Clinical significance of stanniocalcin 2 expression as a predictor of tumor progression in gastric cancer

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Abstract. Stanniocalcin 2 (STC2) is a glycoprotein hormone that plays an important role in calcium and phosphate homeostasis. Furthermore, recent studies have demonstrated that STC2 expression in the primary site is correlated with tumor progression in several types of malignancies. However, few reports have investigated the clinical significance of STC2 expression in the blood of patients with gastric cancer. Therefore, we examined STC2 expression as a molecular blood marker for detection of circulating tumor cells (CTCs) and assessed the relationship between STC2 expression and clinicopathological features including prognosis in patients with gastric cancer. Quantitative PCR assay was used to assess STC2 mRNA expression in 4 gastric cancer cell lines and in blood specimens from 93 patients with gastric cancer and 22 healthy volunteers. The numbers of STC2 mRNA copies were significantly higher in the gastric cancer cell lines and in blood from patients with gastric cancer than in blood from healthy volunteers (P=0.0002 and P=0.01, respectively). STC2 expression was positive in 43 (46.2%) of the 93 patients with gastric cancer, and its expression was significantly correlated with age, depth of tumor invasion, lymph node metastasis, stage and venous invasion (P=0.023, P=0.045, P=0.035, P=0.007 and P=0.027, respectively). The 5-year survival rate was significantly lower in patients with STC2 expression compared to patients without STC2 expression (P=0.014). Our results indicate that STC2 could be a useful molecular blood marker for predicting tumor progression by monitoring CTCs in patients with gastric cancer.

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Introduction

Gastric cancer is the most common malignancy in Asia and is the third leading cause of cancer-related deaths in Japan (1-3). Endoscopic treatments, such as endoscopic mucosal resection and endoscopic submucosal dissection (ESD), have been recently performed in selected patients with early gastric cancer. At present, the expanded ESD indication for patients with early gastric cancer is under discussion (4). In regards to surgical procedures, laparoscopic gastrectomy has been frequently carried out as minimally invasive surgery in patients with early gastric cancer. Furthermore, the 5-year survival rates of patients with mucosal and submucosal gastric cancer are 95-100 and 85-95%, respectively (5-7). Thus, these findings imply that there is a spread of therapeutic options for the clinical management of early gastric cancer patients with good outcome. On the other hand, new anticancer agents for patients with unresectable advanced or recurrent gastric cancer have been developed, and trastuzumab has been focused on as a novel molecular-targeted drug for patients with human epidermal growth factor receptor 2 (HER2)-positive advanced gastric cancer (8). Nevertheless, the prognosis of such patients is still poor even when receiving novel chemotherapy. Therefore, it is important to diagnose patients at an early stage of gastric cancer and to identify post-operative patients at high risk for disease recurrence in clinical management. Although tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are currently used in the blood examination of patients with gastric cancer, the identification of further molecular blood markers is a prerequisite for the clinical management of patients with gastric cancer.

Stanniocalcins (STCs) are glycoprotein hormones that were originally identified in bony fish (9,10). Moreover, several investigators have reported that STCs play an important role in calcium and phosphate homeostasis (11-14). STC2 is one member of the STC family, and recent findings have

demonstrated that it is a hypoxia-inducible factor 1 (HIF-1) target gene that promotes cell proliferation, invasion and epithelial-mesenchymal transition (EMT) in hypoxia (15,16). STC2 is expressed at high levels in tumor cells of malignancies such as neuroblastoma, esophageal, gastric, colorectal, ovarian, prostate, breast and renal cell cancers (16-23). The status of STC2 expression in these tumor cells of the primary site is closely correlated with tumor progression, including prognosis. However, the clinical significance of STC2 expression in the blood of patients with gastric cancer has not yet been determined.

The aim of the present study was to assess STC2 expression in blood specimens from patients with gastric cancer and to investigate the relationship between STC2 expression and clinicopathological factors including prognosis in patients with gastric cancer.

Materials and methods

Gastric cancer cell lines. Four gastric cancer cell lines (MKN-7, MKN-45, MKN-74 and KATO-III) were cultured in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Mitsubishi Kasei, Tokyo, Japan), as well as 100 units/ml each of penicillin and streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2, as described previously (24,25). These cell lines were used for reverse transcription-polymerase chain reaction (RT-PCR) assay.

Patients. Blood specimens were obtained preoperatively from 93 patients (64 men and 29 women; age range, 35-87 years; average age, 68 years) with gastric cancer who underwent curative gastrectomy with lymph node dissection at Kagoshima University Hospital (Kagoshima, Japan) between 2003 and 2005. None of the patients had received endoscopic mucosal resection, palliative resection, preoperative chemotherapy, and/or radiotherapy in the present study. Furthermore, patients who had synchronous or metachronous cancer in other organs were excluded. Patients were classified and staged on the basis of criteria of the tumor-node-metastasis (TNM) classification of gastric carcinoma established by the International Union Against Cancer (UICC) (26). Normal peripheral blood lymphocytes (PBLs) isolated from 22 healthy volunteers were used as a control group. After discharge, all patients were followed up every 3-6 months by blood tumor marker studies (CEA and CA19-9), radiography, ultrasonography and computed tomography at Kagoshima University Hospital. The median follow-up period after surgery was 25 months (range, 1-74 months).

To investigate STC2 protein expression in gastric cancer, 30 paraffin-embedded archival tissue (PEAT) specimens of resected primary gastric tumors from patients enrolled in this study were used for immunohistochemical analysis.

All specimens were collected from patients after informed consent was obtained in accordance with the institutional guidelines of our hospital.

Blood processing and RNA extraction for RT-PCR analysis. Blood specimens (5 ml) from each patient were preoperatively collected in tubes containing sodium citrate, and then blood cells were separated in lymphocyte separation buffer (Gentra Systems, Inc., Minneapolis, MN, USA). Total RNA was extracted from the cell lines and blood specimens using Isogen (Nippon Gene, Toyama, Japan). Total RNA was isolated and purified using phenol-chloroform extraction as previously described (24,25). The concentration and purity of the total RNA were determined using a GeneQuant Pro UV/Vis spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK).

Primers and probes. Primer and probe sequences of STC2 and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) were designed for RT-PCR assays of each marker. The forward primers, fluorescence resonance energy transfer probe sequence, and reverse primers for STC2 and GAPDH were as follows: STC2 forward, 5'-GACTTGCTGCTGCACGAAC-3'; probe, 5'-FAM-ACGTGGACCTCGTGAACTTGCTG-TAMRA-1-3'; reverse, 5'-TGCTCACACTGAACCTGCAC-3' and GAPDH forward, 5'-GGGTGTGAACCATGAGAAGT-3'; probe, 5'-FAM-CAGCAATGCCTCCTGCACCACCAA-TAMRA-1-3'; reverse, 5'-GACTGTGGTCATGAGTCCT-3'. The RT-PCR products for STC2 and GAPDH were resolved as 107- and 136- base pair fragments, respectively. The integrity of the RNA was confirmed by RT-PCR assay using GAPDH.

Quantitative RT-PCR assay. All total RNA samples were reverse-transcribed using the Advantage RT-for-PCR kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) as previously described (24,25). Quantitative RT-PCR (qRT-PCR) proceeded using the LightCycler System (Roche Diagnostics, Mannheim, Germany). The reaction mixtures contained cDNA transcribed from 250 ng of RNA using each primer, probe, $MgCl_2$, and LightCycler FastStart DNA Master hybridization probes (Roche Diagnostics). The amplification profile comprised precycling at 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing for 20 sec (60˚C for STC2, and 55˚C for GAPDH), and extension at 72˚C for 10 sec. Plasmids for each marker were synthesized using pT7Blue-2 T-Vector (Novagen, Madison, WI, USA) according to the manufacturer's instructions. Standard curves for each assay were generated using the threshold cycles of 6 serial dilutions of plasmid templates (10^6-10^1 copies). The mRNA copy number was determined using LightCycler software (Roche Diagnostics). Each assay was repeated in duplicate with positive (cell line), negative (H_2O), and reagent (without cDNA) controls to evaluate the quality of the qRT-PCR assay. Absolute copy numbers in qRT-PCR assays were computed on the basis of standard curves of plasmid templates. Copy numbers of STC2 mRNA were normalized by those of GAPDH mRNA (relative STC2 mRNA copies; absolute STC2 mRNA copies/absolute GAPDH mRNA copies).

Cell spiking study for determining the sensitivity of the RT-PCR assay. Serial dilutions (10^4, 10^3, 10^2, 10^1 and 0) of MKN-74 tumor cells mixed with $1x10^7$ PBLs, which were isolated from a healthy volunteer without STC2 mRNA expression, were used for determining the sensitivity of the qRT-PCR analysis. This *in vitro* assay was repeated 3 times to verify its reproducibility.

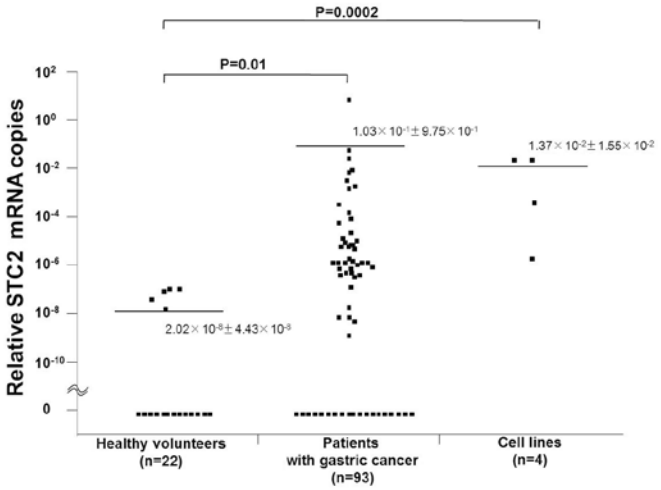

Figure 1. RT-PCR analysis of STC2 mRNA expression in cell lines and in blood specimens from patients with gastric cancer and healthy volunteers. Horizontal bars indicate mean STC2 mRNA copy numbers. STC2, stanniocalcin 2.

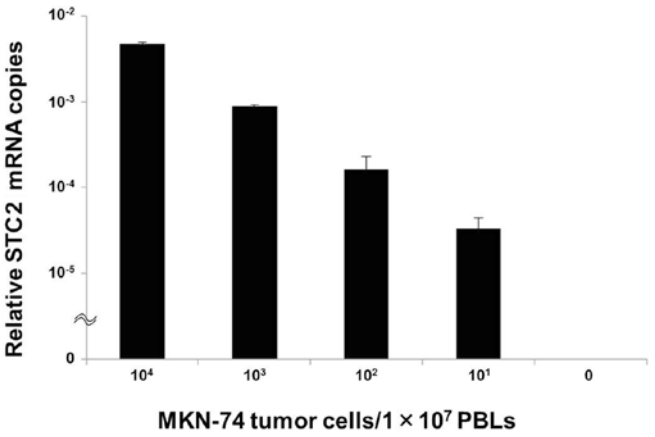

Figure 2. RT-PCR analysis of STC2 mRNA expression in cell spiking study. Serially diluted MKN-74 tumor cells (10^4, 10^3, 10^2, 10^1 and 0) were mixed with normal PBLs. Bars indicate standard deviation (SD). STC2, stanniocalcin 2; PBLs, peripheral blood lymphocytes.

Immunohistochemical staining. The PEAT sections (3 μm) of surgical primary gastric tumors were incubated on slides at 50˚C overnight, deparaffinized with xylene and then rehydrated with a graded series of ethanol. The sections were autoclaved in citrate buffer (0.01 mol/l, pH 6.0) at 120˚C for 10 min to activate the antigen. After cooling at room temperature, endogenous peroxidase was blocked using a peroxidase blocking reagent (DakoCytomation, Carpinteria, CA, USA) for 10 min. Non-specific binding was blocked at room temperature for 30 min with Protein Block Serum-Free (DakoCytomation). The sections were incubated at room temperature for 60 min with an anti-human STC2 polyclonal antibody (Proteintech Group, Inc., Chicago, IL, USA) diluted 1:200 in Dako antibody diluent with background reducing components (DakoCytomation). After three 5-min washes in phosphate-buffered saline (PBS), the reaction for STC2 was developed by the ABC method (Vectastain ABC kit; Vector Laboratories) and visualized using diaminobenzidine tetrahydrochloride (DakoCytomation). Negative controls were treated with PBS without the primary antibody under the same conditions. On the basis of immunostainable intensity, STC2 immunoreactivity was classified into 3 groups: negative, weak, and strong immunoreactions.

Statistical analysis. Differences in STC2 mRNA expression between gastric cancer cell lines and PBLs from healthy volunteers, and between PBLs from patients with gastric cancer and healthy volunteers, were evaluated by the Wilcoxon rank-sum test. The relationship between the status of STC2 expression and categorical clinicopathological factors was assessed by the Chi-square and Fisher's exact tests. Survival curves were generated using the Kaplan-Meier method, and differences in survival were examined using the log-rank test. Prognostic factors were assessed by univariate and multivariate analyses (Cox proportional hazard regression model). All statistical calculations were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). A P-value of <0.05 was considered to indicate a statistically significant result.

Results

STC2 mRNA expression determined by RT-PCR in cell lines and clinical blood specimens. STC2 mRNA expression in 4 gastric cancer cell lines, blood specimens from 93 patients with gastric cancer, and from 22 healthy volunteers without cancers was assessed by qRT-PCR.

The range of relative STC2 mRNA copies was $2.49x10^{-6}$ to $2.75x10^{-2}$ in gastric cancer cell lines, 0 to 9.4 in blood from patients, and 0 to $1.35x10^{-7}$ in normal PBLs from healthy volunteers. The mean relative numbers of STC2 mRNA copies (± SD) were $1.37x10^{-2}$±$1.55x10^{-2}$ in gastric cancer cell lines, $1.03x10^{-1}$±$9.75x10^{-1}$ in blood from patients, and $2.02x10^{-8}$±$4.43x10^{-8}$ in normal PBLs (Fig. 1). Accordingly, the relative numbers of STC2 mRNA copies were significantly higher in the gastric cancer cell lines and in blood from patients than in normal PBLs (P=0.0002 and P=0.01, respectively). STC2 mRNA expression was identified in 43 (46.2%) of the 93 patients with gastric cancer.

STC2 mRNA expression in the cell spiking study. This spiking study was planned to investigate the relationship between STC2 mRNA expression and the number of tumor cells in an *in vitro* assay.

STC2 expression was identified in MKN-74 cells at a density of 10^1 tumor cells/$1x10^7$ PBLs, and the relative numbers of STC2 mRNA copies gradually decreased as the numbers of tumor cells within normal PBLs decreased (Fig. 2).

Relationship between STC2 expression and the clinicopathological features of the gastric cancer patients. All patients were classified into 2 groups based on the status of STC2 expression (positive, n=43; negative, n=50) to evaluate the relationship between STC2 expression and clinicopathological features.

STC2 expression was significantly correlated with age, depth of tumor invasion, lymph node metastasis, stage and venous invasion (P=0.023, P=0.045, P=0.035, P=0.007 and P=0.027, respectively; Table I).

Table I. Relationship between STC2 expression and clinicopathological features in patients with gastric cancer.

Features	STC2 expression, n (%)		P-value
	Negative (n=50)	Positive (n=43)	
Gender			
Male	35 (70.0)	29 (67.4)	0.825
Female	15 (30.0)	14 (32.6)	
Age (years)			
≤70	31 (62.0)	16 (37.2)	0.023
>70	19 (38.0)	27 (62.8)	
Tumor location			
Upper	15 (30.0)	16 (37.2)	0.540
Middle	18 (36.0)	11 (25.6)	
Lower	17 (34.0)	16 (37.2)	
Histological type			
Differentiated	27 (54.0)	19 (44.2)	0.408
Undifferentiated	23 (46.0)	24 (55.8)	
Depth of tumor invasion			
pT1-T2	21 (42.0)	9 (20.9)	0.045
pT3-T4	29 (58.0)	34 (79.1)	
Lymph node metastasis			
Negative	25 (50.0)	12 (27.9)	0.035
Positive	25 (50.0)	31 (72.1)	
Distant metastasis			
Negative	43 (86.0)	36 (83.7)	0.779
Positive	7 (14.0)	7 (16.3)	
Stage			
I-II	32 (64.0)	15 (34.9)	0.007
III-IV	18 (36.0)	28 (65.1)	
Lymphatic invasion			
Negative	20 (40.0)	10 (23.3)	0.119
Positive	30 (60.0)	33 (76.7)	
Venous invasion			
Negative	22 (44.0)	9 (20.9)	0.027
Positive	28 (56.0)	34 (79.1)	

pT1, invasion of lamina propria or submucosa; pT2, invasion of muscularis propria; pT3, invasion of subserosa; pT4, penetration of serosa without invasion of adjacent structures or invasion of adjacent structures. STC2, stanniocalcin 2.

Relationship between STC2 expression and prognosis. The 5-year survival rates were 58.4 and 80.9% in patients with STC2-positive and -negative expression, respectively (Fig. 3). Five-year survival rates were significantly lower in patients with STC2-positive expression than in those with STC2-negative expression (P=0.014; Fig. 3).

Univariate analysis demonstrated that age, depth of tumor invasion, lymph node metastasis, distant metastasis, lymphatic invasion, venous invasion and STC2 expression were

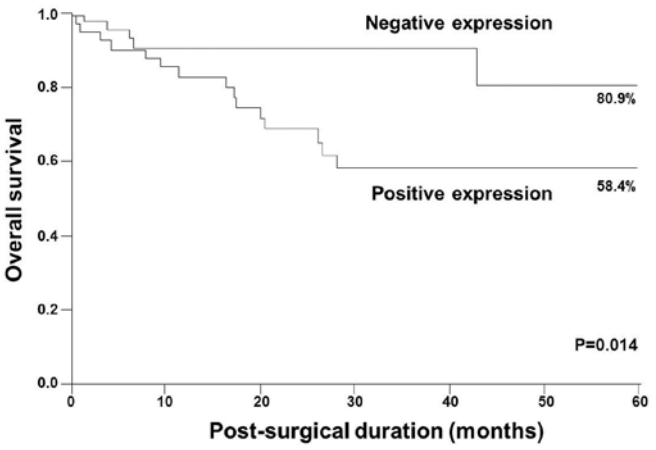

Figure 3. Kaplan-Meier survival curves for patients with gastric cancer based on the status of STC2 expression. Patients with STC2-positive expression had a significantly poorer prognosis than those with STC2-negative expression (P=0.014). STC2, stanniocalcin 2.

significantly correlated with postoperative survival (P=0.020, P=0.003, P=0.002, P=0.046, P=0.030, P=0.041 and P=0.012, respectively; Table II). Multivariate analysis demonstrated that age, lymph node metastasis and distant metastasis were independent prognostic factors (P=0.002, P=0.024 and P=0.047, respectively; Table II). Consequently, STC2 expression was not an independent prognostic factor in the multivariate analysis (P=0.433).

STC2 protein expression as determined by immunohistochemistry in primary tumor specimens. To confirm STC2 expression in primary tumor sites, immunohistochemical evaluation was carried out on 30 surgical PEAT specimens of primary gastric tumors. Immunohistochemical analysis showed that STC2 protein expression was identified in the membrane and/or cytoplasm of gastric tumor cells (Fig. 4). Furthermore, primary gastric tumors had various immunoreactions for STC2 (Fig. 4A-C). Finally, strong immunoreaction was identified in 46.7% (7/15) and 26.7% (4/15) of patients with STC2-positive and -negative mRNA expression, respectively.

Discussion

In the present study, we investigated STC2 mRNA expression in blood from patients with gastric cancer using qRT-PCR assay. Furthermore, we also compared the relationship between the status of STC2 expression and clinicopathological findings to assess the clinical impact of STC2 expression as a useful blood marker in patients with gastric cancer. To our knowledge, this is the first study regarding STC2 expression in circulating tumor cells (CTCs) obtained from patients with gastric cancer.

To date, STC2 expression has been identified in various malignant diseases (16-23). In the present study, STC2 protein expression in gastric tumor cells was verified by immunohistochemical analysis. Furthermore, we confirmed STC2 mRNA expression in all gastric cancer cell lines and demonstrated STC2 mRNA high expression in blood from patients with gastric cancer. Although STC2 mRNA expression was

Table II. Univariate and multivariate analyses of survival in patients with gastric cancer.

	Univariate analysis			Multivariate analysis		
Independent factor	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (years) ≤70/>70	1.719	1.088-2.890	0.020	2.272	1.342-4.110	0.002
Depth of tumor invasion pT1-T2/pT3-T4	2.417	1.300-6.066	0.003	2.281	0.920-7.145	0.080
Lymph node metastasis Negative/positive	2.257	1.306-4.674	0.002	2.812	1.126-9.038	0.024
Distant metastasis Negative/positive	1.700	1.010-2.690	0.046	1.813	1.008-3.186	0.047
Lymphatic invasion Negative/positive	1.821	1.055-3.771	0.030	0.851	0.429-2.035	0.687
Venous invasion Negative/positive	1.763	1.021-3.650	0.041	0.491	0.182-1.486	0.200
STC2 expression Negative/positive	1.820	1.132-3.190	0.012	1.234	0.740-2.225	0.433

pT1, invasion of lamina propria or submucosa; pT2, invasion of muscularis propria; pT3, invasion of subserosa; pT4, penetration of serosa without invasion of adjacent structures or invasion of adjacent structures. STC2, stanniocalcin 2.

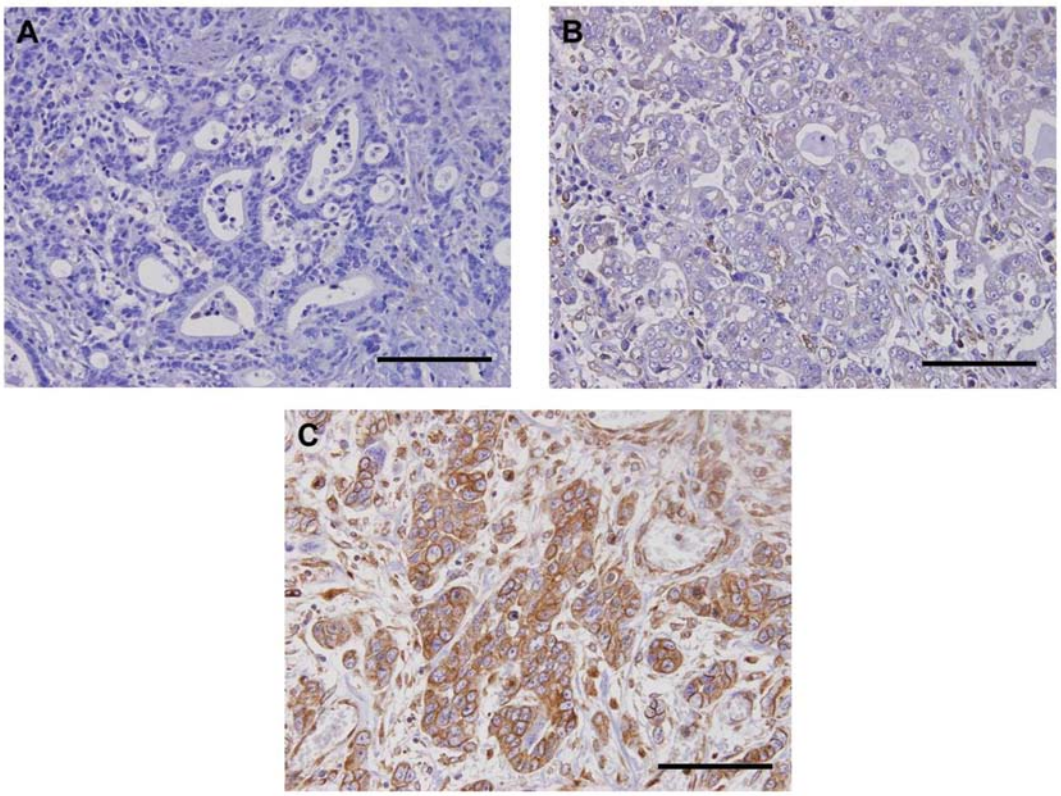

Figure 4. Representative immunohistochemical staining of STC2 expression in primary gastric tumor specimens. (A) Tumor cells with negative expression of STC2. (B) Tumor cells with weak expression of STC2. (C) Tumor cells with strong expression of STC2. Scale bar, 100 μm. Original magnification, x400. STC2, stanniocalcin 2.

detected in a few blood specimens from healthy volunteers, the level of STC2 mRNA expression in these specimens was extremely low in comparison with the level in blood specimens from gastric cancer patients. These results indicate the

clinical utility of RT-PCR assay against the STC2 molecule for discriminating gastric cancer patients from healthy volunteers.

Previous studies have demonstrated the clinical efficacy of CTC detection for predicting the potential for tumor progression and the response to chemotherapy in patients with various types of malignancies (27-30). Furthermore, a CTC assay system using immunomagnetics has been developed as a promising novel tool in recent years (31-33). In gastric cancer, epithelial markers such as cytokeratin and CEA are usually used for CTC detection in RT-PCR assay (28). Miyazono *et al* (28) reported that the CEA mRNA-positive rate in patients with gastric cancer was 36.8%, and CEA mRNA positivity was significantly correlated with the depth of tumor invasion and recurrence. In the present study, we showed the intensive relationship between the status of STC2 expression and tumor progression, such as depth of tumor invasion, lymph node metastasis, stage and venous invasion. Moreover, we verified the positive correlation between STC2 mRNA copies and the number of gastric tumor cells in a cell spiking study. At present, potential blood markers for monitoring CTCs are limited in the clinical management of patients with gastric cancer. Consequently, STC2 could be one of the surrogate markers for predicting tumor progression in patients with gastric cancer.

Although the functional role of STC2 remains unclear, several investigators have demonstrated the positive effects of STC2 induced by hypoxia in an *in vitro* study (15,16). Law and Wong (16) reported that STC2 overexpression enhanced the process of EMT via an increase in N-cadherin and vimentin and a reduction in E-cadherin in hypoxic human ovarian cancer cells. Additionally, a potentially relevant association was found between STC2 expression and matrix metalloproteases involved in tumor invasion in hypoxia (16). In the present study, STC2 overexpression was significantly correlated with tumor aggressiveness and poorer prognosis. These results suggest that STC2 is a promising marker for new targeted therapies that suppress tumor progression in patients with gastric cancer.

In conclusion, we demonstrated that STC2 is overexpressed in blood from patients with gastric cancer and that its expression is positively associated with malignant behavior. Therefore, STC2 is available for predicting tumor progression by monitoring CTCs in patients with gastric cancer. Further understanding of its functional role would allow the development of an attractive treatment strategy for patients with gastric cancer.

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