Connexin 26 expression correlates with less aggressive phenotype of intestinal type-gastric carcinomas

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Abstract. Connexin 26 (Cx26), one of the gap junction-forming family members, is more controversial than other members, as a tumor suppressor. Here, we assessed Cx26 expression in gastric carcinoma, which has not been investigated before, and its clinical significance including survival analyses. Cx26 expression was assessed in 205 tissue samples from gastric carcinoma by immunohistochemistry. Of 205 gastric carcinoma cases, 79 (38.5%) were positive for Cx26 with mainly cytoplasmic localization compared to sporadic membranous staining in normal epithelium, and the expression levels were confirmed by Western blotting and real-time PCR. Negative associations were revealed between Cx26 expression and most clinicopathologic features (all $P<0.05$). Notably, high Cx26 expression was associated with histological intestinal-type ($P=0.017$) and early stage of gastric carcinoma. The multivariate regression analysis revealed that positive Cx26 expression was an independent prognostic predictor of intestinal-type GC ($P=0.023$, HR=2.019). Our findings suggest that aberrant expression of Cx26 in cytoplasm plays a tumor-suppressor role in gastric carcinoma and is an independent biomarker for favorable prognosis in intestinal-type gastric carcinoma.

Introduction

Connexin 26 (Cx26) is one of the connexin (Cx) family members, with the smallest molecular weight (26 kDa). Cx proteins are encoded by a multigene family and so far 20 different human Cx genes have been identified (1). These transmembrane proteins in humans, comprise the main subunits of gap junctions (GJs), the connexon which is a specialized cluster of intercellular channels that allow adjacent cells to directly share ions and hydrophilic molecules of up to ~1 kDa in size (2). GJs may be composed of identical Cx isotypes (homotypic) or of more than one Cx isotype (heterotypic) (3). Gap junctional intercellular communication (GJIC), meditated by GJs, is believed to be involved in the regulation of cell proliferation, migration and differentiation. GJIC also plays an important role in the maintenance of tissue homeostasis, probably by regulating the proliferation and apoptosis processes (3-5). These different connexins can have distinct or overlapping functions depending on the level of their expression in various tissues and cells (1).

It has been shown that abnormal function of GJIC is generally implicated in the progression of a variety of tumors, usually by down-regulation of Cxs. Reduced expression of Cx43 in human lung and breast cancers (7,8), Cx32 in human lung and gastric cacncers (7,9), and Cx43 in prostatic adenocarcinoma and human brain glioma cells have been reported (10,11). In addition, in cell lines defective of Cx expression, transfection of cDNA encoding connexin protein causes the suppression of tumor growth (12,13). Thus, genes encoding Cx proteins have been classified as tumor suppressors. Analogous to other Cxs, Cx26 has been considered to serve as a tumor suppressor gene, however, it is more controversial than other members in cancer progression. Lee *et al* have observed that induced expression of Cx26 in a rodent mammary tumor cell-line (BICR-M1R_K) restrains cell growth, however, without increased GJIC, which means an antiproliferate effect of exogenous Cx26 in a GJIC-independent manner (13). Kanczuga-Koda *et al* revealed that loss of normal intercellular Cx26 occurred during colorectal carcinogenesis and predicted a different role of Cx26 in neoplastic cells (14). On the contrary, accumulating evidence indicates that Cx26 is overexpressed in carcinomas of the prostate and skin (15,16). Increased Cx26 expression was also observed in invasive breast carcinoma (17) and metastatic lymph nodes and the invasive front of malignant melanoma of the skin (18). Moreover, other studies demonstrate that high Cx26 expression is associated with poor prognosis of lung squamous cell and colorectal cancers with lung metastasis (19,20). Together, these strands of evidence show the contradictory roles of Cx26 in malignant cells.

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Presently, little is known about the expression pattern of Cx26 and its roles in human gastric carcinoma (GC). In this study, we focused on the expression pattern of Cx26 in gastric cancer to explore its clinical significance and prognostic analysis which has not yet been systematically analyzed.

Materials and methods

Patient population and clinical data. Tissue samples were obtained with written consent from 213 patients with gastric carcinoma, who underwent a curative gastrectomy in the Tsushimi Hospital, Hagi, Yamaguchi (1990-1997). Of the 213 patients, 127 (59.6%) were men and 86 (40.4%) women, with a median age of 68 (range, 31-93 years). None of the patients received either chemotherapy or radiation therapy before surgery.

All histologic slides were re-evaluated by two pathologists (Z.D. Xu and X.P. Liu). Gastric carcinomas were classified histologically as diffuse type (DI), intestinal type (IT) or mixed type (MT) according to Lauren's criteria (21). Eight cases of MT-GC were excluded, which left 205 cases for this study. Pathological stage was determined into early and advanced groups according to the wall penetration. Early was defined as carcinoma confined to the mucosa and submucosa, and advanced was defined as a more invasive carcinoma. Patient characteristics were according to the general rules of gastric cancer outlined by the Japanese Research Society for Gastric Cancer (22), including the depth of invasion, lymph node metastasis, venous invasion, and lymphatic invasion. The clinicopathological data of patients are shown in Table I.

All patients were followed up after the surgery until 31 December, 2004 with detailed and complete clinicopathological data. At the end of the follow-up period, 120 patients were still alive, and 70 had died of the disease. During the follow-up period, 15 cases were excluded, of whom 14 died of unrelated causes and one died within 30 days after surgery, and the remaining 190 patients were subjected to survival analyses.

Antibodies. Mouse anti-Cx26 monoclonal antibody 13-8100, obtained from Zymed Laboratories, Inc. (Invitrogen Corporation), recognizes the human Cx26 protein (molecular weight, 26.5 kDa). Mouse anti-human actin antibody was purchased from Sigma-Aldrich.

Inmmunohistochemistry and evaluation of staining result. Paraffin-embedded tissue sections 4-μm thick, were subjected to immunohistochemistry of Cx26 protein with the avidin-biotin-peroxidase method (SP staining kit, Shanghai Changdao Biotech Com.). The sections were first treated with 3% H_2O_2 in methanol for 15 min at room temperature to inhibit endogenous peroxidase activity. Antigen retrieval was performed with 10 mmol/l sodium citrate buffer (pH 6.0) at 95˚C for 20 min and was allowed to cool in room temperature for 50 min. After blocking reagent was added for 5 min, the sections were incubated with the diluted primary antibody at a dilution of 1:200 overnight, followed by incubation with biotinylated anti-mouse IgG and avidin-biotin-peroxidase at room temperature, for 30 min each, and 3,3-Diaminobenzidine tetrahydrochloride (DAB) from Dako for 10 min. Finally, sections were counterstained with Mayer's hematoxylin and mounted.

Table I. Correlation of Cx26 and clinicopathological features in GC (n=205).

Feature	Total cases	Cx26 expression group		P-value[a]
		Negative	Postive	
Age				
<6	73	47	26	
≥6	132	79	53	0.523
Sex				
Female	84	51	33	
Male	121	57	46	0.854
Lauren's criteria				
Intestinal-type	127	70	57	
Diffuse-type	78	56	22	0.017
Depth of invasion				
T1	83	37	46	
T2	64	45	19	
T3+T4	58	44	14	1.77×10^{-4}
Lymph node				
Negetive	114	59	55	
Positive	91	69	24	0.001
Stage				
Early	112	57	55	
Advanced	93	69	24	6.43×10^{-4}
Lymphatic invasion				
ly0	69	32	37	
ly1	53	32	21	
ly2	52	40	12	
ly3	31	22	9	0.004
Vein invasion				
Negetive	109	57	52	
Positive	96	69	27	0.004
Disease-specific survival rate	63.20%	54.70%	76.70%	0.002

[a]P<0.05 was considered statistically significant.

The positive control for Cx26 was used with sections of formalin-fixed, paraffin-embedded human normal colon mucosa, as indicated by Kanczuga-Koda (13). Negative controls were incubated with immunoglobulin fraction (TBS) in place of the polyclonal primary antibody in the positive tissues mentioned above.

Stained slides were evaluated independently by two pathologists (X.P. Liu and Z.D. Xu). For each specimen, the evaluation of Cx26 expression was analyzed over ten different visual fields at a power of x400 (Carl Zeiss, Germany), and mean percentage of interceller staining cells (membranous staining cells) or cytoplasmic staining cells were respectively calculated irrespective of stained intensity (13). For Cx26 staining of tumor sections, it was determined as positive when at least 10% of cancer cells exhibited positive cytoplasmic staining (14,23).

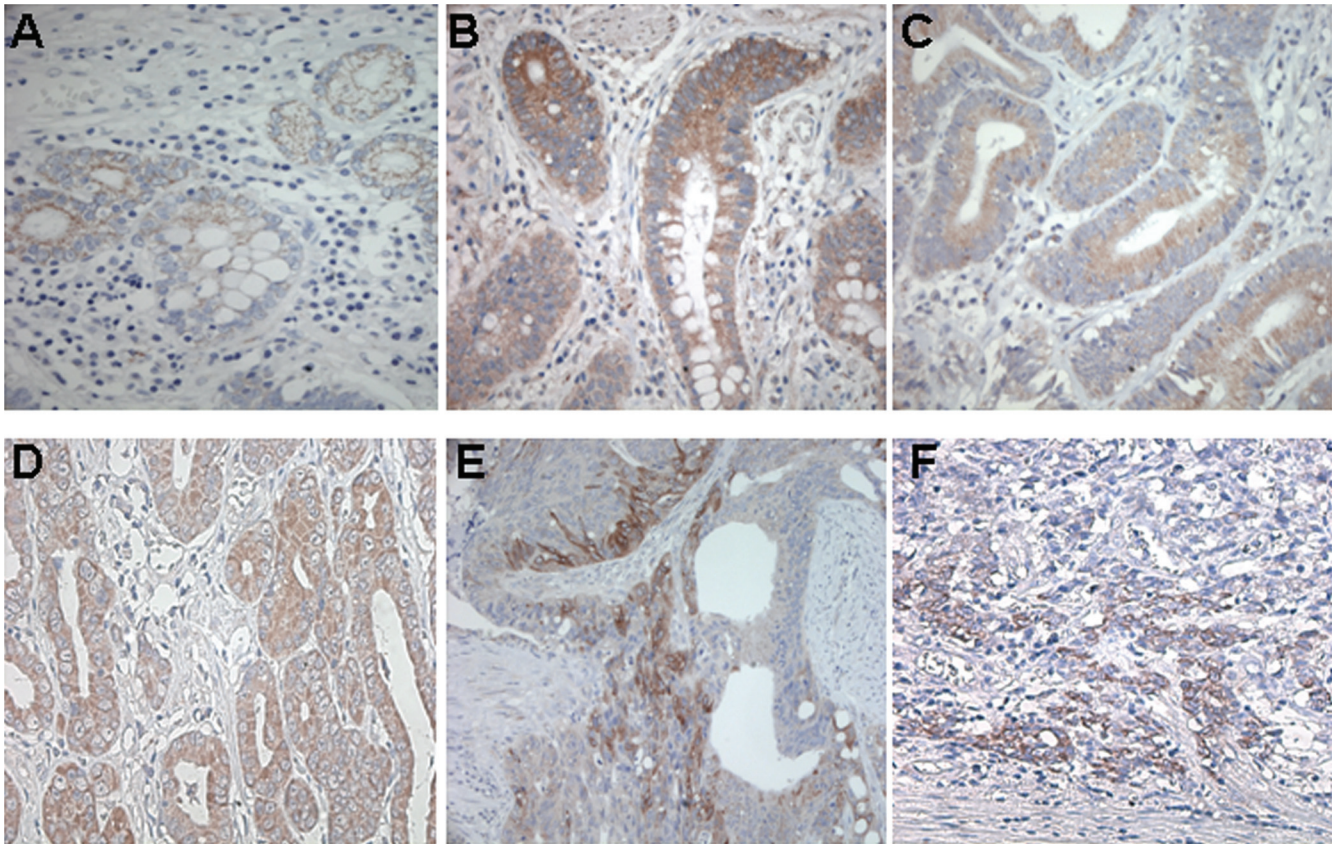

Figure 1. Representative results of immunohistochemical staining of Cx26. A. Normal gastric epithelium, intercellular granular staining of Cx26 between epithelial cells was observed. B. Moderate dysplasia glands, both membranous and cytoplasmic staining were observed. C. Severe dysplasia glands, maily diffuse cytoplasmic staining was observed. D. Intestinal-type GC (early stage), strongly positive in cytoplasm of most carcinoma cells. E. Intestinal-type GC (advanced stage), part of carcinoma cells had cytoplasmic staining. F. Diffuse-type GC (advanced stage), minor carcinoma cells adjacent to periphery were positive for Cx26.

Western blot analysis. Based on the distinct expression of Cx26 in gastric cancers by inmmunohistochemistry, 8 fresh gastric cancer tissues were randomly chosen from the Cx26 positive-staining cases (n=4) and negative-staining cases (n=4), and Western blot analysis was done on these samples. Briefly, protein samples (100 Ag) were separated by 12% SDS-PAGE. For Western blot analysis, proteins were transferred to poly-vinylidene fluoride (PVDF) membranes, blocked in 5% non-fat milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (w/v) Tween-20 for 1 h. Membranes were incubated with primary antibodies against Cx26 (1:500) and ß-actin (1:1,000) for 1 h, followed by 1 h incubation with the appropriated secondary antibody consisting of horseradish peroxidase (HRP). Detection by enzyme-linked chemiluminescence (ECL; Pierce, Rockford, USA) was performed according to the manufacturer's protocol. Controls for protein loading were identified by ß-actin as the internal standard.

Reverse transcription and quantitative real-time PCR. Total RNA was extracted from 8 case-tissues used in above Western blot analysis, by the Trizol method. cDNA was generated from 1 μg RNA with a PrimeScript RT Reagent Kit (Takara, Otsu, Japan). Quantitative real-time polymerase chain reaction (RT-PCR) was then performed using SYBR Premix Ex Taq II (Takara). The transcription value of Cx26 was determined by plotting against the standard curve constructed using MKN45 gastric cancer cells (20). Relative gene expression was determined by the fluorescence intensity ratio of the target gene to GAPDH from the same sample. Primers are as follows, Cx26 forward primer, 5'-CGGAATTCAGATGGATTGGG GCACG-3', reverse primer, 5'-CGGGATCCACTGGCTTTT TTGACTT-3' (19); GAPDH forward primer: 5'-AACGGAT TTGGTCGTATTG-3', reverse primer: 5'-GGAAGATGGTG ATGGGATT-3'. The PCR amplification products were visualized after electrophoresis on 1% agarose gel.

Statistical analysis. The Cx26 immunohistochemistry results of 205 gastric cancer cases and the clinical significance were assessed using statistical analyses. Pearson's Chi-Square tests were used to compare the protein expression level of Cx26 within clinicopathological features. Kaplan-Meier survival analysis was used to estimate the prognostic relevance of Cx26 and the survival difference between groups was assessed by the log-rank test. Univariate and multivariate Cox regression analysis was performed to evaluate differences of all possible factors in the risk of death. SPSS 15.0 software (Chiago, IL, USA) was used for all statistical analyses. For all tests, a $P<0.05$ was defined as statistically significant.

Results

Cx26 expression in non-carcinoma gastric epithelium by immunohistochemistry. The Cx26 protein was scarcely expressed in the epithelium of normal gastric mucosa. Only

Table II. Correlations of Cx26 and clinicopathological features in two types of GC (n = 205).

	Intestinal-type Cx26				Diffuse-type Cx26			
Variable	Cases	Negative	Positive	P-value[a]	Cases	Negative	Positive	P-value[a]
Age (year)								
<6	39	23	16		34	24	10	
≥65	88	47	41	0.561	44	32	12	0.835
Sex								
Male	79	44	35		42	31	11	
Female	48	26	22	0.867	36	25	11	0.669
Lymph node								
Negative	82	35	47		32	24	8	
Positive	45	35	10	$1.43x10^{-4}$	46	32	14	0.600
Vein invation								
Negative	80	36	44		29	23	6	
Positive	47	34	13	0.003	49	38	11	0.856
Lymph invasion								
Negative	53	22	31		16	13	3	
Positive	74	48	26	0.009	62	48	14	0.519[b]
Depth of invasion								
T1	66	26	40		17	11	6	
T2	35	23	12		29	22	7	
T3+T4	26	21	5	$5.26x10^{-4}$	30	23	9	0.719
Clinical stage								
Early	84	36	48		28	21	7	
Advanced	43	34	9	$1.03x10^{-4}$	50	35	15	0.638

[a]P<0.05 was considered statistically significant; [b]Fisher's test, the other data, χ^2 (Chi-square) test.

epithelial cells of fundic glands in the lamina propria of the mucosa layer were inclined to be positive for Cx26 protein (Fig. 1A), mainly granular intercellular staining (membranous staining). In mild or moderate dysplasia glands, Cx26 protein was expressed in both cell membrane and cytoplasm (Fig. 1B). In lesions with severe dysplasia, mainly diffuse or occasionally granular cytoplasmic staining was clearly observed (Fig. 1C).

Cx26 expression in gastric carcinomas by immunohistochemistry and its correlations with clinicopathological features. We systematically analyzed Cx26 expression in 205 primary gastric carcinomas, and 79 cases (38.5%) were positive for Cx26 protein. In gastric carcinoma cells, Cx26 immunoreactivity mainly revealed cytoplasmic localization, with diffuse or occasionally a granular immunostaining pattern. Representative Cx26 positive immunostaining is shown in Fig. 1D-F. However, intercellular staining pattern (cell membranous staining), was sporadically and focally seen in some tumor sections.

Between two Cx26 expression groups (positive and negative groups), we found no significant differences in distribution according to age and sex. However, we did observe significant negative correlations of Cx26 expression with tumor invasion depth (T) (P<0.001), lymph node metastasis (N) (P=0.001), pathologic stage (P<0.001), lymphatic invasion (P=0.004), venous invasion (P=0.004), and Cx26 positive expression was observed more frequently in intestinal-type gastric carcinomas (IT-GC) than in diffuse-types (DT-GC) (P=0.017) (Table I).

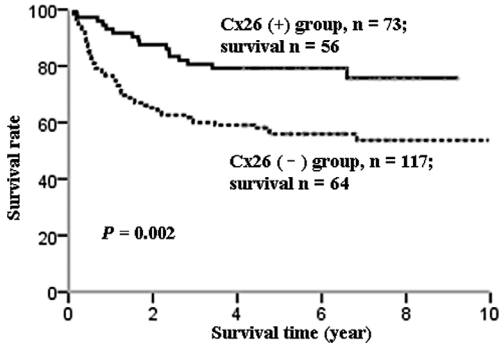

Figure 2. Survival rate of Cx26 expression group data (n=190). Cx26 expression group data were analyzed by Kaplan-Meier analysis and log-rank test, a negative association between the Cx26-positive group and the decreased survival rate of GC (P=0.002).

Moreover, 56 of 73 cases exhibiting Cx26 positive-staining were survivors (76.7%), whereas 64 of 117 cases exhibiting Cx26 negative-staining were survivors (54.7%) (Table I). Kaplan-Meier survival analysis of 190 cases revealed a negative correlation between Cx26 positive expression and shorter disease-specific survival times (P=0.002) (Fig. 2).

Table III. Comparison of Cx26 expression between IT-GC and DT-GC.

Pathologic stages		Cx26(-) (cases)	Cx26(+) (cases)	P-value[a]
Early stage	IT-GC	31	43	
	DT-GC	19	6	0.003
Advanced stage	IT-GC	33	9	
	DT-GC	34	15	0.322

[a]P<0.05 was considered statistically significant.

Table IV. Comparison of Cx26 expression in lymph node metastasis sub-groups.

Lymphatic sub-types		mCx26(-) (cases)	Cx26(+) (cases)	P-value[a]
Lymph node-positive	IT-GC	33	10	
	DT-GC	31	14	0.408
Lymph node-negative	IT-GC	31	42	
	DT-GC	22	7	0.002

[a]P<0.05 was considered statistically significant.

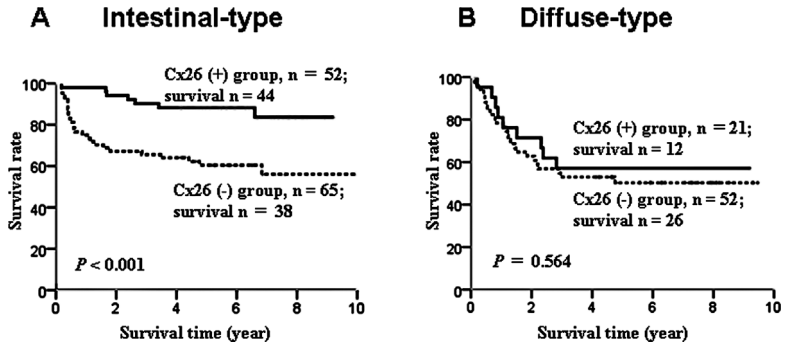

Figure 3. Survival rate of Cx26 expression groups in IT-GC and DT-GC separately. A. IT-GC, Cx26-positive group showed a higher survival rate compared to Cx26-negative group. B. DT-GC, two Cx26 expression groups showed no difference in survival rate.

Cx26 expression in IT-GC and DT-GC by immunohistochemistry. Cx26 positive staining was observed more frequently in IT-GC than in DT-GC [Table I; 57/127 cases (44.9%) vs. 22/78 cases (28.2%); P=0.017]. Furthermore, similar correlations of Cx26 expression with clinicopathological features observed in all 205 gastric cancers were detected also in IT-GC (all P<0.05), but not in DT-GC (Table II). A significant difference was found in the timing of Cx26 positive (cytoplasmic) expression in the two types. Cx26 positive occurred often in the early stage of IT-GC, but not in that of DT-GC (P=0.003; Table III). In contrast, it decreased progressively as tumor progressed to an advanced stage in both IT-GC and DT-GC (P=0.322; Table III).

Similarly, a significant difference was also found in Lymph node metastasis-negative subgroup (N-negative group) of Cx26 positive expression in two types. Cx26 positivity occurred often in N-negative group of IT-GC, but not in that of DT-GC (P=0.002; Table IV).

Next, the relationships of Cx26 positive expression with the disease-specific survival rates were analyzed separately in IT-GC and DT-GC. In IT-GC, the disease-specific survival rate of patients with Cx26-positive expression was significantly higher than that of those with Cx26-negative (84.6% compared to 58.4%; P<0.001, Fig. 3). However, no significant difference of disease-specific survival rate was found between the Cx26-positive and -negative group for DT-GC (P=0.564).

Western blot analysis and RNA level analysis of Cx26 in gastric carcinomas. Western blot analysis from eight gastric cancer cases (4 positive and 4 negative cases for Cx26 expression) showed that level of Cx26 protein was well-correlated with the Cx26 expression as detected by immunohistochemistry (Fig. 4A).

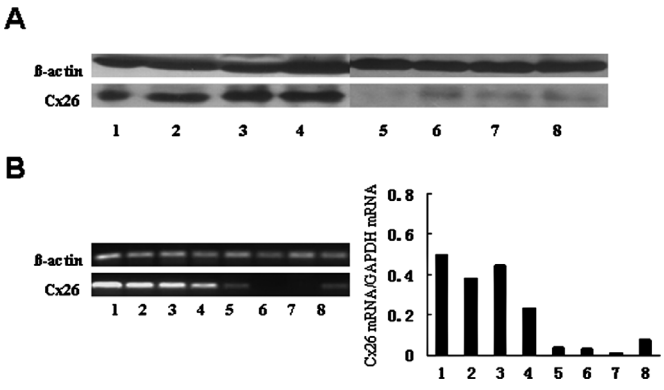

Figure 4. Cx26 expression in gastric carcinoma tissues. A. Cx26 protein expression by Western blotting; B. Cx26 mRNA expression by real-time PCR; cases 1-8, eight different specimens of GC tissues; cases 1-4 from Cx26-positive group and cases 5-8 from Cx26-negative group.

The 8 tumor tissues mentioned above were subjected to quantitative reverse transcription real-time PCR analysis of Cx26 RNA levels. We confirmed that the tumor samples expressing high levels of Cx26 protein generally showed high levels of Cx26 RNA, whereas samples with low Cx26 protein levels exhibited low levels of Cx26 RNA (Fig. 4B).

Univariate and multivariate survival analyses. Using the log-rank test, disease-specific survival time of patients with gastric

Table V. Univariate and multivariate analysis in overall cases (n=190).

Features	Univariate	Multivariate		
	P-value[a]	HR[b]	P-value	95% CI[c]
Age	0.482	1.559	0.091	0.931-2.611
Sex	0.141	1.196	0.496	0.715-2.001
Lauren's histological type	0.007	1.088	0.736	0.666-1.778
Depth of invasion	$4.28x10^{-19}$	1.702	0.015	1.108-2.613
Lymph node	$1.45x10^{-14}$	1.091	0.858	0.419-2.842
Lymphatic invasion	$5.89x10^{-10}$	1.847	0.179	0.756-4.516
Venous invasion	$9.96x10^{-7}$	0.809	0.51	0.430-1.521
Clinical stage	$8.12x10^{-18}$	1.892	0.027	1.181-2.734
Cx26	0.002	0.645	0.123	0.363-1.128

[a]P<0.05 was considered statistically significant; [b]hazard ratio; [c]confidence interval.

Table VI. Univariate and multivariate analysis in IT-GC (n=117).

Features	Univariate	Multivariate		
	P-value[a]	HR[b]	P-value[a]	95%CI[c]
Age	0.449	2.187	0.073	0.929-3.148
Sex	0.25	1.626	0.232	0.732-2.612
Depth of invasion	$9.67x10^{-14}$	1.467	0.017	1.158-2.436
Lymph node	$5.19x10^{-9}$	1.937	0.062	0.947-2.736
Lymphatic invasion	0.001	1.025	0.936	0.566-1.856
Vessel invasion	$8.31x10^{-5}$	1.190	0.582	0.640-2.212
Stage	$1.86x10^{-9}$	0.886	0.696	0.482-1.628
Cx26	$1.04x10^{-4}$	2.019	0.023	1.358-2.936

[a]P<0.05 was considered statistically significant; [b]hazard ratio; [c]confidence interval.

carcinoma having Cx26 positive-expression was longer than that of patients with Cx26 negative-expression tumors (P=0.002) (Fig. 2).

Univariate survival analysis revealed that in addition to Cx26, depth of invasion, lymph node metastasis, venous invasion, lymphatic invasion and the pathological stage were significantly associated with disease-specific survival, but age and sex did not have a statistically significant effect on survival. The results of univariate survival analysis are shown in Table V.

Additionally, in order to get a more precise combined analysis of all the factors and control confounding factors more effectively, all factors in univariate analyses were entered in a Cox proportional hazards model for multivariate survival analysis. When the effect of covariates was adjusted, only depth of invasion (T) (P=0.015) and clinical stage (P=0.027) could independently influenced the probability of prognosis overall in 190 cases (Table V).

Furthermore, intestinal-type group in which Cx26-positive expression has a significant effect on disease-specific survival, we adjusted the covariates, including Cx26 expression in another Cox proportional hazards model, and investigated that Cx26 was a significantly independent prognostic predictor in IT-GC (P=0.023). In addition, tumor invasion depth (T) (P=0.017) also independently influenced the probability of prognosis (Table VI).

Discussion

Cx26 has been shown to be induced in some gastrointestinal malignancies, including squamous esophageal and pancreatic cancers (24,25). In our study, we systematically analyzed Cx26 expression in 205 primary gastric carcinomas for the first time, and evaluated its role in predicting patients' prognosis for patients with gastric carcinoma. Our data showed that cytoplastic expression of Cx26 protein plays an important role, and is an independent biomarker for favorable prognosis in intestinal-type gastric carcinomas.

We found that in the normal gastric epithelium, Cx26 protein was mainly located on the cellular membrane, which represents the normal functional gap junction (20). On the contrary, in gastric cancer cells, we found that Cx26 protein was mainly located in the cellular cytoplasm, as previously reported in colorectal and human breast cancers (20,26). We observed that an increase of Cx26 protein expression in cellular cytoplasm of the gastric cancer cells are in contrast to the noncarcinoma gastric epithelium. These results indicate a

possible involvement of Cx26 protein expression on the cellular cytoplasm in the carcinogenesis of GC. In a parallel study, Kanczuga-Koda and colleagues analyzed Cx26 expression during colorectal carcinogenesis and found cytoplasmic Cx26 staining in carcinomas, consistent with our results (14). The altered form of Cx26 was possibly due to transcriptional or post-transcriptional defects of this protein (14). With real-time PCR and Western blotting, we confirmed that the abnormal amplification of Cx26 occurred in gastric carcinomas, which has not been detected before. This suggests that a large amount of Cx26 is concentrated in the endoplasmic reticulum and Golgi apparatus where Cx26 synthesis occurs to perform their physiological functions. The synthesis of another connexin member, Cx43 in astrocytes, has been shown to be directly stimulated by IGF-I (27), and cytoplasmic Cx43 is a result of Cx43 induced by IGF-I in lens epithelial cells without assembled GJ plaques (28). Such modification (phosphorylation) may also be applicable to Cx26 in gastric cancer. The IGF-I system, including IGF-I, IGF-I receptor (IGF-IR), and IGF binding proteins (IGFBPs) may be involved in changes of Cx26 expression in gastric cancer. Meanwhile, we observed correlation of Cx26 with IGF-IR expression in gastric carcinomas by immunohistochemistry, and the results revealed that there is a positive correlation between expression of both proteins (data not shown), which might explain our hypothesis, however, further investigations are required.

We observed in this study negative significant associations between Cx26 expression and tumor invasion depth (T), lymph node metastasis (N), clinical stage, lymphatic invasion, and venous invasion. Our results revealed that Cx26 protein expression seems an early event in malignant transformation and is associated with a biologically less aggressive phenotype, and these data suggest that this protein plays a tumor-suppressor role in gastric carcinomas. It has been observed that Cx26 is often up-regulated in hyperplastic tissues including benign prostatic hyperplasia and mouse skin hyperplasia and neoplasia (29,30). Presently, we observed that Cx26 was also highly expressed in lesions with severe dysplasia of gastric mucosa (Fig. 1C). In this study, the cytoplasmatically localized Cx26 specifically perform a tumor-suppressor role in gastric cancer, which may be independent of GJIC. Olbina *et al* showed that mutations of Cx43 inhibited localization to the plasma membrane but do not decrease the ability of Cx43 to suppress tumor cell growth *in vitro* (31). It was confirmed that the control of cell growth by Cxs does not always require GJIC. Besides the tumor-suppressor role, cytoplasmic Cx26 might be drawn to modulate certain signal pathways and abnormally affect the cell growth and apoptosis as well (32). Our recent study revealed that MGC-803 gastric cancer cells (low expression of Cx26) transfected with Cx26 have shown increased synthesis of both proapoptotic Bax and antiapoptotic Bcl-xL (data not shown). Such dual opposing effects of Cx26 on cell apoptosis had been reported in colorectal cancer by Kanczuga-Koda (32). These data suggested the possibility that regulation of apoptosis by Cx26 could be a result of a control of the ratio between pro- and anti-apoptotic proteins.

In addition, positive staining for Cx26 in gastric carcinoma was characteristic, especially in the tumors histologically classified as IT-GC. The higher Cx26 expression was significantly correlated with IT-GC compared with DT-GC. Interestingly, similar significant correlations of Cx26 expression with clinicopathological features (including survival analysis) observed in all 205 gastric carcinomas were detected also in IT-GC, but not in DT-GC. It has been well known that these two subtypes of gastric carcinoma are of different origin and undertake different molecular changes in carcinogenesis (21). Thus Cx26 may play a different role in tumor carcinogenesis and progression between these two histological subtypes of gastric carcinoma.

Our results show that cytoplasmic expression of Cx26 in human gastric cancer was negatively correlated with lymph node metastasis, lymphatic and venous invasion. These results seemed opposite to those which suggested a metastasis-promoting function of Cx26 in several forms of cancer, including lung and breast (17,19). The above findings suggest that Cx26 has a specific role that facilitates invasion and metastasis of tumor cells through heterologous gap junction formation with surrounding endothelial cells. Competent gap junction through membranous Cx26 was a necessity for its metastasis-promoting function (15,18,19). Considering the absence of Cx26 protein in plasma membrane of cancer cells in our study, we supposed that metastatic-promoting heterotypic or homoeotypic GJs fail to form in gastric carcinoma, thus metastasis-promoting function of Cx26 was not observed in this form of cancer. Alternatively, Cx26 expression pattern in metastatic lesions was not detected in our study. Ito *et al* investigated Cx26 expression in lung cancer and found that in metastatic lesions its expression corresponded with that in primary lesions (19). To explore whether Cx26 enhances invasive ability in gastric cancer, further detections of regional and even distant metastatic lesion are still needed.

In conclusion, the present study analyzed for the first time the abberant presence of Cx26 in human gastric carcinoma. Our results suggest that cytoplasm-localized Cx26 protein plays a tumor-suppressor role and is associated with biologically less aggressive phenotype and pathologic early stage of gastric carcinoma, especially in IT-GC. Moreover, Cx26 protein expression in cytoplasm is an independent biomarker for favorable prognosis in IT-GC.

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