Myofibroblasts are associated with the progression of scirrhous gastric carcinoma

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Received April 8, 2010; Accepted May 31, 2010

DOI: 10.3892/etm_00000086

Abstract. Fibroblasts, particularly myofibroblasts, affect the malignant progression of cancer cells *in vitro*. However, to date few reports have addressed the clinical significance of myofibroblasts in the gastric cancer microenvironment. This study examined the correlation between myofibroblast expression and clinicopathological features in gastric carcinoma. A total of 265 primary gastric tumors resected by gastrectomy were stained with antibodies against α-smooth muscle actin and vimentin. Stromal cells positive for vimentin were considered to be fibroblasts. Myofibroblasts were defined as fibroblasts positive for α-smooth muscle staining. Myofibroblast-positive gastric carcinoma was established when myofibroblasts accounted for more than 25% of fibroblasts in the cancer stroma. Myofibroblast expression was positive in 92 (35%) of the 265 gastric carcinomas. Myofibroblast expression showed a significantly ($p<0.001$) high frequency in advanced gastric cancers (76 of 146), in comparison to the early stage cancers (16 of 119). Taken together, there was a statistically significant correlation between myofibroblast expression and scirrhous type gastric cancer ($p<0.001$), lymph node metastasis ($p<0.001$), lymphatic invasion ($p<0.001$) and peritoneal dissemination ($p=0.005$). The prognosis of patients with tumors positive for myofibroblast expression was significantly ($p<0.001$) worse, while a multivariate analysis revealed that myofibroblast expression was not an independent prognostic factor. These findings suggest that myofibroblasts are associated with scirrhous gastric cancer. Overexpression of myofibroblasts may therefore be a useful prognostic indicator of gastric carcinoma.

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Key words: myofibroblasts, scirrhous gastric carcinoma

Introduction

There is increasing evidence that the stroma is involved in the growth and metastasis of several types of cancer, including gastric (1), breast (2,3) and prostate (4) cancer. The stroma consists of a variety of components, including fibroblasts, macrophages, and the extracellular matrix (5,6). Among these cells, fibroblasts constitute a major stromal compartment and play a critical role in the regulation of tumor growth. Among the various types of fibroblasts, myofibroblasts, which are distinct from normal fibroblasts in their expression of both vimentin and α-smooth muscle actin (α-SMA), have recently been implicated to have important functions in epithelial solid tumor biology, such as neoplastic progression, tumor growth and metastasis *in vitro* (7-9). De Wever *et al* emphasized the myofibroblast as a driver of invasive cancer growth; the role of myofibroblasts in tissue development was also proposed (10).

Scirrhous gastric cancer cells proliferate and extensively invade the submucosa in the gastric submucosa accompanied by abundant fibrosis (11). Interactions have been reported to exist between scirrhous gastric cancer cells and orthotopic fibroblasts, thus suggesting that the proliferation of scirrhous gastric carcinoma is related to growth factor production by gastric fibroblasts (12). Myofibroblasts have also been shown to be present in gastric cancer, but their histogenesis remains unclear. Despite increasing reports illustrating the role of tumor-stroma in cancer progression, no consensus has been reached regarding whether myofibroblasts regulate tumor development positively or negatively. Few reports of clinical studies of scirrhous gastric cancer discuss the significance of myofibroblasts. Therefore, the present study was performed to investigate the significance of myofibroblast expression in gastric carcinomas.

Materials and methods

Clinical materials. A total of 265 patients who had undergone resection of a primary gastric tumor at our institute were enrolled in this study. Tumor specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Sections (4-μm) were cut and mounted on glass slides. The pathologic diagnoses and classifications were made according to the

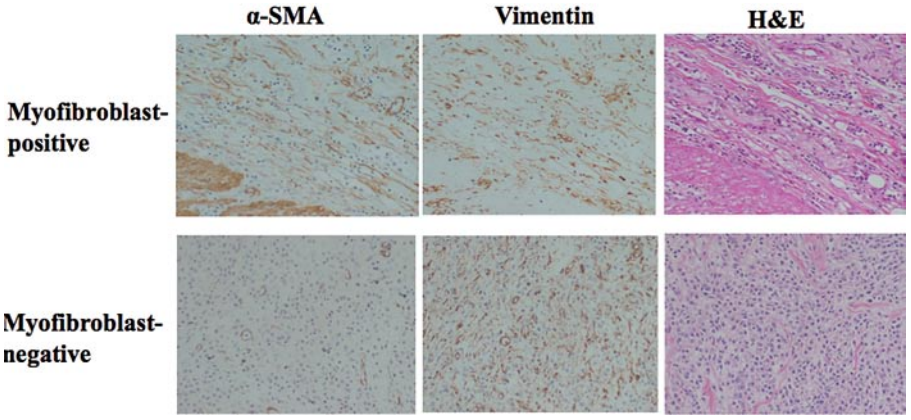

Figure 1. Myofibroblast expression in stromal cells. Expression of α-smooth muscle actin was observed in the stroma in a diffuse-type carcinoma in original magnification, x200. Expression of vimentin was observed at the stroma.

Japanese Classification of Gastric Carcinoma (13). The median follow-up time for all 265 patients was 58 months (range, 1-177 months). The median follow-up time for the patients that succumbed to the disease was 25 months (n=88) compared with 75 months for surviving patients (n=177). Thirty-one patients were lost during more than 60 months of follow-up. Kaplan-Meier overall survival curves were calculated from the date of surgery.

Antibodies and reagents. A mouse monoclonal antibody which recognizes α-SMA (clone 1A4) and a mouse monoclonal antibody which recognizes vimentin (clone Vim 3B4) were purchased from DakoCytomation (Cambridge, UK). Normal rabbit serum, normal mouse immunoglobulin G, biotinylated rabbit anti-mouse immunoglobulin G, streptavidin-peroxidase reagent and diaminobenzidine were purchased from Nichirei Corp. (Tokyo, Japan).

Immunohistochemical techniques. Since there is no myofibroblast-specific immunocytochemical marker, characterization of human tumor-associated myofibroblasts is based on a combination of positive markers such as vimentin and α-SMA. The methods for the immunohistochemical determination of α-SMA and vimentin are described in detail in the manufacturer's instructions. Briefly, the slides were deparaffinized in xylene and hydrated in decreasing concentrations of ethyl alcohol. The tissues were heated for 20 min at 105˚C and at 0.4 kg/cm^2 by autoclave in Target Retrieval Solution (Dako Co., Carpinteria, CA). The sections were then dewaxed and incubated with 3% hydrogen peroxide v/v in methanol for 15 min to block endogenous peroxidase activity. Next, the sections were washed in phosphate-buffered saline (PBS) and incubated in 10% normal rabbit serum v/v for 10 min to reduce non-specific antibody binding. The specimens were incubated with α-SMA antibodies (1:200) or vimentin antibodies (1:200) for 1 h at room temperature followed by three washes with PBS. Sections were incubated with biotinylated rabbit anti-mouse immunoglobulin G for 30 min, followed by three washes with PBS. Slides were treated with streptavidin-peroxidase reagent for 15 min and washed with PBS three times. Finally, the slides were incubated in PBS diaminobenzidine and 1% hydrogen peroxide v/v for 20 sec, counterstained with Mayer's hematoxylin and mounted.

Immunohistochemical determination of α-smooth muscle actin and vimentin. The tumor specimens showed various staining patterns against the anti-α-SMA and anti-vimentin antibodies. Vimentin-positive stromal cells were considered to be fibroblasts. Myofibroblasts were defined as fibroblasts which were positive for α-SMA staining. Smooth muscle was defined as being α-SMA-positive and vimentin-negative. The myofibroblast expression level was semi-quantitatively analyzed according to the percentage of fibroblasts showing α-SMA positivity: 0, 0%; 1+, 1-24%; 2+, 25-49%; 3+, ≥50%. Myofibroblast expression was considered positive when scores were ≥2+, and negative when scores were ≤1+ (Fig. 1). The slides were interpreted by two investigators without knowledge of the corresponding clinicopathological data.

Statistical analysis. The χ^2 test was used to determine the significance of the differences between the covariates. Survival durations were calculated using the Kaplan-Meier method and were analyzed by the log-rank test to compare the cumulative survival durations in the patient groups. The Cox proportional hazards model was used to compute univariate and multivariate hazards ratios for the study parameters. For all tests, a p-value <0.05 was defined as statistically significant. The SPSS software program (SPSS Japan, Tokyo, Japan) was used for the analyses.

Results

Correlation between the clinicopathological factors and myofibroblast expession. Myofibroblast expression was positive in 92 (35%) of the 265 gastric carcinoma specimens, in 16 (13%) of the 119 early stage gastric carcinoma specimens, and in 76 (52%) of the 146 advanced gastric carcinoma specimens. The relationships between myofibroblast positivity and clinicopathological features of the tumors are shown in Table I. A significantly (p<0.001) high frequency of myofibroblast positivity was observed in the advanced gastric cancers (76 of 146) in comparison to the early stage cancers (16 of 119). Therefore, a statistically significant

Table I. Correlation between clinicopathological factors and myofibroblast expression.

Clinicopathological factors	Myofibroblast expression[a] Positive n=92 (35%)	Negative n=173 (65%)	p-value
Invasion depth			
Early stage cancer	16 (13%)	103 (87%)	
Advanced cancer	76 (52%)	70 (48%)	<0.001
Macroscopic type[b]			
Type 0, 1, 2, 3	65 (29%)	157 (71%)	
Type 4 (scirrhous type)	27 (63%)	16 (37%)	<0.001
Histological type			
Differentiated	34 (30%)	79 (70%)	
Undifferentiated	58 (38%)	94 (62%)	0.172[c]
Peritoneal metastasis			
Positive	12 (57%)	9 (43%)	
Negative	80 (33%)	164 (67%)	0.024
Venous invasion			
Positive	25 (45%)	31 (55%)	
Negative	67 (32%)	142 (68%)	0.079
Lymph node metastasis			
Positive	57 (51%)	54 (49%)	
Negative	35 (23%)	119 (77%)	<0.001
Lymphatic invasion			
Positive	56 (48%)	60 (52%)	
Negative	36 (24%)	113 (76%)	<0.001
Cytology[c]			
Positive	19 (56%)	15 (44%)	
Negative	73 (32%)	158 (68%)	0.005

[a]Myofibroblasts were defined as fibroblasts positive for α-smooth muscle staining. [b]Classification was according to the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer. Type 0 is defined as superficial flat tumors with or without minimal elevation or depression. Type 1 is defined as a polypoid tumor, sharply demarcated from the surrounding mucosa and usually attached on a wide base. Type 2 is defined as polypoid tumor with ulceration and with sharply demarcated margins. Type 3 is defined as ulcerated carcinoma with cancer infiltration into the surrounding wall. Type 4 is defined as diffusely infiltrating flat carcinoma in which ulceration is usually not a marked feature. [c]'Cytology' is defined as peritoneal lavage cytology at laparotomy as a standard method for the detection of free tumor cells.

correlation was found between myofibroblast expression and scirrhous type gastric cancer (p<0.001). Myofibroblast positivity was also significantly present in patients with lymph node metastasis (p<0.001), positive cytology (p=0.005) and lymphatic invasion (p<0.001). 'Cytology' is defined as peritoneal lavage cytology at laparotomy as a standard method for the detection of free tumor cells. There was no statistically significant association between myofibroblast positivity and histological type, peritoneal dissemination and venous invasion.

Table II. Univariate analysis with respect to overall survival in gastric cancer.

Parameter	Risk ratio	95% CI	p-value
Myofibroblast expression Positive vs. negative	1.482	1.146-1.917	0.003
Invasion depth Early stage vs. advanced cancer	1.692	1.323-2.163	<0.001
Macroscopic type Type 1, 2, 3 vs. Type 4 (scirrhous type)	3.366	2.389-4.742	<0.001
Histological type Diffuse vs. intestinal	1.084	0.848-1.386	0.520
Peritoneal metastasis Positive vs. negative	6.462	3.926-10.638	<0.001
Cytology Positive vs. negative	4.417	3.002-6.501	<0.001
Venous invasion Positive vs. negative	1.363	1.011-1.838	0.042
Lymph node metastasis Positive vs. negative	1.911	1.490-2.450	<0.001
Lymphatic invasion Positive vs. negative	1.663	1.299-2.130	<0.001

CI, confidence interval.

Survival. The prognosis of patients with tumors positive for myofibroblast expression was significantly (p<0.001) worse than that of patients with tumors negative for myofibroblast expression (Fig. 2A). The 5-year survival of the patients with myofibroblast-positive tumors was 57% in comparison to 79% for those patients with negative tumors. The prognosis of patients who underwent a curative resection (R0) with myofibroblast-positive tumors was significantly (p<0.0210) worse than the prognosis of patients with tumors negative for myofibloblast expression (Fig. 2B). Univariate analysis revealed that myofibroblast expression (p=0.003), advanced cancer (p<0.001), peritoneal dissemination (p<0.001), venous invasion (p=0.042) and lymph node metastasis (p<0.001) were significantly correlated with patient survival (Table II). Multivariate analysis indicated that the macroscopic type and peritoneal dissemination were independent prognostic factors (Table III), while myofibroblast expression (p=0.290) was not an independent prognostic factor.

Discussion

The present study demonstrated that myofibroblast expression was significantly associated with advanced stage diffuse-type gastric cancer, particularly scirrhous type and distant metastasis. In the present study, 43 cases of Type 4 showed diffusely infiltrating carcinoma accompanied by extensive

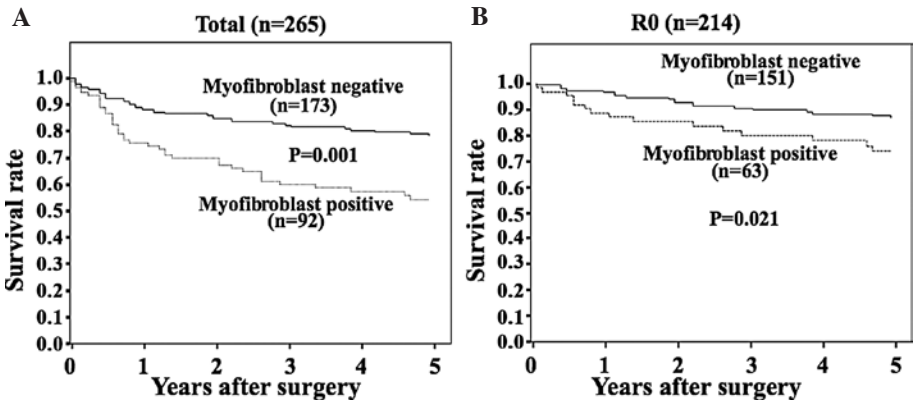

Figure 2. Patient survival. (A) Overall survival of the studied patients based on myofibroblast expression. (B) Overall survival of the patients with curative resection (R0) based on myofibroblast expression.

Table III. Multivariate analysis with respect to overall survival in gastric cancer.

Parameter	Risk ratio	95% CI	p-value
Myofibroblast expression			
Positive vs. negative	1.076	0.664-1.744	0.766
Invasion depth			
Early stage vs. advanced cancer	3.366	1.401-8.088	0.007
Macroscopic type			
Type 1, 2, 3 vs. Type 4 (scirrhous type)	4.344	2.136-8.838	<0.001
Histological type			
Diffuse vs. intestinal	1.118	0.586-2.123	0.735
Peritoneal metastasis			
Positive vs. negative	2.184	1.162-4.105	0.015
Venous invasion			
Positive vs. negative	1.543	0.869-2.741	0.139
Lymph node metastasis			
Positive vs. negative	1.308	0.675-2.533	0.426

CI, confidence interval.

stromal fibroblasts, which is distinguished as scirrhous gastric carcinoma (11). This type of carcinoma accounts for approximately 10% of all gastric carcinomas, and patients presenting with this type are associated with a poorer prognosis in comparison to other types of gastric carcinomas, thus reflecting a rapid proliferation of cancer cells (14). Interactions between scirrhous gastric cancer cells and orthotopic fibroblasts have been previously reported (12). Myofibroblasts, among orthotopic stromal cells, might thus play an important role in cancer progression in the development of scirrhous type gastric cancer. Orimo *et al* found that myofibroblasts exhibited significant positive signals of various growth factors in breast cancer (9,15). Our previous study indicated that keratinocyte growth factor, transforming growth factor-β and hepatocyte growth factor secreted by human gastric fibroblasts might stimulate proliferation and invasion of human scirrhous gastric carcinoma cells (16,17). Myofibroblasts are thought to accelerate the aggressive phenotype of scirrhous gastric carcinoma via these growth factors.

The prognosis of patients with myofibroblast-positive tumors such as colorectal cancer (18) was reported to be significantly worse than the prognosis of patients with myofibroblast-negative tumors, thus suggesting that myofibroblast expression is indicative of high malignancy and might also be associated with diffusely invasive growth and poor prognosis. Overexpression of myofibroblasts might be a useful prognostic indicator, while multivariate analysis revealed that myofibroblast expression was not an independent prognostic factor.

Differentiation from resident stromal fibroblasts into myofibroblasts is induced by paracrine signals generated by repairing or inflamed tissues. Among these signals, TGF-β is a well-known inducer of myofibroblasts which stimulates fibroblasts to differentiate into myofibroblasts in cancer tissues (2,19,20). Overexpression of TGF-ß is reported to accelerate metastasis and is thus correlated with the poor prognosis of gastric tumors, particularly for scirrhous gastric carcinoma (21,22). Moreover, TGF-β is produced to a greater extent by most scirrhous gastric cancer cells than by other types of gastric cancer cells (23). These findings suggest that myofibroblasts induced by TGF-β from scirrhous gastric cancer cells might thus be responsible for the poor prognosis observed for scirrhous gastric cancer.

In conclusion, myofibroblasts in the microenvironment of gastric cancer were found to be significantly associated with an advanced stage, particularly for the macroscopically scirrhous type gastric carcinoma. Overexpression of myofibroblasts may therefore be a useful prognostic indicator for such cases.

Acknowledgements

We thank Masako Shinkawa (Osaka City University Graduate School of Medicine) for the technical advice on the immunohistochemical staining. This study was supported, in part, by Grants-in-Aid for Scientific Research (nos. 18591475, 20591073 and 18390369) from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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