Decreased ST2 expression is associated with gastric cancer progression and pathogenesis

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Abstract. Gastric cancer is a type of cancer with increasing incidence and high mortality rates, but molecular biomarkers of diagnostic and therapeutic value are currently lacking. The aim of the present study was to examine the expression pattern of the interleukin 1 receptor-like 1 (ST2) protein and assess its clinicopathological significance in gastric cancer. Western blot analysis of 12 gastric cancer specimens and paired adjacent tissues demonstrated that the protein levels of 2 isoforms of ST2, soluble secreted ST2 and the ST2 variant without the third immunoglobulin motif and splicing in the C-terminal, were markedly decreased in cancer tissues compared with non-cancerous tissues. Immunohistochemical analysis demonstrated that ST2 protein expression was markedly decreased in primary gastric cancer tissues (39.1%, 90/230) compared with adjacent non-cancerous tissues (60.7%, 54/89) ($P<0.05$). Statistical analysis demonstrated that decreased ST2 expression was significantly associated with advanced tumor node metastasis stage ($P<0.001$) and tumor differentiation ($P<0.001$). These data suggest that ST2 protein may be a valuable biomarker of gastric cancer progression and pathogenesis.

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Introduction

Gastric cancer has a poor prognosis that is largely attributable to early and frequent metastasis (1-3). Surgery and combined radio-chemotherapeutic regimens are associated with modest survival benefits in advanced gastric cancer, with an overall 5-year survival rate of <24% (3). At present, the mechanisms underlying the initiation and progression of gastric cancer remain unclear and molecular markers for gastric cancer remain to be identified. It is imperative to identify the biological and molecular changes that frequently occur during gastric carcinogenesis, in order to elucidate cancer pathology and identify new diagnostic markers, in order to individualize treatment for patients with gastric cancer.

Interleukin 1 receptor-like 1 (ST2/IL1RL1), also known as T1, DER4 or FIT-1, which was originally identified as a primary responsive gene, is a member of the interleukin (IL)-1 receptor family and is highly induced by growth stimulation and oncogenic Ras-induced signaling (4). Based on alternative splicing and processing of mRNA, ST2 exists as 4 isoforms, including a transmembrane full-length form (ST2L), also known as IL1RL1-b, a soluble secreted form (sST2), also known as IL1RL1-a, a variant without the third immunoglobulin motif and splicing in the C-terminal (ST2v) and a fourth that has yet to be fully characterized (4-6). ST2 is produced by various types of immune cells produce ST2 including mast cells, macrophages and dendritic cells, and non-immune cells, including endothelial, epithelial, smooth muscle and fibroblast cells (7). The widespread expression of the ST2 gene in different cell types indicates that it may have important functions across a broad spectrum of biological systems. A recent functional study demonstrated that ST2 is the ligand-binding component of the interleukin-33 (IL-33) receptor, whereas sST2 is considered as a decoy receptor preventing IL-33 signaling (8). ST2L stimulated by IL-33 was demonstrated to activate the transcription factor through common signaling molecules to interleukin-1 receptor

(IL-1R), including myeloid differentiation primary response protein MyD88 (MyD88), MyD88-adapter-like, the Toll/IL-1R domain of the cytoplasmic region of IL1RL1, IL-1R-associated kinase, tumor necrosis factor receptor-associated factor 6 and NF-κB, and the mitogen-activated protein kinase family (8,9). ST2/IL-33 signaling has also been identified to be involved in T-cell mediated immune responses, particularly Th2 cells and Th2-associated cytokines (9). IL-33 binds to ST2 and induces the expression of interferon, IL-4, IL-5 and IL-13, thereby leading to severe pathological changes in mucosal organs. ST2L has previously been used as a marker for Th2 cells. In addition, sST2 may also serve a key role in downregulating the inflammatory response (10). It has also been suggested that ST2L/IL-33 participates in a number of inflammatory immune response processes associated with asthma, allergic diseases, autoimmune diseases, cardiovascular diseases and other conditions associated with acute heart failure, myocardial infarction, respiratory failure and acute trauma (11,12). Previous studies revealed that ST2 was involved in the pathogenesis and prognosis of multiple types of cancer, including glioblastoma, breast and pancreatic cancer, and leukemia (13-16). However, to the best of our knowledge, the role of ST2 in gastric cancer remains to be elucidated. The aim of the present study was to investigate the expression status of the 2 primary splice variants, sST2 and ST2v, by western blot analysis, and to evaluate ST2 protein expression in gastric cancer using immunohistochemistry and determine its clinicopathological significance.

Patients and methods

Patient information and tissue specimens. A microarray chip containing 178 samples of gastric cancer tissues from Beijing Hua Nuo Aomei Biotechnology, Inc. was utilized. A second cohort of 52 formalin-fixed paraffin-embedded tumor specimens was collected from patients who underwent surgery at the Xijing Hospital Affiliated to The Fourth Military Medical University (Xi'an, China) between September 2006 and January 2012. There were 230 patients in total, including 69 females and 161 males (median age, 54 years; range, 31-75 years) included in the present study. The patients were diagnosed as follows: 104 cases of gastric antrum carcinoma; 76 cases of carcinoma of the gastric cardia; and 50 cases of gastric body carcinoma. A total of 116 adjacent non-cancerous tissue specimens were also collected as controls. Tumor grade and stage were classified in accordance with the International Union against Cancer/American Joint Committee on Cancer pathological Tumor-Node-Metastasis (TNM) classification, 7th edition (2010) (17). In addition, 12 independent primary gastric cancer tissues and matched adjacent non-cancerous samples were frozen and stored in liquid nitrogen for western blot analysis. Signed informed consent was obtained from the patients prior to tissue sample collection. The study protocol conformed to the ethical guidelines outlined in the Declaration of Helsinki and was approved by The Institutional Review Board (approval no. 07-170) of Ningxia Hui Autonomous Region People's Hospital (Ningxia Hui Autonomous Region, China).

Western blot analysis. This procedure was performed as described previously (18-21). Frozen tissue samples, including gastric cancer tissues and paired adjacent non-cancerous tissues obtained from 12 patients were prepared in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). A total of 20 μg proteins were separated by 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore). The membranes were incubated in blocking buffer (TBS with 0.1% Tween and 5% non-fat dry milk) for 1 h at 37˚C and then incubated with a rabbit polyclonal anti-ST2 antibody (cat. no. 11920-1-AP; dilution, 1:500; ProteinTech Group, Inc.) in blocking buffer overnight at 4˚C, followed by a horseradish peroxidase-conjugated secondary antibody against rabbit IgG (cat. no. sc-2004; dilution, 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C. Signals were visualized with the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham; GE Healthcare). The blots were probed with an anti-β-actin monoclonal antibody (cat. no. ab8226; dilution, 1:2,000; Abcam) as the control. Densitometric analyses of protein expression levels were performed using Bio-Rad Quantity One software (version 4.5.2; Bio-Rad Laboratories, Inc.). Expression was considered to be decreased when the ratio of expression in tumor and paired non-cancerous tissue was <2.

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as described previously (18,19). Sections (4 μm) cut from tissue specimens fixed in 10% buffered formalin and embedded in paraffin were deparaffinized, rehydrated (anhydrous ethanol gradient, 100, 95, 80 and 70%) and incubated with 0.3% H_2O_2 for 20 min at 37˚C. Non-specific blocking was performed by using 10% goat serum (Sigma-Aldrich; Merck KGaA) containing 0.05% Tweeen-20 for 30 min at 37˚C. Glass slides were coated with a rabbit polyclonal anti-ST2 antibody (cat. no. 11920-1-AP; dilution, 1:50; ProteinTech Group, Inc.) overnight at 4˚C. The tissue sections were washed with PBS containing 0.05% Tween-20 and then incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (cat. no. sc-2012; dilution, 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The sections were counterstained with propidium iodide (PI; Sigma-Aldrich; Merck KGaA) for 5 min at 37˚C in the dark (final concentration of PI, 50 μg/ml). Finally, the slides were washed twice with PBS and were examined under an Olympus Flouview FV1000 confocal laser scanning microscope (Olympus Corporation) at x400 magnification.

Immunohistochemistry and evaluation. Immunohistochemical analysis was performed as described previously (20,21). Sections (4 μm thick) were cut from tissue specimens fixed in 10% buffered formalin and embedded in paraffin. After deparaffinization (xylene, 2 times and 10 min each at 37˚C) and rehydration (alcohol gradient, 100, 95, 80 and 70%), endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 min at room temperature. For antigen retrieval, tissue sections were autoclaved at 121˚C in citrate buffer (10 mM, pH 6.0) for 10 min. The sections were blocked for 1 h in 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in PBS at 37˚C, and were subsequently incubated with a rabbit polyclonal anti-ST2 antibody (cat. no. 11920-1-AP; dilution, 1:50; ProteinTech Group, Inc.) overnight at 4˚C. Staining was visualized using an EnVision antibody complex method. An EnVision kit (OriGene Technologies, Inc.) was used and

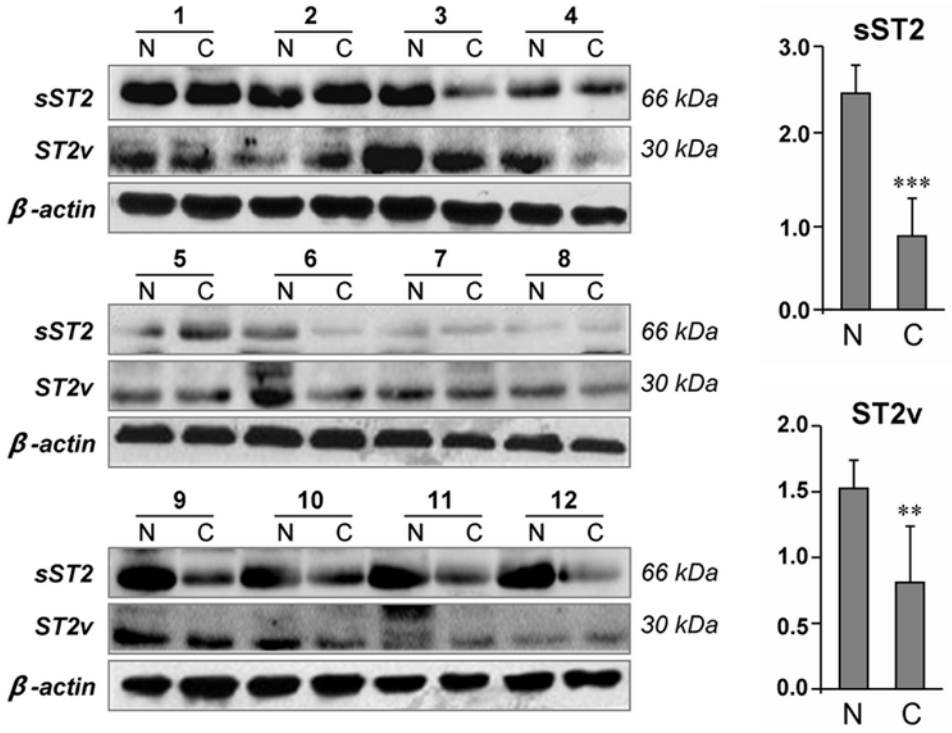

Figure 1. ST2 protein levels were detected by western blot analysis in gastric cancer tissues compared with paired adjacent non-cancerous tissues. Of the samples analyzed 75% (9/12) and 66.7% (8/12) of gastric cancer tissues exhibited decreased sST2 or ST2v expression compared with the adjacent non-cancerous tissues, respectively. C, gastric cancer tissues; N, paired adjacent non-cancerous tissues. ST2, interleukin 1 receptor-like 1; sST2, soluble secreted ST2; ST2v, ST2 variant without the third immunoglobulin motif and splicing in the C-terminal. **P<0.01, ***P<0.001 as compared with the N group.

3,3'-diaminobenzidine was used as the chromogen. Nuclei were counterstained with 0.5% hematoxylin for 2 min at room temperature. Sections immunostained with the normal rabbit IgG (cat. no. PP501P; dilution, 1:50; OriGene Technologies, Inc.) as the primary antibody were used as negative controls. A total of 10 random microscopic fields/slide, were evaluated by 2 independent observers who were blinded to the clinical information, at a magnification of x400 using a light microscope (Carl Zeiss AG, Oberkochen, Germany). ST2 staining was assessed using a semi-quantitative approach, which combined the staining intensity and proportion of positive cells. The mean percentage of positively-stained cells was scored as follows: 0, 0-5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. Staining intensity was categorized as follows: 0, Absent; 1, weak; 2, moderate; and 3, strong. The multiplication of staining intensity and percentage of positive cells was used as the final staining score. For statistical evaluation, the tumor samples with final staining scores of <3 were classed as negative ST2 expression and those with scores ≥3 as positive ST2 expression.

Statistical analysis. Statistical analysis was performed using the SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA). The association between the expression levels of ST2 with different clinical variables was assessed using Fisher's exact test or Pearson's χ^2 test as appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

Protein expression levels of ST2 in cancer and non-cancerous tissues. In light of the data indicating that ST2 is a frequently silenced candidate tumor suppressor gene during tumorigenesis (14), the present study first examined whether the expression of ST2 was altered at the protein level in tumors compared with normal tissues. In 12 pairs of gastric cancer tissues and their adjacent non-cancerous tissues, 2 bands were observed with molecular weights corresponding to the size of sST2 (30 kDa) and ST2v (66 kDa) (Fig. 1). Notably, 75% (9/12) of gastric cancer tissues exhibited decreased sST2 expression compared with the adjacent non-cancerous tissues (P<0.001). Similarly, decreased ST2v expression levels were also identified in 66.7% (8/12) of the tumors compared with the adjacent non-cancerous tissues (P<0.01).

The expression profile of ST2 in a cohort of 230 primary gastric cancer tissues and 116 adjacent non-cancerous tissues was additionally investigated by immunohistochemistry. ST2 expression was observed primarily in the cytoplasm of neoplastic cells, although it was also observed to a certain extent in the cell membrane (Fig. 2). Positive expression of the ST2 protein was observed in 39.1% (90/230) of tumor specimens and in 60.7% (54/89) of adjacent non-cancerous tissues (P<0.05). Taken together, these results clearly indicate that ST2 expression is frequently downregulated in gastric cancer tissues compared with normal controls.

Cellular localization of ST2 expression in gastric cancer. To determine the cellular localization of ST2 expression, immunofluorescence analysis was performed. Consistent with the results of the immunohistochemistry analysis, positive immunostaining for ST2 was observed in the cytoplasm and the membrane of cancer cells, whereas marked ST2 immunoreactivity was predominantly identified in

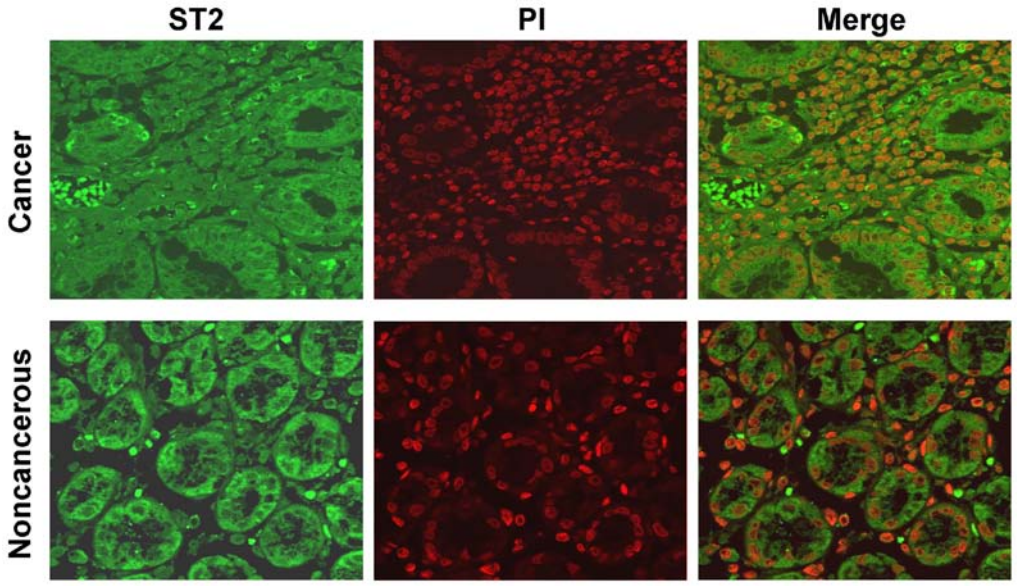

Figure 2. Analysis of ST2 expression in gastric cancer and non-cancerous tissues by immunofluorescence confocal microscopy. Tissue sections were incubated with an anti-ST2 antibody, followed by a fluorescein isothiocyanate-conjugated secondary antibody (green) and counterstaining with propidium iodide (red) to visualize the nuclei. The slides were observed under a confocal microscope. Magnification, x400. ST2, interleukin 1 receptor-like 1.

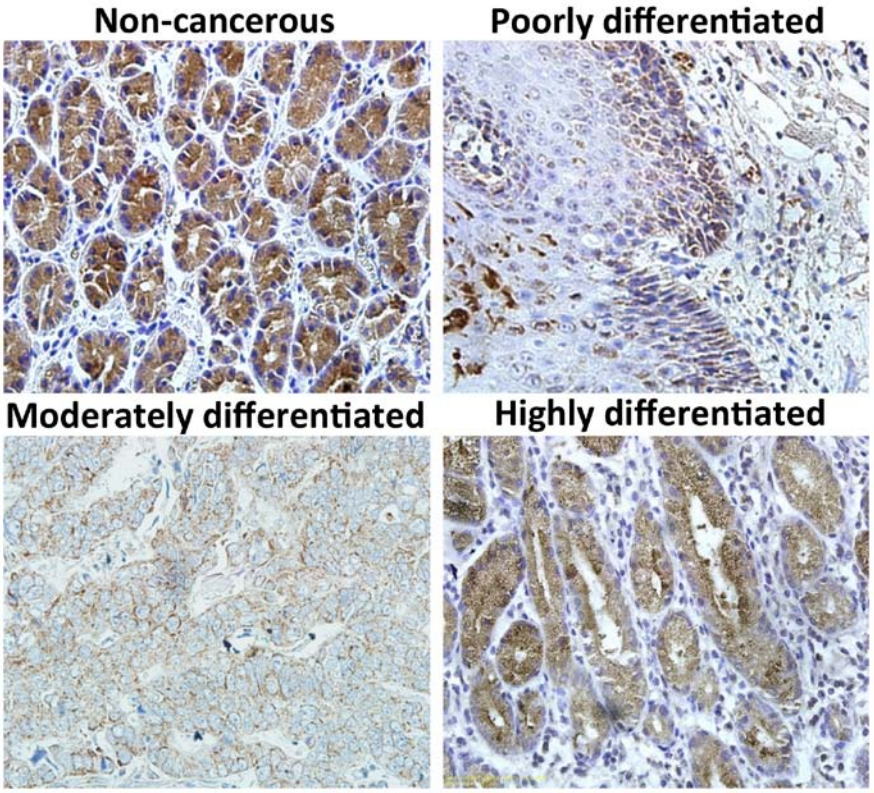

Figure 3. Immunohistochemical analysis of interleukin 1 receptor-like 1 protein levels in gastric cancer tissues. Representative samples of non-cancerous tissues, and well-differentiated, moderately differentiated and poorly differentiated tumors are presented (original magnification, x200).

the cytoplasm of epithelial cells in non-cancerous tissues (Fig. 3).

Clinicopathological implications of ST2 expression. The potential associations between ST2 protein levels and the clinicopathological characteristics of 230 gastric cancer specimens were next assessed. As demonstrated in Table I, decreased ST2 expression was identified to be significantly associated with decreased differentiation ($P<0.001$) and advanced TNM stage ($P<0.001$). No significant association was observed between ST2 expression and the other clinicopathological factors, including patient age and sex. In summary, these data indicated that decreased ST2 expression is closely associated with parameters implicated in gastric cancer progression and pathogenesis.

Table I. Associations between ST2 expression and clinicopathological variables.

Variables	Patients, n	ST2 expression		P-value
		Negative, n	Positive, n	
Sex				
Male	161	101	60	0.38
Female	69	39	30	
Age, years				
≤61	134	85	15	0.44
>61	96	55	20	
Differentiation[a]				
Well	37	9	28	<0.001
Moderate	72	38	34	
Poor	94	71	23	
TNM staging				
I/II	143	76	67	<0.001
III/IV	87	64	23	

[a]27 cases of mucinous adenocarcinoma were censored. ST2, interleukin 1 receptor-like 1; TNM, tumor node metastasis.

Discussion

ST2 has been identified as a regulatory effector for Th2-type responses, and as a negative feedback regulator in pro-inflammatory responses (4). ST2 also has a significant effect on the pathogenesis of several diseases, including inflammation, allergies, fibrillation, cardiac hypertrophy and rheumatoid arthritis (22). Numerous studies have suggested that ST2 expression is associated with carcinogenesis and tumor progression in multiple cancer types (13-16,22). ST2 has been shown to regulate innate and acquired immunity in tumors. The biological functions of ST2 in cancer growth and progression are primarily mediated by the IL-33/ST2 signaling pathway, which compromises the integrity of the intestinal barrier and promotes the production of pro-tumorigenic IL-6 by immune cells (22).

However, the exact physiological and pathological functions of ST2 in cancer development and progression remain to be elucidated (23,24). In the present study, the protein expression of the ST2 gene in human gastric cancer tissues and their matched non-cancerous tissues was analyzed by western blot analysis and immunohistochemistry. A total of 2 variant subtypes of the ST2 protein were also identified in gastric cancer tissues, sST2 and ST2v, with corresponding molecular weights of 30 and 66 kDa, respectively. Notably, the expression levels of ST2 among normal tissues adjacent to the tumor were variable across the patient cohort. This discrepancy may be due to the difference between individuals. However, 75% (9/12) of gastric cancer tissues exhibited a significantly decreased sST2 expression compared with the adjacent non-cancerous tissues. This observation suggested that ST2 protein expression was markedly decreased in cancer tissues compared with non-cancerous tissues. The present analysis in gastric cancer indicated an association between negative ST2 expression and tumor progression phenotype, including advanced tumor stage and poor tumor differentiation. These results suggested that ST2 may act as a tumor suppressor gene in gastric cancer, consistent with a previous study in glioblastoma cells (24), but are inconsistent with other studies in breast and colorectal cancer (23,25). Notably, a previous study involving gastric cancer revealed that IL-33 enhanced tumor cell invasion and migration via the ST2-ERK1/2 pathway, and knockdown of the IL-33 receptor ST2 attenuates the IL-33-mediated malignant phenotypes (26). It may be concluded that this disparity may be due to the intrinsic differences among tumor types.

It has been demonstrated that the ST2 protein may be involved in the immunity of the tumor microenvironment. IL-33 combined with IL-1 and other inflammatory cytokines enhance the expression of ST2, resulting in the activation of oncogenes (27). It has been suggested that knockdown of ST2L in mice may lead to the inhibition of mammary tumor growth and metastasis, followed by enhanced circulating levels of pro-inflammatory cytokines and activation of natural killer and $CD8^+$ T cells (28). Accumulating evidence suggests that ST2 may serve as an inflammatory cytokine in promoting cancer development and the production of Th2 cell-associated cytokines, including IL-4, IL-5 and IL-13. These cytokines have been detected in the microenvironment of several tumors (29-31). Taking into consideration the complexity of the tumor microenvironment, the physiological and molecular mechanisms of action of the ST2 protein in gastric cancer remain to be elucidated.

There were certain limitations to the present study. Additional validation of the results in a larger series of patients with gastric cancer will strengthen the results of the present study and improve the understanding of the clinical behavior of ST2. In addition, a number of factors are able to contribute to abnormal gene silencing in cancer, and not all aberrant gene

silencing is involved in tumor development. Therefore, candidate tumor suppressor genes, the expression levels of which are downregulated in tumors, require additional investigation for their biological roles in cancer development and progression.

In the present study, several methods were used to characterize the ST2 expression profile in gastric cancer. Downregulated ST2 expression is a molecular signature of gastric cancer progression and pathogenesis. Therefore, it may be concluded that ST2 not only appears to be a promising diagnostic biomarker, but also a potential treatment target in gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding authors on reasonable request.

Authors' contributions

FBai, YY and YN conceived, designed the experiments and drafted the manuscript. FBa, YF, WT, CW and MJ performed the experiments. YF and WT analyzed the data.

Ethics approval and consent to participate

Signed informed consent was obtained from the patients prior to tissue sample collection. The study protocol conformed to the ethical guidelines outlined in the Declaration of Helsinki and was approved by the Institutional Review Board (approval no. 07-170) of Ningxia Hui Autonomous Region People's Hospital.

Patient consent for publication

Signed informed consent was obtained from the patients prior to tissue sample collection.

Competing interests

The authors declare that they have no competing interests.

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