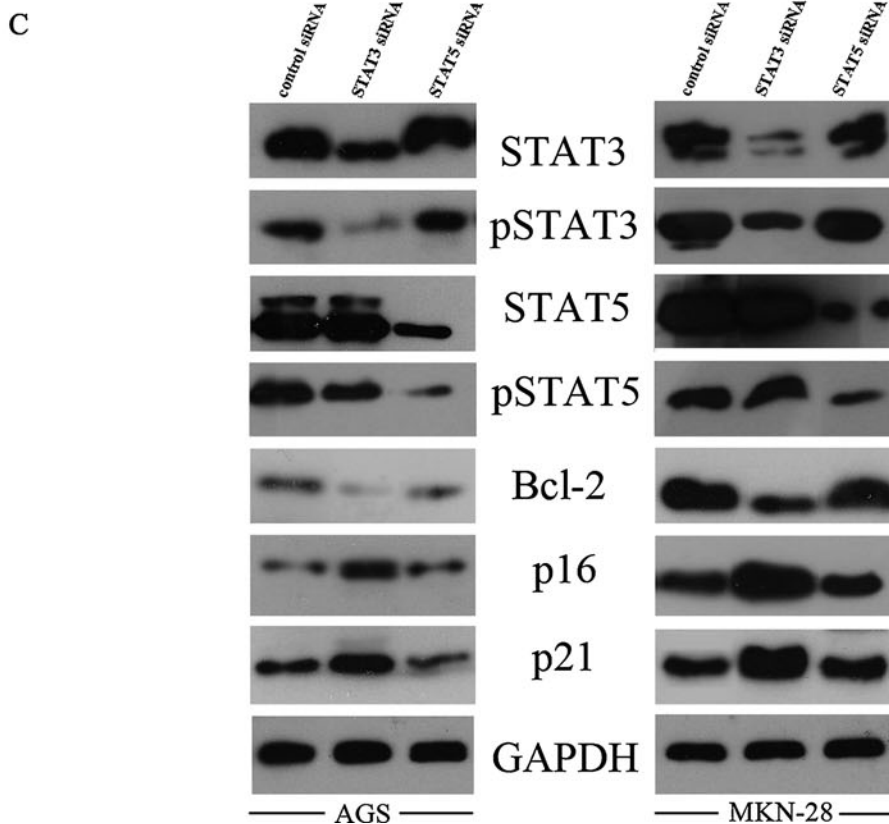
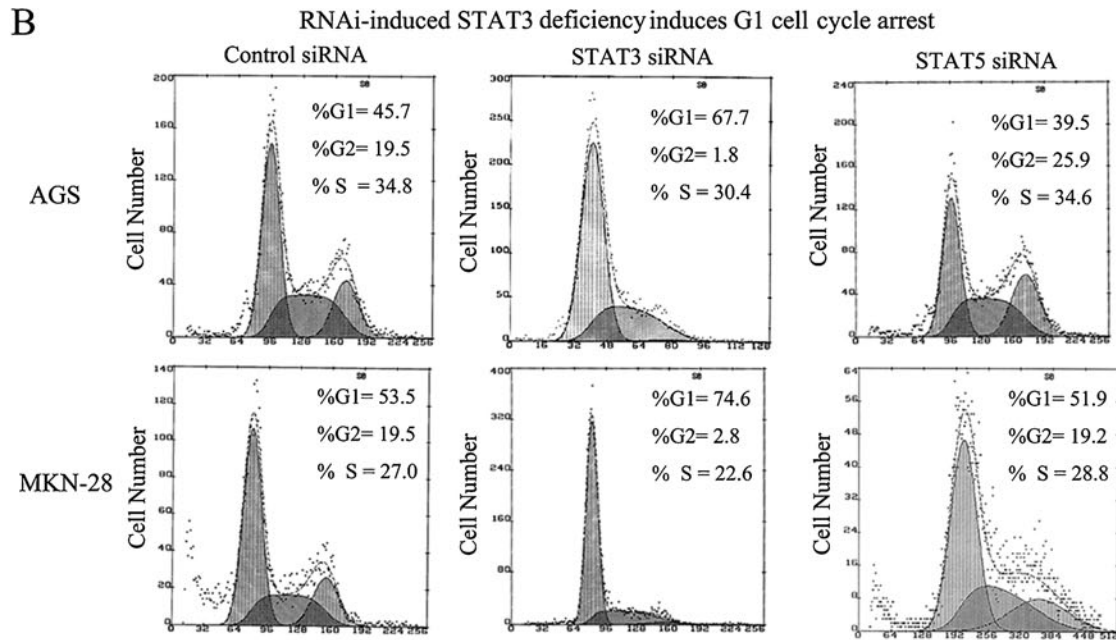
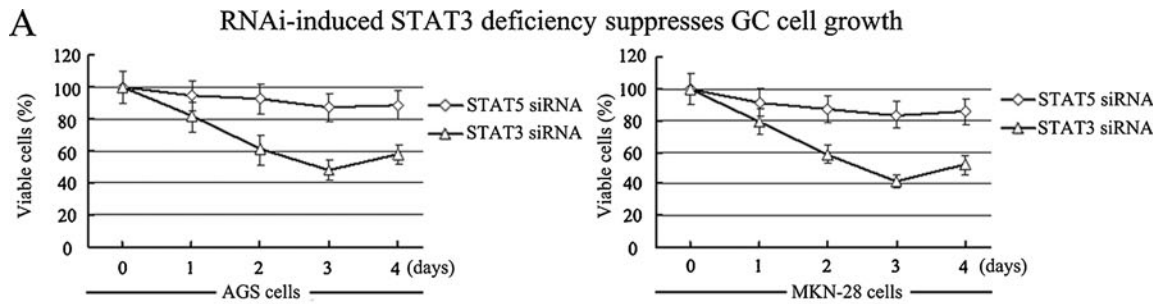


Fig. 1 GO enrichment and pathway analysis of STAT3 and STAT5 target genes. **a**, **b** The significant gene ontology (GO) categories for **a** STAT3 target genes and **b** STAT5 target genes. The vertical axis represents the GO category, and the horizontal axis represents GO enrichment. **c**, **d** The significant pathways for **c** STAT3 target genes

and **d** STAT5 target genes. The vertical axis represents the pathway category, and the horizontal axis represents the pathway enrichment. *P* values of <0.05 and false discovery rates (*FDR*) of <0.05 were used as the thresholds for GO and KEGG pathway selection. *LgP*, logarithm of the *P* value to the base 10



target genes were the Wnt signaling pathway, cell cycle, apoptosis, adherens junction, and focal adhesion (Fig. 1d).

Silencing of STAT3, but not STAT5, inhibits growth and induces G1 cell cycle arrest in gastric cancer cells

Using the CCK-8 assay, we observed that RNAi-induced STAT3 silencing, but not STAT5 silencing, inhibited GC cell growth. In STAT3-silenced cells, the lowest cell viability was detected 3-day post-transfection, suggesting that STAT3 plays a significant role in GC cell growth (Fig. 2a). To further explore the effect on cell viability, we examined the effect of STAT3 and STAT5 silencing on cell cycle progression and apoptosis. As shown in Fig. 2b, transfection of GC cells with STAT3 siRNA blocked cells in the G1 phase of the cell cycle. In the AGS cell line, the G0/G1-phase fraction 72-h post-transfection with 50 nM of STAT3 siRNA was 67.7%, compared to 45.7% in untreated cells. In MKN-28 cells, the G0/G1-phase fraction was 74.6% 72-h post-transfection with 50 nM of STAT3 siRNA, compared to 53.5% in untreated cells. No significant change in the cell cycle was observed in AGS or MKN-28 cells transfected with STAT5 RNAi. Additionally, flow cytometry analysis was performed to measure apoptosis; however, there was no significant increase in apoptosis in either STAT3 or STAT5 siRNA transfected cells compared with the nonspecific siRNA control transfected cells (data not shown). Therefore, these results indicate that STAT3, but not STAT5, inhibits GC cell growth and induces G1 cell cycle arrest.

We measured the expression of various apoptosis and cell cycle-regulatory proteins to investigate the molecular basis of cell growth inhibition, cell cycle arrest and apoptosis in GC. As shown in Fig. 2c, RNAi-induced STAT3 silencing, but not STAT5 silencing, induced Bcl-2 downregulation with simultaneous upregulation of p16^{ink4a}, p21^{waf1/cip1} at 72-h post-transfection in both GC cell lines. These results demonstrate that STAT3 may be involved in the modification of Bcl-2, p16^{ink4a} and p21^{waf1/cip1} expression in GC cells.

Activated STAT3 correlates with TNM stage and differentiation in gastric cancer

A total of 305 patients were recruited for this study; 43 cases were withdrawn due to loss during follow-up ($n=21$), death due to other causes ($n=8$), and poor slide quality ($n=14$). In the remaining population ($n=262$) of 176 males and 86 females, the mean age was 59.3 years (range, 23–79 years).

We determined STAT3, pSTAT3^{Tyr705}, STAT5, and pSTAT5^{Tyr694/Tyr699} expression in the human GC samples using immunohistochemistry. STAT3, STAT5, and pSTAT5^{Tyr694/Tyr699} staining were detected mainly in the cytoplasm, with occasional nuclear staining, while pSTAT3^{Tyr705} expression was mostly detected in the nucleus (Fig. 3). In

STAT3-, STAT5-, and pSTAT5^{Tyr694/Tyr699}-positive and negative patients, there was no significant difference in gender, age, tumor size, TNM stage, or differentiation. However, positive pSTAT3^{Tyr705} was detected in 38/93 (40.9%) of the TNM stage I/II specimens and 98/169 (58.0%) of the TNM stage III/IV specimens. Furthermore, pSTAT3^{Tyr705} staining was observed in 38.8% (31/80) of the well differentiated (I/II) GC samples and 57.7% (105/182) of the poorly differentiated (III/IV) GC samples. Therefore, the results demonstrate that pSTAT3^{Tyr705} levels were associated with TNM stage ($P=0.008$) and differentiation in GC ($P=0.005$), but not age, gender, or tumor size (Table 1).

Expression of activated STAT3 correlates with poorer prognosis in gastric cancer

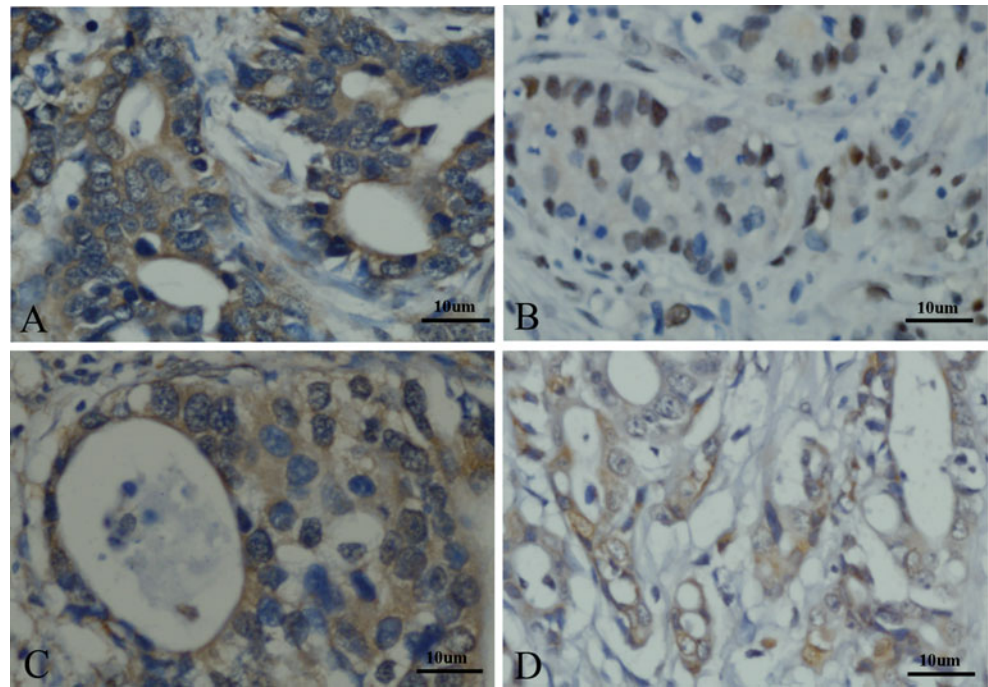
To explore whether STAT3, STAT5 and their activated proteins could determine the clinical outcome of GC, we investigated the correlation of protein expression and activation with patient prognostic data. Patients who expressed pSTAT3^{Tyr705} had a significantly poorer prognosis than pSTAT3^{Tyr705}-negative patients. The 3- and 5-year OS rates for pSTAT3^{Tyr705}-positive patients were 25.7% and 11.8%, respectively, versus 65.9% and 45.9%, in pSTAT3^{Tyr705}-negative patients ($P<0.001$, log-rank test; Fig. 4b). Similarly, the median OS time of pSTAT3^{Tyr705}-positive patients (21.3 months) was significantly shorter than pSTAT3^{Tyr705}-negative patients (57.0 months). STAT3, STAT5, and pSTAT5^{Tyr694/Tyr699} were not associated with differences in survival ($P=0.167$, 0.253, and 0.115, respectively, log-rank test; Fig. 4).

Cox's proportional hazards regression analysis was performed to determine independent prognostic factors in GC. In univariate analysis (Table 2), a larger tumor size, higher TNM stage, poorly differentiated tumors, and positive pSTAT3^{Tyr705} expression were confirmed as independent risk factors for OS. Age, gender, STAT3, STAT5, and pSTAT5^{Tyr694/Tyr699} expression had no prognostic significance for OS. Multivariate analysis confirmed that positive pSTAT3^{Tyr705} expression was independently associated with OS (hazards ratio, 2.101; 95% confidence interval, 1.529–2.891; $P<0.001$; Table 2). Tumor size, TNM stage, and tumor differentiation were also independent survival predictors ($P=0.021$, 0.000, and 0.037, respectively).

Discussion

Despite improved knowledge of the molecular mechanisms during GC tumorigenesis, the signal transduction pathways involved have not been fully characterized. Recently, frequent constitutive activation of STATs proteins has been reported in several malignant neoplasms, and STAT3 and STAT5 are reported to have carcinogenic effects.

Fig. 3 Immunohistochemical staining of human gastric cancer tissue. **a** STAT3 expression is detected in the cytoplasm. **b** pSTAT3^{Tyr705} expression is mainly observed in the nucleus. **c** STAT5 and **d** pSTAT5^{Tyr694/Tyr699} expression are mainly detected in the cytoplasm, ×200 magnification



STATs are a family of transcription factors which are involved in the regulation of numerous tumor associated genes and act as important cellular mediators in response to various cytokines and growth factors [16, 17]. During recent years, evidence has suggested that constitutive activation of STAT3 and STAT5 are involved in tumor formation and progression; however, the entire range of STAT3 and STAT5 downstream targets have not been identified. In this study, we

used ChIP-on-chip to identify the genes which STAT3 and STAT5 bind to in genomic DNA from AGS cells. A total of 2,514 and 1,314 genes were identified as STAT3 and STAT5 target genes, respectively. Moreover, GO and pathway analysis revealed that STAT3 and STAT5 target genes have a variety of functions in diverse biological processes and signaling pathways. The target gene functions were mainly related to GC cell growth (including proliferation, death, apoptosis, and

Table 1 Correlation between STAT3, pSTAT3^{Tyr705}, STAT5, pSTAT5^{Tyr694/Tyr699}, and clinicopathological characteristics in gastric cancer patients

	STAT3		P value	p-STAT3 ^{Tyr705}		P value	STAT5		P value	pSTAT5 ^{Tyr694/Tyr699}		P value
	Positive	Negative		Positive	Negative		Positive	Negative		Positive	Negative	
Gender												
Male	168	8	0.411	89	87	0.535	94	82	0.850	81	95	0.941
Female	80	6		47	39		47	39		40	46	
Age												
≤50	73	4	1.000	39	38	0.792	39	38	0.507	30	47	0.130
>50	175	10		97	88		102	83		91	94	
Tumor diameter												
≥5 cm	124	5	0.298	71	58	0.318	73	56	0.375	58	71	0.696
<5 cm	124	9		65	68		68	65		63	70	
Differentiation												
I/II	75	5	0.665	31	49	0.005*	47	33	0.288	40	40	0.411
III/IV	173	9		105	77		94	88		81	101	
TNM stage												
I/II	85	8	0.082	38	55	0.008*	55	38	0.200	45	48	0.596
III/IV	163	6		98	71		86	83		76	93	

pSTAT3^{Tyr705} levels are associated with TNM stage ($P=0.008$) and tumor differentiation ($P=0.005$), suggesting that constitutive activation of STAT3 may be associated with poorer prognosis in gastric cancer

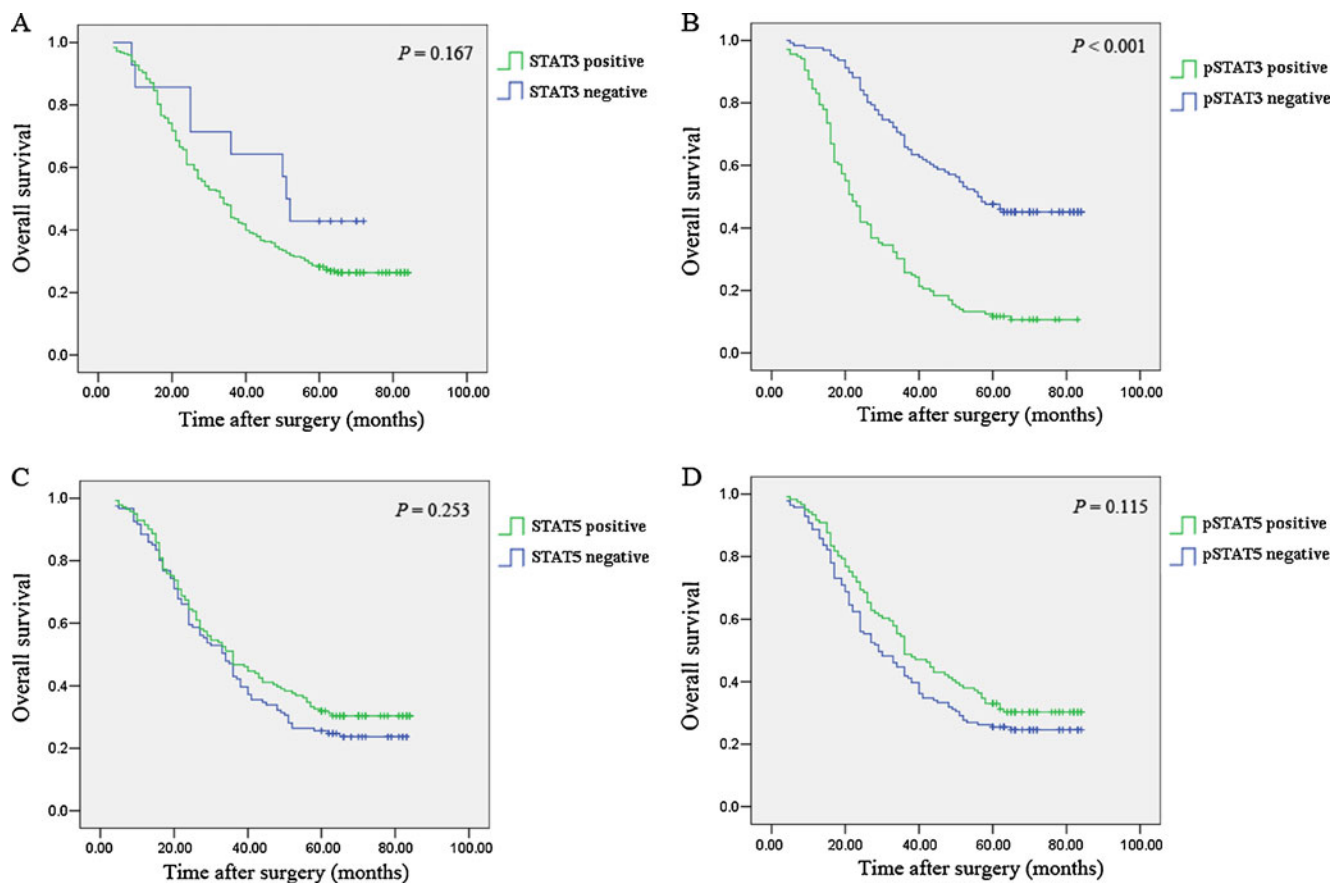


Fig. 4 Kaplan–Meier survival curves and log-rank test for gastric cancer patients stratified by pSTAT3^{Tyr705} expression. Gastric cancer patients with positive pSTAT3^{Tyr705} expression (b) have a poorer overall survival ($P < 0.001$)

the cell cycle), metabolism, differentiation, adhesion, immune response, and stress responses, suggesting that STAT3 and STAT5 may play roles in many areas of GC progression. The significant pathways involving STATs target genes include the Wnt signaling pathway, MAPK signaling pathway and mTOR signaling pathway, suggesting cross-talk may exist between

these pathways and STAT signaling in GC tumorigenesis and progression. These findings strongly suggest that STAT signaling plays a significant role in GC malignant progression.

We also evaluated the biological significance of STAT3 and STAT5 in the pathogenesis of GC cells. Down-regulation of STAT3, but not STAT5, was associated with

Table 2 Cox’s proportional hazards regression of independent prognostic factors in gastric cancer

Variables	Overall survival		
	Univariate (<i>P</i> value)	Multivariate analysis	
		Hazard ratio (95% CI)	<i>P</i> value
Age (years; ≤50 versus >50)	0.222		
Gender (male versus female)	0.115		
Tumor diameter (cm; ≥5 versus <5)	<0.001	1.439 (1.057–1.958)	0.021
Differentiation (I/II versus III/IV)	<0.001	1.436 (1.021–2.020)	0.037
TNM stage (I/II versus III/IV)	<0.001	3.351 (2.236–5.022)	<0.001
STAT3 (positive versus negative)	0.177		
pSTAT3 (positive versus negative)	<0.001	2.101 (1.529–2.891)	<0.001
STAT5 (positive versus negative)	0.260		
pSTAT5 (positive versus negative)	0.121		

pSTAT3^{Tyr705} is a significant prognostic factor, results were considered significant if the *P* value was less than 0.05

a reduction in the number of viable cells in two GC cell lines. Additionally, we observed mechanistic evidence that the decreased cell viability in GC cells after silencing of STAT3 was attributed to G1 cell cycle arrest, consistent with up-regulation of p16^{ink4a} and p21^{waf1/cip1}, suggesting STAT3 is involved in cell cycle regulation. However, despite decreased expression of Bcl-2, STAT3 silencing did not induce a significant degree of apoptosis in GC cells, indicating that STAT3 may play a minor role in GC cell survival. STAT5 had no significant effect on cell viability or cell cycle regulation in GC cells.

To determine whether STAT signaling was associated with the clinical outcome of GC, we examined STAT3 and STAT5 expressions and activation in 262 human GC samples. Our data did not confirm the findings of Kim et al. [18], who reported that total STAT3 expression correlated with TNM stage and survival in GC patients; however, the authors did not measure expression of activated STAT3. In our study, there was no significant correlation between total STAT3 expression and clinical characteristics such as TNM stage and differentiation; however, we observed a positive correlation between expression of pSTAT3^{Tyr705} and TNM stage and differentiation, suggesting that activation of STAT3 may contribute to a poor prognosis in human GC. Additionally, patients with positive pSTAT3^{Tyr705} expression had a significantly poorer prognosis than pSTAT3^{Tyr705}-negative patients, as the 3- and 5-year OS rates for pSTAT3^{Tyr705}-positive patients (25.7% and 11.8%) were poorer than pSTAT3^{Tyr705}-negative patients (65.9% and 45.9%). Additionally, univariate and multivariate analyses revealed that pSTAT3^{Tyr705} expression is a powerful independent prognostic factor of survival in GC patients. Taken together with the results of our in vitro cell experiments, these findings suggest that abnormal activation of STAT3 plays a significant role in GC oncogenesis, and that STAT3 could provide a potential therapeutic target for the treatment of GC.

Under normal circumstances STAT5 is involved in the growth of mammalian glands and milk secretion [19]. Recently, high levels of STAT5 expression have been observed in hematopoietic malignancies [20, 21] and certain solid cancers, such as prostate cancer [7]; however, the contribution of STAT5 to GC progression was unknown. In this study, neither total nor activated STAT5 expressions demonstrated a significant correlation with reliable GC prognostic factors, such as TNM stage or differentiation. Moreover, expression of STAT5 or pSTAT5^{Tyr694/Tyr699} did not contribute to a poor prognosis in GC. These data confirm the results of the in vitro GC cell line experiments which showed that STAT5 has no significant effect on viability or cell cycle regulation in GC cells.

In conclusion, this study suggests that expression of pSTAT3^{Tyr705} is a significant prognostic factor in GC, and

STAT3 may provide a potential target for molecular therapy in GC. In addition, our study had yielded a list of STAT3 and STAT5 target genes, which may provide insight into the mechanisms of GC tumorigenesis and progression. Additionally, since the function of STATs' upstream genes, such as JAKs, has not been fully elucidated, further studies regarding their biological significances in GC should be performed.

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Conflict of interest The authors have declared that no conflict of interest exists.

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