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The antagonistic effect between STAT1 and Survivin and its clinical significance in gastric cancer

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Abstract. In previous studies, we observed that STAT1 and Survivin correlated negatively with gastric cancer tissues, and that the functions of the IFN-γ-STAT1 pathway and Survivin in gastric cancer are the same as those reported for other types of cancer. In this study, the SGC7901 gastric cancer cell line and 83 gastric cancer specimens were used to confirm the relationship between STAT1 and Survivin, as well as the clinical significance of this relationship in gastric cancer. IFN-γ and STAT1 and Survivin antisense oligonucleotides (ASONs) were used to knock down the expression in SGC7901 cells. The protein expression of STAT1 and Survivin was tested by immunocytochemical and image analysis methods. A gastric cancer tissue microarray was prepared and tested by immunohistochemical methods. Data were analyzed by the Spearman's rank correlation analysis, the χ^2 test and Cox's multivariate regression analysis. Upon knockdown of IFN-γ, STAT1 and Survivin expression by ASON in the SGC7901 cell line, an antagonistic effect was observed between STAT1 and Survivin. In gastric cancer tissues, STAT1 showed a negative correlation with depth of invasion ($p<0.05$) in gastric cancer tissues exhibiting a negative Survivin protein expression. Furthermore, in tissues exhibiting a negative STAT1 protein expression, Survivin correlated negatively with N stage ($p<0.05$). Pathological and molecular markers were used to conduct Cox's multivariate regression analysis, and depth of invasion and N stage were found to be prognostic factors ($p<0.05$). On the other hand, in tissues exhibiting a negative Survivin protein expression, Cox's multivariate regression analysis revealed that the differentiation type and STAT1 protein expression were prognostic factors ($p<0.05$). There is an antagonistic effect between STAT1 and Survivin in gastric cancer, and this antagonistic effect is of clinical significance in gastric cancer.

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Introduction

Gastric cancer accounts for a large proportion of malignancies and gastric cancer-related deaths account for the largest proportion of deaths from cancer (1). Disordered apoptosis has been linked to cancer development, and repression of apoptosis has been observed in gastric cancer (2). STATs and Survivin, as significant apoptosis-regulated molecules, play a pivotal role in oncogenesis (3-8).

The JAK/STAT-pathway was originally observed in studies of interferon-unresponsive cells (9). This pathway is known to be involved in two types of proteins. One of these types is the receptor pre-associated tyrosine kinases, termed Janus kinases (JAKs); the other is latent cytosolic transcription factors, termed signal transducers and activators of transcription (STATs). The dimerisation of cell surface receptors induces the mutual phosphorylation of receptor-preassociated JAK proteins, after which JAK proteins recruit and phosphorylate STAT proteins in the cytoplasm. The phosphorylated STATs form dimers, migrate into the nucleus, binding to specific DNA response elements in gene promoters, and regulate gene transcription (10). The STAT protein family comprises at least seven members, i.e. STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (11). STAT1 was the first member of this family to be identified. As a tumor surveillance gene, it plays a significant role in IFN-γ-induced biological responses (4-8), the immune response (12,13) and cell growth control (14,15). It has been suggested that STAT1 serves as a tumor suppressor by promoting the expression of $p21^{waf}$, caspase 3 and caspase 7 to activate pro-apoptotic pathways (16).

The inhibitor of the apoptosis (IAP) family of proteins was originally named due to its physical ability to inhibit caspases (17). IAPs present an approximately 70 amino acid baculovirus IAP repeat (BIR) (18). Survivin is a protein of 142 amino acids and is the smallest mammalian member of the IAP family (18). It is an established cancer gene, as it was found to be overexpressed in almost all human tumors, whereas it is largely undetectable or minimally expressed in normal mature tissues (19). Due to its differential distribution of other IAPs, which are typically found in normal tissues and occasionally up-regulated in cancer (19), Survivin was regarded as one of the most prominent cancer genes (20). Survivin blocks apoptosis induced by various stimuli, including chemotherapeutic drugs (3,21), FAS/CD95 (22) and irradiation (23). Its ability

to inhibit apoptosis is believed to lie in its binding directly to $p21^{waf}$, caspase 3 and caspase 7 and preventing their activation (22,24).

Little research has been conducted into the functions of STAT1 and Survivin and their clinical characteristics in gastric cancer. In previous studies, we observed that the IFN-γ-STAT1 pathway, which adjusts $p21^{waf}$ and caspase 7 expression, was present in the SGC7901 gastric cancer cell line and human tissues and that STAT1 initiates advanced gastric cancer (25,26). Meanwhile, we observed that Survivin inhibits $p21^{waf}$ and caspase 7 expression, whereas IFN-γ inhibits Survivin expression in SGC7901 cells and initiates lymph node metastasis of gastric cancer (26,27). In addition, we noted that STAT1 protein expression was negatively correlated with Survivin protein expression in human gastric cancer tissues (26).

In the present study, we treated the SGC7901 cell line with IFN-γ, STAT1 antisense oligonucleotides (ASONs) and Survivin ASONs prior to performing immunocytochemistry and image analysis to detect the expression regulation of STAT1 and Survivin protein, and analyzed the antagonistic effect between STAT1 and Survivin. We then performed immunohistochemistry to analyze the expression of STAT1 and Survivin protein in 83 resected human gastric cancer tissue samples, evaluated the clinicopathological and prognostic significance of STAT1 and Survivin expression in gastric cancer tissues, and analyzed the clinical characteristics of the antagonistic effect between STAT1 and Survivin in gastric cancer.

Materials and methods

Cell culture, IFN-γ treatment and ASON treatment. The SGC7901 human gastric adenocarcinoma cell line, obtained from the Chinese Academy of Medical Sciences Cell Center of Basic Medicine (Beijing, China), was maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS) at 37˚C in a 5% CO_2 atmosphere.

For IFN-γ treatment, cells were plated as slides in a 6-well plate for 24 h, then placed in RPMI-1640 medium containing 10% FCS and 1,000 U/ml concentration IFN-γ (28) (02CY27; Peprotech EC) for 24 h.

The phosphorothioate oligonucleotides used as antisense for STAT1 and Survivin were 5'-CCACTGAGACATCCTGC CACC-3' (29) and 5'-CCCAGCCTTCCAGCTCCTTG-3' (30), respectively. SGC7901 cells cultured on 6-well plates to reach a confluence of 70-80% were incubated with STAT1 and Survivin ASONs using Transfectin (TianGENE) at a charge ratio of 3:1 (Transfectin/ASON) in serum-free medium. At the end of a 6-h incubation period, RPMI-1640 containing 10% FCS was added. A combination of IFN-γ (1,000 U/ml) and STAT1 ASON (600 and 800 nM) was administered to SGC7901 for 24 h. Survivin ASON (200 and 400 nM) was administered to SGC7901 for 24 h alone.

Pathological examination. The 83 human gastric cancer tissue samples, which were histological and clinically verified between 1998 and 2003, were collected at the Jiangda Pathology Institution, China. For the use of these clinical materials for research purposes, prior patient consent and approval from the Institute Research Ethics Committee were obtained. Of the 83 patients included in this study, 60 patients were male and 23 were female, age range 26-82 years (mean 58). Routine pathological examination was performed to establish the depth of invasion and histological classification of gastric cancer. All lymph nodes were found using the clearing fat method (>15/case) (31). Depth of invasion and lymph node metastasis were staged according to the standards of the WHO, sixth edition. Histological classification was divided into two types according to the standards of the WHO, sixth edition. The well-differentiated type included well-differentiated adenocarcinoma. The poorly differentiated type included poorly differentiated adenocarcinoma, mucinous adenocarcinoma and signet-ring cell carcinoma. Tumor size was calculated using the largest diameter of tumor. Heterogeneity defines a tumor that has more than two histological types. Clinicopathological characteristics were recorded for all cancer patients (Table I). Pathological status was classified according to the sixth edition of the TNM classification of the WHO (2003). None of the patients received chemotherapy or radiation therapy prior to surgery. No patients were lost prior to follow-up. Median time of follow-up was 28.8 months (range 1-159).

Immunohistochemistry, immunocytochemistry and image analysis. The primary monoclonal antibody for STAT1 P84/P91 (C-136; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was purchased from Beijing ZhongShan Ltd. (China). The primary polyclonal antibody for Survivin (RAB-0536; NeoMarkers) and SP kit was purchased from Fujian Maxin Ltd. (China). The immunohistochemical and immunocytochemical staining was performed according to the manufacturer's instructions. Diaminobenzedin (DAB) was used for color development. The positive results of STAT1 were present in the cytoplasm or/and nuclei of tumor cells exhibiting brown coloration. The positive result of Survivin were present in cytoplasm of tumor cells exhibiting brown coloration. In the immunocytochemical staining process, all stainings were carried out under identical conditions and simultaneously, using image analysis software (Motic) to collect immunocytochemical staining images and detect their average optical density (OD) values.

Statistical analysis. The statistical software package SPSS 12.0 was used. The average OD values of image were analyzed by the Student's t-test analysis. The correlations of STAT1 and Survivin expression and clinicopathological factors were analyzed by the Spearman's rank correlation analysis and the χ^2 test. Survival curves were plotted according to the Kaplan-Meier method, and the generalized log-rank test was applied to compare the survival curve. Multivariate survival analysis was performed on all of the parameters that were found to be significant on the univariate analysis using Cox's regression model. The statistical significance of differences was determined by one-way analysis of variance. $P<0.05$ was considered to indicate statistical significance.

Results

IFN-γ inhibits Survivin protein expression by promoting STAT1 protein expression in SGC7901 cells. STAT1 and Survivin expression was examined in cells treated with IFN-γ

Table I. Correlation between STAT1 and Survivin expression and clinicopathological factors of gastric cancer.

Variables	STAT1 protein expression		P-value	Survivin protein expression		P-value
	Negative (n=51)	Positive (n=32)		Negative (n=40)	Positive (n=43)	
Gender						
Male	40	20	0.12	28	32	0.660
Female	11	12		12	11	
Age						
≤60	29	18	0.96	22	25	0.780
>60	22	14		18	18	
Size (diameter)						
<5 cm	27	17	0.99	20	24	0.600
≥5 cm	24	15		20	19	
Depth of invasion						
T1	2	2	0.01[a]	3	1	0.530
T2	3	6		3	6	
T3	17	14		17	14	
T4	29	10		17	22	
Histological type						
Well-differentiated	23	8	0.07	11	20	0.080
Poorly differentiated	28	24		29	23	
Heterogeneity						
Yes	34	17	0.22	25	26	0.850
No	17	15		15	17	
Lymph node metastasis						
N0	7	6	0.49	3	10	0.002[a]
N1	16	3		7	12	
N2	12	12		11	13	
N3	16	11		19	8	

[a]P<0.05.

Table II. IFN-γ and ASONs induced STAT1 protein expression changes in SGC7901 cells.

	IFN-γ	IFN-γ + STAT1 ASON 600 nM	IFN-γ + STAT1 ASON 800 nM	Survivin ASON 200 nM	Survivin ASON 400 nM
	P-value	P-value	P-value	P-value	P-value
Control	0.04[a]	0.18	0.200	0.030[a]	0.0100[a]
IFN-γ	-	0.28	0.009[a]	0.500	0.6000
IFN-γ + STAT1 ASON 600 nM	-	-	0.040[a]	0.400	0.3000
IFN-γ + STAT1 ASON 800 nM	-	-	-	0.001[a]	<0.0001[a]
Survivin ASON 200 nM	-	-	-	-	0.5000

[a]P<0.05.

(1,000 U/ml) for 24 h. We observed that STAT1 protein expression was increased and Survivin protein expression was decreased (Figs. 1-3, Tables II and III). Cells that had been treated with IFN-γ (1,000 U/ml) and STAT1 ASON (600 and 800 nM) for 24 h were selected to confirm whether IFN-γ inhibited Survivin protein expression by promoting STAT1 protein expression. We noted that when SGC7901 cells were treated with IFN-γ (1,000 U/ml) and the concentration of STAT1 ASON ranged from 600 to 800 nM, STAT1 protein expression was gradually decreased. Simultaneously, Survivin protein expression was gradually increased (Figs. 1-3, Tables II and III).

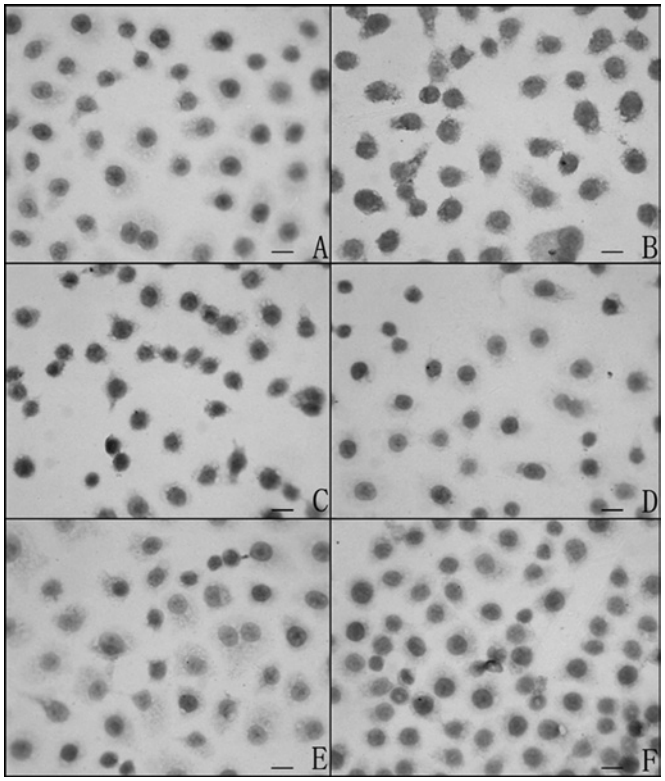

Figure 1. IFN-γ and ASON-induced STAT1 protein expression variations in SGC7901 cells. (A) Control group; (B) IFN-γ (1,000 IU/ml) group; (C) IFN-γ (1,000 IU/ml) + STAT1 ASON (600 nM) group; (D) IFN-γ (1,000 IU/ml) + STAT1 ASON (800 nM) group; (E) Survivin ASON (200 nM) group; (F) Survivin ASON (400 nM) group. Scale bar, 5 μm; magnification, x400.

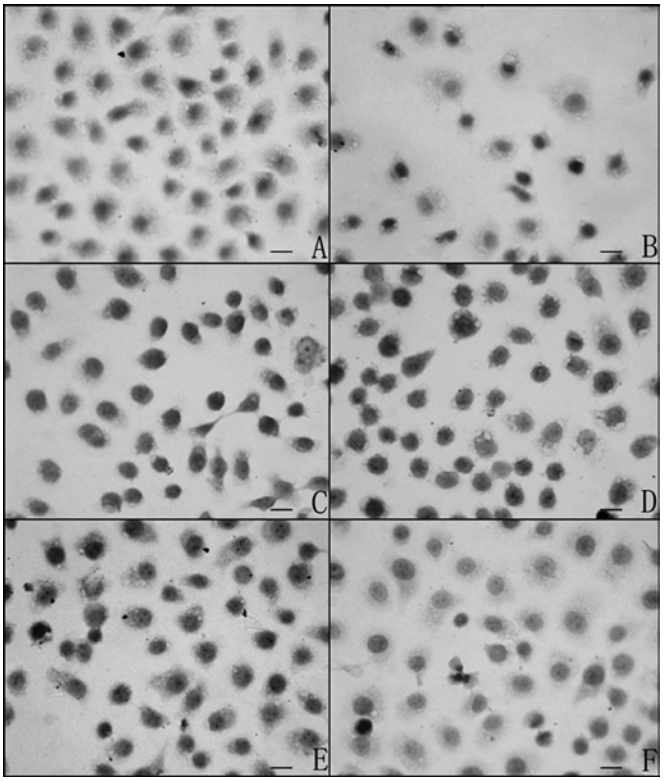

Figure 2. IFN-γ and ASON-induced Survivin protein expression variations in SGC7901 cells. (A) Control group; (B) IFN-γ (1,000 IU/ml) group; (C) IFN-γ (1,000 IU/ml) + STAT1 ASON (600 nM) group; (D) IFN-γ (1,000 IU/ml) + STAT1 ASON (800 nM) group; (E) Survivin ASON (200 nM) group; (F) Survivin ASON (400 nM) group. Scale bar, 5 μm; magnification, x400.

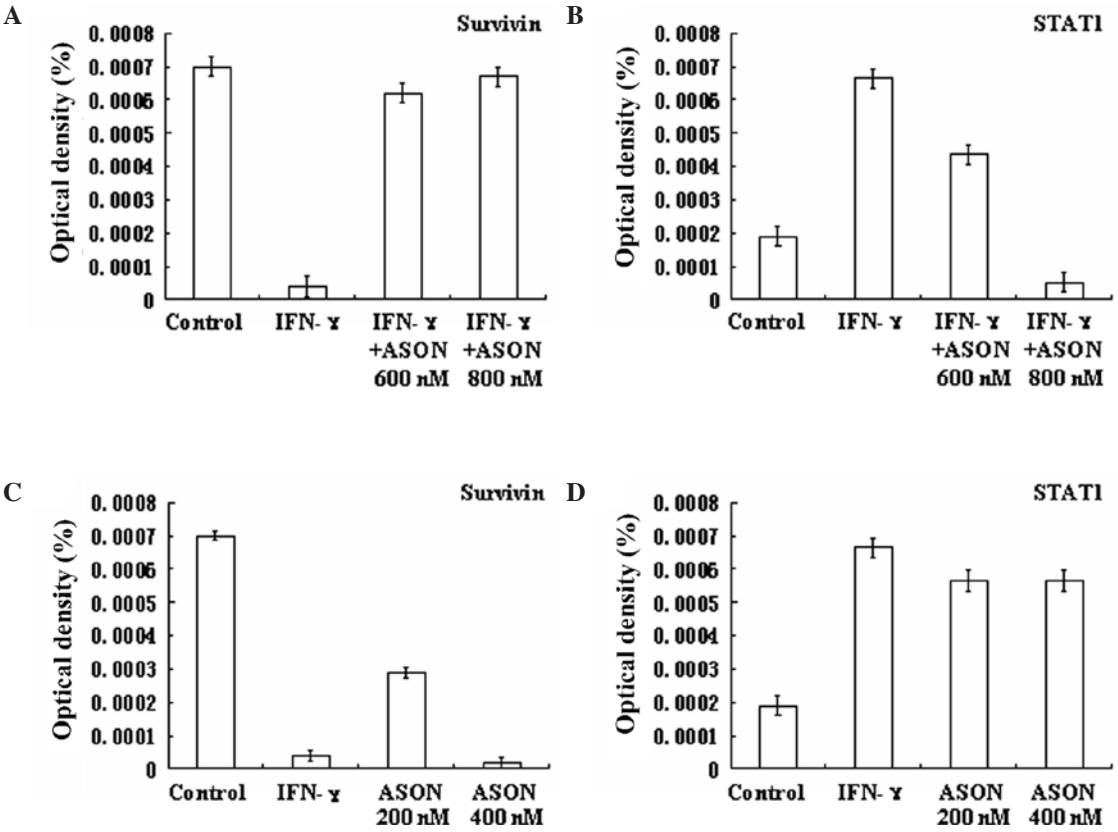

Figure 3. IFN-γ and ASON-induced STAT1 and Survivin protein expression variations in SGC7901 cells. (A) IFN-γ and STAT1 ASON-induced Survivin protein expression variations. (B) IFN-γ and STAT1 ASON-induced STAT1 protein expression variations. (C) IFN-γ and Survivin ASON-induced Survivin protein expression variations. (D) IFN-γ and Survivin ASON-induced STAT1 protein expression variations. Magnification, x400.

Table III. IFN-γ and ASONs induced Survivin protein expression changes in SGC7901 cells.

	IFN-γ	IFN-γ + STAT1 ASON 600 nM	IFN-γ + STAT1 ASON 800 nM	Survivin ASON 200 nM	Survivin ASON 400 nM
	P-value	P-value	P-value	P-value	P-value
Control	<0.0001[a]	0.0050[a]	0.1200	0.0005[a]	<0.0001[a]
IFN-γ	-	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.2100
IFN-γ + STAT1 ASON 600 nM	-	-	0.0400[a]	<0.0001[a]	<0.0001[a]
IFN-γ + STAT1 ASON 800 nM	-	-	-	<0.0001[a]	<0.0001[a]
Survivin ASON 200 nM	-	-	-	-	0.0005[a]

[a]P<0.05.

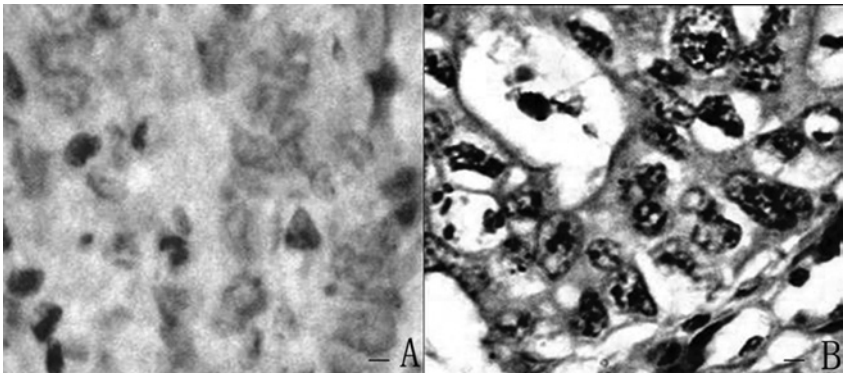

Figure 4. Immunohistochemical staining results of gastric cancer tissues. Immunohistochemical result of (A) STAT1 and (B) Survivin. Scale bar, 5 μm; magnification, x400.

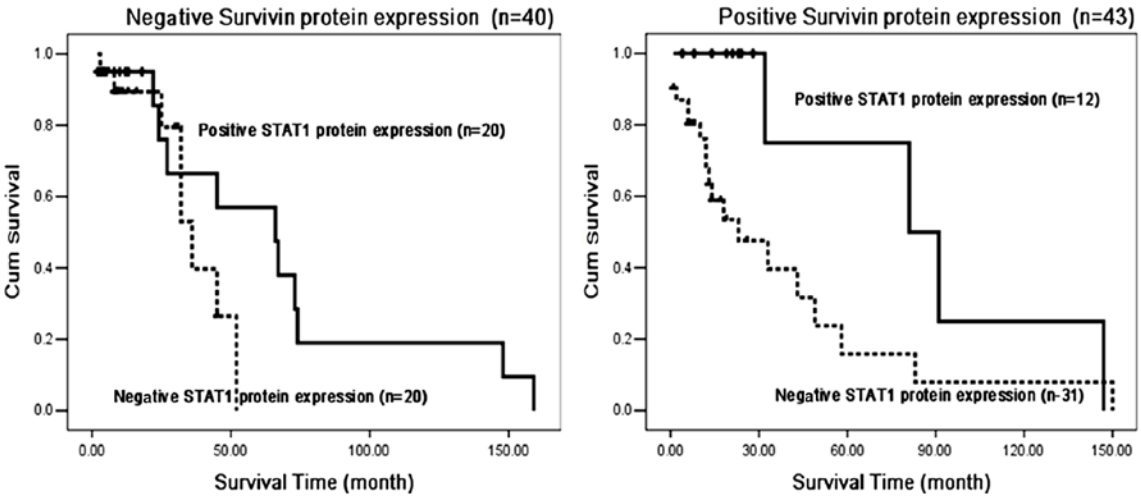

Figure 5. Kaplan-Meier statistical analyses showing a correlation of STAT1 protein levels with overall survival rates among the positive and negative Survivin protein expression groups. Patients with positive STAT1 protein expression showed significantly more favorable survival rates than those with negative STAT1 protein expression in the positive Survivin protein expression group (P=0.033).

Survivin inhibits STAT1 protein expression in SGC7901 cells. Survivin protein expression was at a high level in the SGC7901 cell line (35). Cells that had been treated with Survivin ASON (200 and 400 nM) for 24 h were selected to confirm whether or not Survivin protein expression inhibits STAT1 protein expression. We noted that when SGC7901 cells were treated with a concentration of Survivin ASON ranging from 200 to 400 nM, Survivin protein expression was gradually/decreased. Simultaneously, STAT1 protein expression was gradually increased (Figs. 1-3, Tables II and III).

STAT1 exhibited a negative correlation with depth of invasion in Survivin protein-negative gastric cancer tissues. Additionally, exhibited a negative correlation with N stage in STAT1 protein-negative tissues.

In 83 human gastric cancer tissues, the positive rate of STAT1 protein expression was 38.6% (32/83) (Fig. 4A) and that of Survivin protein expression was 51.8% (43/83) (Fig. 4B). STAT1 expression exhibited a negative correlation with depth of invasion (P=0.01, r=-0.27). Survivin expression exhibited a negative correlation with N stage (P=0.002, r=-0.34). A significant negative correlation was observed between STAT1 expression and Survivin expression (P=0.04, r=-0.23).

To confirm whether or not the antagonistic effect between STAT1 and Survivin was capable of affecting their correlations with clinicopathological characteristics, a positive and negative expression of STAT1 and Survivin proteins was used to divide 83 human gastric cancer tissues into four groups. We found that STAT1 exhibited a negative correlation with depth of invasion in Survivin protein-negative tissues (P=0.04, r=-0.30) and no correlation in Survivin protein-positive tissues (P=0.16, r=-0.22). Survivin exhibited a negative correlation with N stage in STAT1 protein-negative tissues (P=0.009, r=-0.36), and no correlation with N stage in STAT1 protein-positive tissues (P=0.18, r=-0.24).

STAT1 protein expression is an independent prognostic factor in Survivin protein-negative gastric cancer tissues. Univariate and multivariate analyses indicated that STAT1 protein expression (P=0.008, χ^2=6.98), depth of invasion (P=0.014, χ^2=6.00) and N stage (P=0.026, χ^2=4.99) were independent prognostic factors of survival for gastric cancer patients.

We also observed that STAT1 protein expression was an independent prognostic factor in the negative Survivin protein expression group (P=0.033, χ^2=4.55), and had no correlation with survival in the positive Survivin protein expression group (P=0.17, χ^2=1.92) (Fig. 5).

Discussion

Survivin is expressed in almost all human malignancies as well as embryonic and fetal tissues, but is almost undetectable in adult tissues (32). Overexpression of Survivin in cancer invariably provides a survival advantage in tumor cells. Therefore, lack of Survivin or disruption of the Survivin function causes cell death, such as apoptosis and mitotic catastrophe (33). Limited studies have focused on the mechanisms by which Survivin protein expression is regulated in gastric cancer. Findings of a recent study showed that IFN-γ was capable of down-regulating Survivin protein expression in gastric cancer cells (27). STAT1 is an important molecule in the IFN-γ-JAK/STAT-pathway. STAT1 and Survivin regulate apoptosis, but their biological effects are adverse. As a transcription factor, STAT1 up-regulates the expression of caspases 3 and 7 simultaneously with the enzymatic substrate of caspases 3 and 7. Survivin is capable of binding with caspases 3 and 7 to inhibit their activity. The relationship of STAT1 and Survivin in gastric cancer, and whether or not they adjust the expression of one another, has yet to be elucidated. We confirmed that IFN-γ down-regulates Survivin protein expression and simultaneously up-regulates STAT1 protein expression in gastric cancer cells. When IFN-γ and STAT1 ASON were administered to the cell line together, we observed that STAT1 protein expression was gradually increased and that Survivin protein expression was gradually decreased in a concentration-dependent manner. These results indicate that IFN-γ inhibits Survivin protein expression via the IFN-γ-STAT1 signal pathway in gastric cancer.

Recently, another STATs family member, STAT3 protein, has been shown to correlate with Survivin and to have a clear bearing on gastric cancer progression, although the detailed mechanism for this relationship has yet to be clarified (34). The SGC7901 cell line that we used in this study was derived from poorly differentiated and metastatic gastric adenocarcinoma from the Chinese population, and exhibited a high Survivin expression in the protein level (35). Our results showed that when the SGC7901 cell line was treated with Survivin ASON, Survivin protein expression was gradually increased and STAT1 protein expression was gradually decreased in a manner dependent on the concentration of Survivin ASON. These results indicate that Survivin may also inhibit STAT1 protein expression in gastric cancer cells, and that there is an antagonistic effect between STAT1 and Survivin in gastric cancer cells.

STAT1 and Survivin are important apoptosis regulators and have important clinical significance in gastric cancer. STAT1 is a molecular marker involved in the prediction of advanced gastric cancer and Survivin is a molecular marker of lymph node metastasis in gastric cancer (26). In this study, STAT1 was found to be negatively correlated with depth of invasion in Survivin protein-negative tissues, and Survivin exhibited a negative correlation with N stage in STAT1 protein-negative tissues. In addition, STAT1 was an independent survival factor only in Survivin protein-positive tissues. These results confirmed that there is antagonistic effect between STAT1 and Survivin in gastric cancer tissues, and that this antagonistic effect had clinical significances in gastric cancer.

In conclusion, our study indicates that there is an antagonistic effect between STAT1 and Survivin in gastric cancer, and that this effect is of clinical significance. Thus, STAT1 and Survivin may be potential molecular targets for cancer therapy, which may allow for more individualized treatments of gastric cancer patients.

Acknowledgements

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