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Prognostic significance of connective tissue growth factor expression in stromal cells in patients with diffuse-type gastric cancer

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Abstract. Connective tissue growth factor (CTGF) is a target gene of the Hippo signaling pathway. Its differential role in the histological types of gastric cancer (GC) remains unknown; therefore, the present study aimed to confirm the clinical significance of CTGF expression in cancer and stromal cells in patients with GC depending on the histological type. The present study enrolled 589 patients with GC. Immunohistochemistry was used to analyze CTGF expression in cancer and stromal cells. CTGF mRNA expression data and the corresponding clinical information of GC samples were collected from The Cancer Genome Atlas (TCGA) database. Subsequently, the associations between CTGF expression and several clinicopathological factors were investigated. In the present study, CTGF expression was mainly observed in the cytoplasm of cancer and stromal cells. CTGF expression in stromal cells was significantly associated with CTGF expression in cancer cells (P<0.001). CTGF positivity in stromal cells was also significantly associated with intestinal type, non-scirrhous type, tumor depth (T1-2), lymph node metastasis (negative), lymphatic invasion (negative) and tumor size (<5 cm). Low CTGF expression in stromal cells was independently associated with worse overall survival (OS). Furthermore, the OS of patients with low CTGF expression in stromal cells, especially in patients with diffuse-type GC, was significantly worse than patients with high CTGF expression (P=0.022). This trend was similar to that revealed by TCGA data analysis. In conclusion, low CTGF expression was associated with a significantly worse OS in patients with diffuse-type GC. These data indicated that CTGF, and its control by the Hippo pathway, may be considered potential treatment targets in diffuse-type GC.

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Key words: connective tissue growth factor, gastric cancer, diffuse type

Introduction

Gastric cancer (GC) is one of the most prevalent cancers, and it still accounts for over one million new cases worldwide (1). Several new treatments, including immune checkpoint inhibitors, have improved the survival outcomes of GC (2-4). However, the prognosis of patients with advanced GC, especially stage IV, remains dismal, and the underlying molecular mechanism of its progression should be uncovered for treating these patients.

Deregulation of the Hippo pathway has been reported in different cancer types, and the role of the Hippo signaling pathway is attracting attention in terms of cancer progression (5). The Hippo pathway is an evolutionally conserved regulator of tissue growth and comprises a kinase cassette (MST and LATS). LATS phosphorylates Yes-associated protein (YAP), which is the main effector in this pathway. Unphosphorylated YAP enters the nucleus and promotes tissue growth and cell viability by regulating the activity of different transcription factors. One of the targeted genes of this transcription regulation is connective tissue growth factor (CTGF) (6).

CTGF is a secretory protein that belongs to the CCN family, consisting of six members: CTGF, nephroblastoma overexpressed (NOV), cysteine-rich angiogenic protein 61 (CYR61), WNT1-inducible signaling pathway protein 1 (WISP1), and WISP2. CCN proteins are biologically active when binding and/or activating cell surface integrins, and they are related to cell proliferation, migration, adhesion, and extracellular matrix formation in tumor tissues (7). CTGF in breast cancer improves the motility of cancer cells via an integrin-αvβ3-ERK1/2-dependent S100A4-upregulated pathway (8). Conversely, CTGF is a favorable prognostic factor in GC because it inhibits peritoneal metastasis by blocking integrinα3β1 dependent adhesion (9). These data indicate the variable functions of CTGF in different cancer types.

This report focused on the prognostic significance of CTGF in GC. GC has been histologically classified into intestinal and diffuse types by Lauren (10), and diffuse-type GC usually has a lot of stromal components in the tumor tissue (11-13). Generally, the interaction between cancer and stromal cells is crucial for the progression of diffuse-type GC (14,15). However, Chen *et al* (9) did not pay attention to the difference in histological type. Furthermore, the differential role of CTGF expression in cancer and stromal cells remains unknown. Thus, this study aimed to clarify the clinical significance of CTGF expression in cancer and stromal cells in patients with GC, depending on histological type, using the Lauren classification.

Patients and methods

Patients. A total of 589 patients who underwent resection of primary GC from January 2000 to December 2006 at Department of Gastroenterological Surgery, Osaka City University (currently, Osaka Metropolitan University; Osaka, Japan) were retrospectively reviewed. The age range of the recruited patients was 21-88 years old, and median age was 67. We generated tissue microarrays (TMA) from these patients and used them for immunohistochemical (IHC) staining. Each TMA core was selected at the invasion front of the cancer. The pathologic diagnoses and classifications were made according to the Japanese Classification of Gastric Carcinoma, fifteenth edition. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. The Osaka Metropolitan University ethics committee approved this study in 2022 [approval number (approval date): 2022-077 (2022/08/10)]. Informed consent was obtained in the form of opt-out.

IHC determination of the CTGF. IHC staining was performed using 589 GC samples. Slides were deparaffinized and then heated for 10 min at 105˚C in an autoclave in Target Retrieval Solution (Dako, Carpinteria, CA). After blocking endogenous peroxidase activity using 3% hydrogen peroxide, the specimens were incubated with CTGF antibody (1:200; Life Technologies) for 1 h at room temperature and were incubated with biotinylated goat antirabbit IgG for 10 min. The slides were treated with streptavidin–peroxidase reagent, followed by counterstaining with Mayer's hematoxylin. Slides were scanned using a Leica Aperio CS2 scanner (Leica Biosystems), and subsequent expression evaluations at each core were performed using the open-source software QuPath (http://qupathe.github.io). Cell detection and IHC quantification were performed using 30 cores (Fig. 1). Furthermore, an objective classifier was trained to classify individual cells as stromal or epithelial cells. After these trainings, CTGF expression in all TMA cores was assessed in every tumor and stromal cell. The percentage of positive cells among total tumor and stromal cells was automatically calculated using the threshold value of 0.15. We diagnosed a case as CTGF positive when the positive percentage of tumor and stromal cells was more than the first quartile of all analyzed cases.

TCGA data. CTGF mRNA expression data and corresponding clinical information of GC samples were collected from The Cancer Genome Atlas (TCGA) database (http://tcga-data.nci.nih.gov/tcga/). We used data from the 'tcga_pan_cancer_atlas_2018' study. The third quartile of the mRNA expression level was defined as the cut-off, and we categorized patients into high- and low-expression groups.

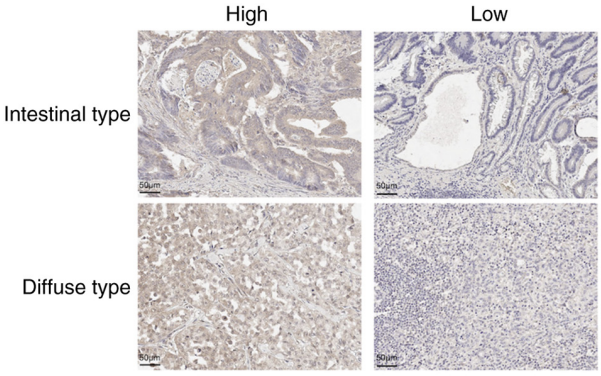

Figure 1. CTGF expression. Representative pictures of CTGF high and low expression depending on diffuse and intestinal type gastric cancer. CTGF was mainly expressed in the cytoplasm. CTGF, connective tissue growth factor.

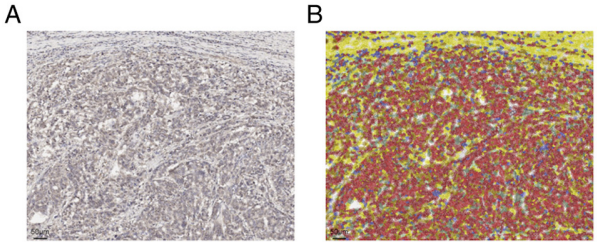

Figure 2. Connective tissue growth factor expression analysis by software. Sample images (A) before and (B) after QuPath analyses. (B) Red cells indicate positive cancer cells, while green cells indicate negative cancer cells. Blue cells indicate positive stromal cells, while yellow cells indicate negative stromal cells.

Statistical analysis. Associations between CTGF expression and clinicopathological results were analyzed using the chi-square test. Overall survival (OS) was the time from surgery to death from any cause. The Kaplan-Meier method and the log-rank test were used to estimate and compare the OS, respectively. Disease-free survival was the time from surgery to recurrence or death from any cause. Multivariate analysis was performed using the Cox proportional hazards model. Statistical Package for the Social Sciences statistical software (version 29.0; IBM) was used for all statistical analyses. Two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Association between CTGF expression and clinicopathological factors in GC. CTGF expression was mainly observed in the cytoplasm of cancer and stromal cells (Fig. 1). We investigated the CTGF expressions of cancer and stromal cells in 589 GC tissues using software (Fig. 2). Of 589 patients, 444 and 442 had CTGF-positive cancer and stromal cells, respectively.

Table I. Association between CTGF expression and clinicopathologic factors in 589 patients with gastric cancer.

	CTGF expression in tumor cells			CTGF expression in stromal cells		
Clinicopathological factors	Low (n=145)	High (n=444)	P-value	Low (n=147)	High (n=442)	P-value
Age, years			0.414			0.121
<70	94 (25.8%)	271 (74.2%)		99 (27.1%)	266 (72.9%)	
≥70	51 (22.8%)	173 (77.2%)		48 (21.4%)	176 (78.6%)	
Sex			0.034			0.058
Female	53 (20.4%)	207 (79.6%)		55 (21.2%)	205 (78.8%)	
Male	92 (28.0%)	237 (72.0%)		92 (28.0%)	237 (72.0%)	
Macroscopic type			0.154			0.003
0-3	126 (23.8%)	404 (76.2%)		123 (23.2%)	407 (76.8%)	
4 (scirrhous type)	19 (32.2%)	40 (67.8%)		24 (40.7%)	35 (59.3%)	
Histologic type			0.119			0.034
Intestinal	63 (21.8%)	226 (78.2%)		61 (21.1%)	228 (78.9%)	
Diffuse	82 (27.3%)	218 (72.7%)		86 (28.7%)	214 (71.3%)	
Tumor depth			0.013			<0.001
T1-2	71 (20.9%)	269 (79.1%)		65 (19.1%)	275 (80.9%)	
T3-4	74 (30.7%)	175 (69.3%)		82 (32.9%)	167 (67.1%)	
Lymph node metastasis			0.047			0.008
N0-1	88 (22.2%)	309 (77.8%)		86 (21.7%)	311 (78.3%)	
N2-3	57 (29.7%)	135 (70.3%)		61 (31.8%)	131 (68.2%)	
Lymphatic invasion[a]			0.071			0.014
Negative	55 (21.1%)	206 (78.9%)		52 (19.9%)	209 (80.1%)	
Positive	90 (27.5%)	237 (72.5%)		94 (28.7%)	233 (71.3%)	
Venous invasion			0.054			0.282
Negative	112 (23.0%)	374 (77.0%)		117 (24.1%)	369 (75.9%)	
Positive	33 (32.0%)	70 (68.0%)		30 (29.1%)	73 (70.9%)	
Tumor size[a]			0.001			0.002
<3 cm	38 (17.4%)	181 (82.6%)		39 (17.8%)	180 (82.2%)	
≥3 cm	107 (29.2%)	260 (70.8%)		107 (29.2%)	260 (70.8%)	
CTGF expression in stromal cells			<0.001			
Negative	79 (53.7%)	68 (46.6%)				
Positive	66 (14.9%)	376 (85.1%)				

[a]There are some missing data. CTGF, connective tissue growth factor.

Table I shows the association between CTGF expression and clinicopathological factors. CTGF expression in stromal cells was significantly associated with CTGF expression in cancer cells (P<0.001). CTGF positivity in cancer cells was significantly associated with sex (female), tumor depth (T1-2), lymph node metastasis (N0-1), and tumor size (<3 cm). CTGF positivity in stromal cells was significantly associated with intestinal type, non-scirrhous type, tumor depth (T1-2), lymph node metastasis (N0-1), lymphatic invasion (negative), and tumor size (<3 cm).

Multivariate analysis. Table II shows the univariate and multivariate analyses using the proportional hazards model. Univariate analysis revealed that the OS of patients was significantly associated with CTGF expression in stromal cells. Multivariate logistic regression analysis revealed that CTGF expression in stromal cells as well as age, tumor depth (T3-4), lymph node metastasis (N2–3), and tumor size (≥3 cm) were independent predictive parameters for OS.

Survival analysis of the subgroups. Fig. 3 shows the OS curves of patients by CTGF expression using the Kaplan-Meier method. The 5-year OS rates of patients with high and low CTGF expression in cancer cells were 69.3 and 67.6%, respectively. Regarding the analyses of cancer cells, OS outcomes between patients with high and low CTGF expression were not statistically significant (log-rank; P=0.684). Conversely, the analyses of stromal cells revealed that patients with low CTGF expression demonstrated significantly worse OS than those with high CTGF expression (log-rank; P=0.006). The 5-year

Table II. Univariate and multivariate analyses with respect to overall survival after surgery in 597 patients with gastric cancer.

	Univariate		Multivariate	
Variable	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
CTGF in tumor cells (High)	0.93 (0.65-1.32)	0.68	1.36 (0.91-2.02)	0.125
CTGF in stromal cells (High)	0.63 (0.45-0.87)	0.006	0.68 (0.47-0.99)	0.048
Age (≥70 years)	1.71 (1.25-2.34)	<0.001	1.76 (1.27-2.44)	<0.001
Sex (Male)	1.26 (0.91-1.75)	0.15	0.98 (0.70-1.36)	0.75
Lymph node metastasis (≥N2)	5.72 (4.11-7.97)	<0.001	3.36 (1.87-6.02)	<0.001
Distant metastasis (Positive)	6.06 (3.49-10.5)	<0.001	3.59 (2.49-5.17)	<0.001
Tumor size (≥3 cm)	5.68 (3.47-9.29)	<0.001	2.79 (1.63-4.78)	<0.001

CTGF, connective tissue growth factor.

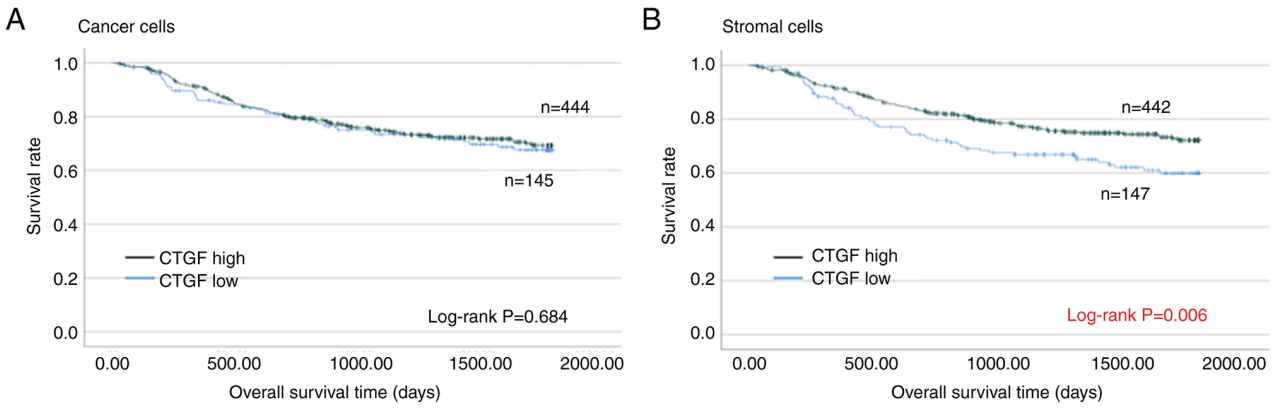

Figure 3. Survival of patients with gastric cancer depending on CTGF expression (immunohistochemistry). (A) Survival of gastric cancer patients depending on CTGF expression in cancer cells. (B) Survival of gastric cancer patients depending on CTGF expression in stromal cells. CTGF, connective tissue growth factor.

OS rates of patients with high and low CTGF expression in stromal cells were 72.1 and 59.8%, respectively.

Fig. 4 shows the analyses for each histological type. The OS rates in terms of the intestinal case were not significantly different depending on CTGF expression in cancer and stromal cells (P=0.183 and P=0.230, respectively). In contrast, among the cases with diffuse type, the OS of patients with low CTGF expression in stromal cells was significantly worse than that of patients with high CTGF expression (P=0.036). The cancer cell analysis in the diffuse type revealed no statistically significant difference, but CTGF-negative cases demonstrated a worse prognosis (P=0.175).

Fig. 5 shows the analyses for each macroscopic type. Among the scirrhous type GC, patients with CTGF-negative cases demonstrated worse survival in both cancer and stromal cell analyses although the difference was not significant.

TCGA data. Fig. 6 shows the survival analyses using TCGA. Among all cases, CTGF positivity did not affect disease-free survival (log-rank; P=0.941). Low CTGF expression cases demonstrated worse prognosis than high CTGF expression cases in genomically stable cases and diffuse type, although the differences were not significant (log-rank; P=0.431 and P=0.169).

Discussion

This study revealed that low CTGF expression of stromal cells in diffuse GC was significantly associated with a worse prognosis. This trend is similar in the scirrhous type, which is a special subgroup in the diffuse type.

CTGF expression was mainly observed in the cytoplasm of both cancer and stromal cells. Notably, CTGF expression in stromal cells significantly affected survival outcomes. The main component of stromal cells is generally cancer-associated fibroblasts (CAFs), and our data reveal that CTGF expression of CAFs and its secretion to the stroma is strongly related to cancer progression. To the best of our knowledge, this is the first study to investigate the significance of CTGF expression in stromal cells.

CTGF expression in stromal cells was significantly associated with that in cancer cells. CTGF is known to be controlled by the Hippo pathway (6). Surrounding mechanotransduction controlled the pathway (16). As described, diffuse-type GC consists of many stromal components, which are composed of stromal cells and fibrosis by excessive collagen deposition. This feature is less distinct in the intestinal type. Thus, the different extracellular matrix stiffness may be associated with the different roles of CTGF

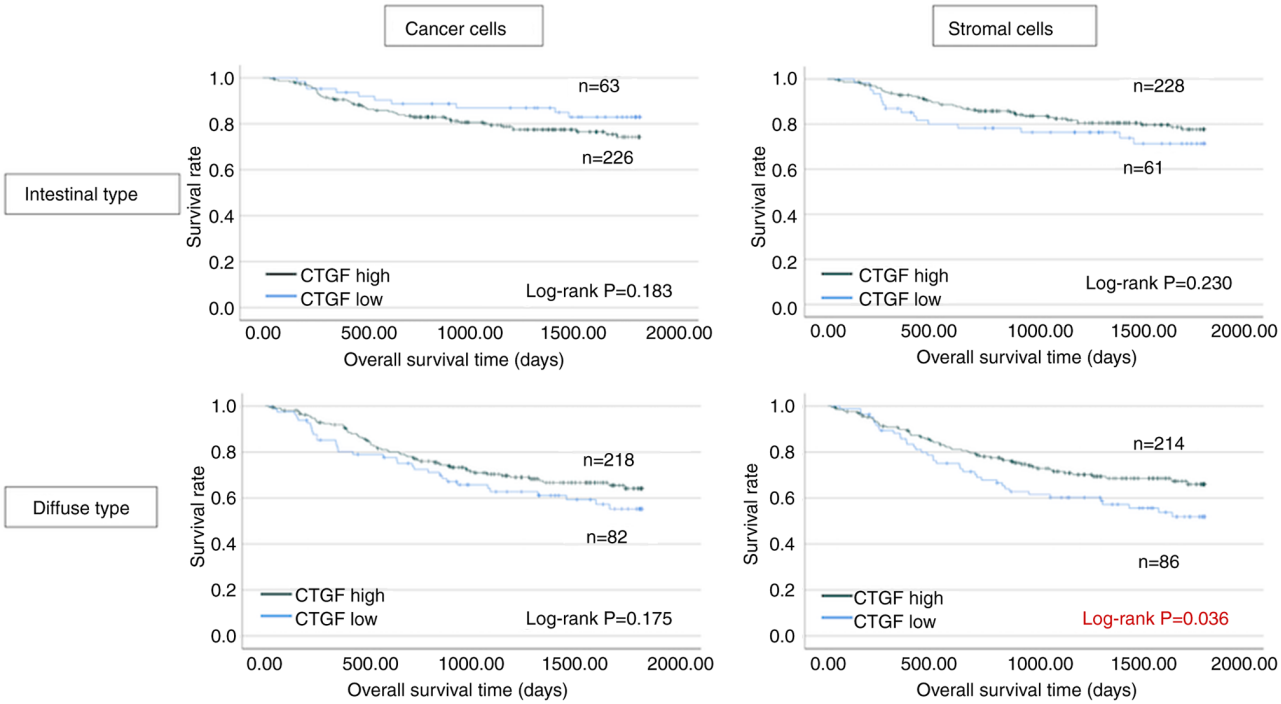

Figure 4. Survival of patients with gastric cancer depending on CTGF expression (immunohistochemistry) and histological types. CTGF, connective tissue growth factor.

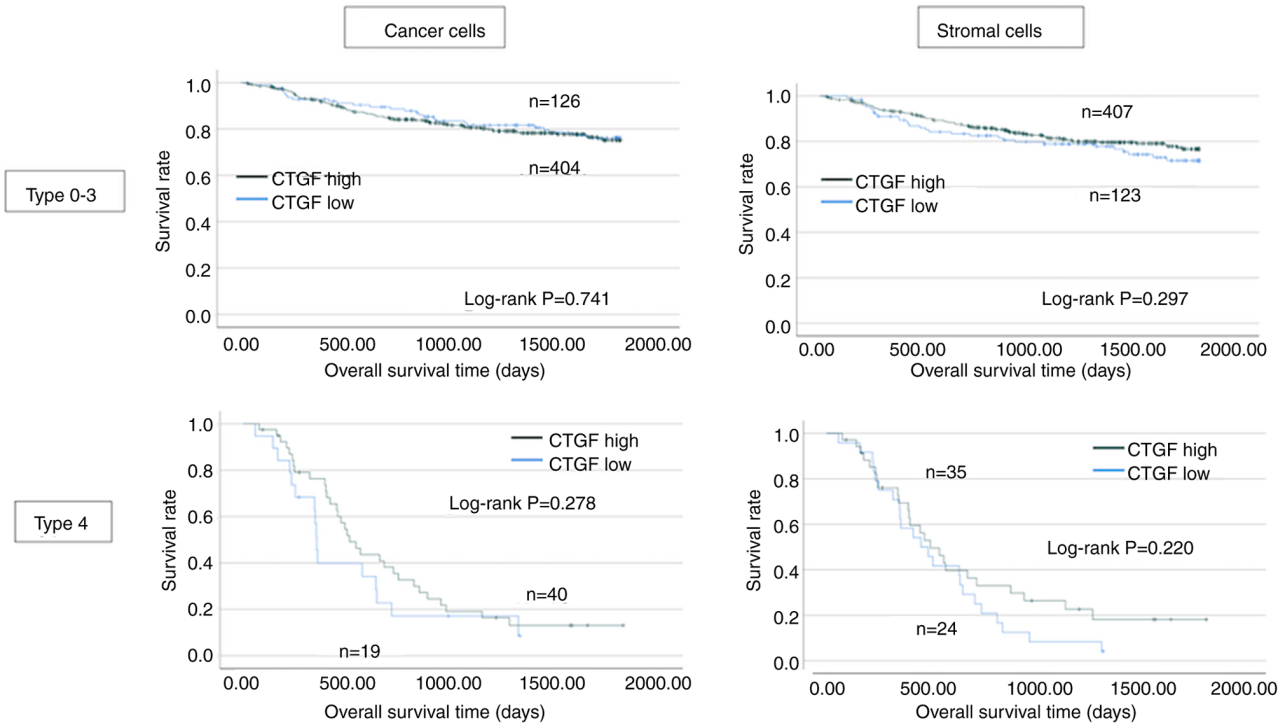

Figure 5. Survival of patients with gastric cancer depending on CTGF expression (IHC) and macroscopic types. CTGF, connective tissue growth factor.

expression in diffuse and intestinal types. The detailed mechanism remains unknown, and factors that affect the Hippo pathway in CAFs and cancer cells should be investigated in the future.

Previously, Chen *et al* (9) revealed that CTGF expression was significantly associated with early TNM staging and better survival. This is consistent with our current data. However, we reveal the more prominent effect of CTGF on survival in the diffuse type. Furthermore, we revealed that CTGF expression on stromal cells was independently associated with a worse prognosis. Reportedly, CTGF inhibits cell adhesion through integrin α3β1 and decreases the incidence of peritoneal

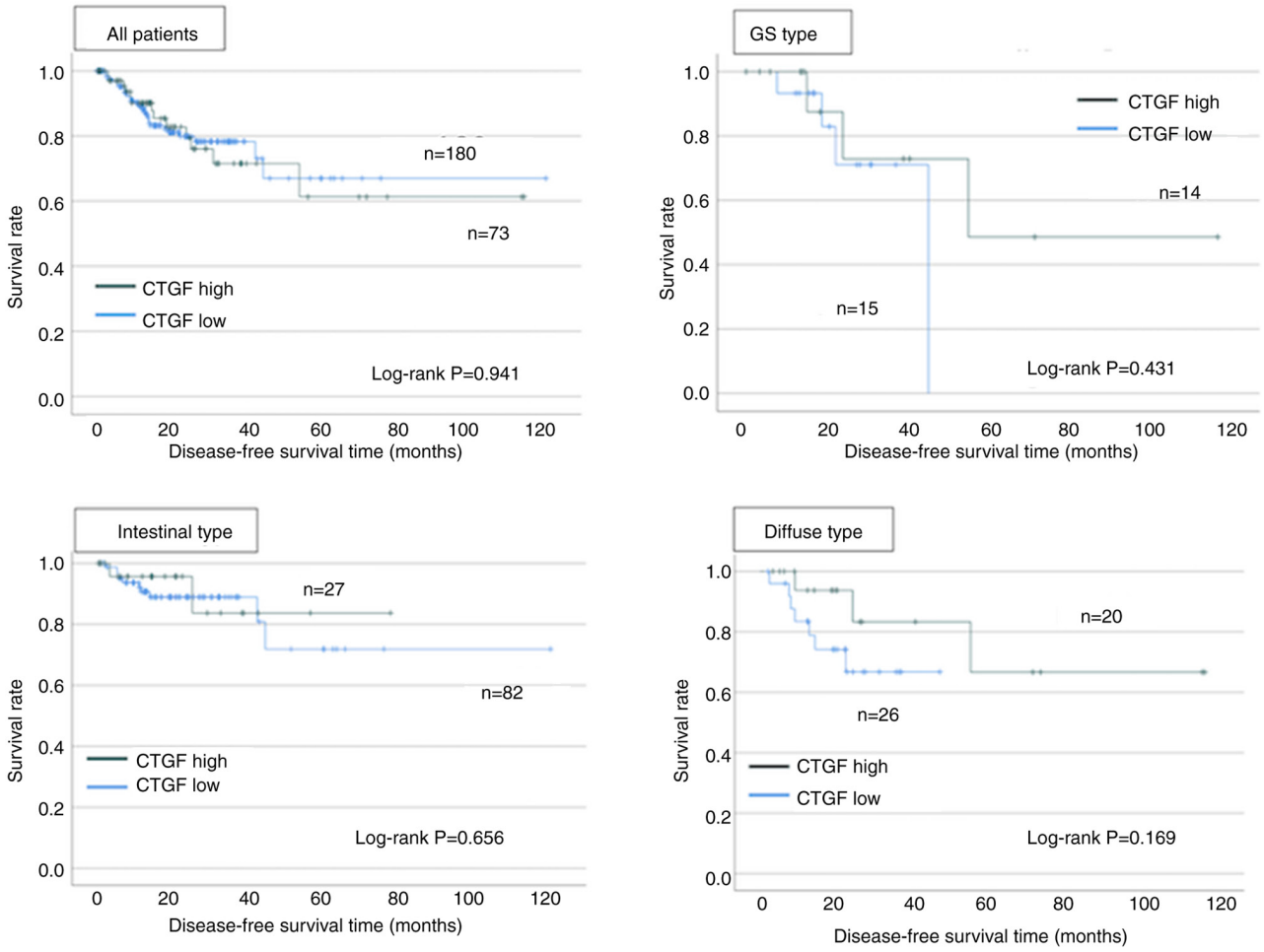

Figure 6. Survival of patients with gastric cancer depending on CTGF mRNA expression by using The Cancer Genome Atlas data. CTGF, connective tissue growth factor; GS, genomically stable.

metastasis. Diffuse-type GC and its special subtype scirrhous type often metastasize to the peritoneum. Therefore, CTGF function regarding cell adhesion during peritoneal metastasis formation explained the poor survival of low stromal CTGF expression in the diffuse type.

We used the downloaded data from the TCGA database to validate our IHC data. The survival difference between the CTGF high and low groups was not statistically significant although the trend is similar to our IHC data. The TCGA data does not completely include the histological type, so the number of cases we could include was relatively small. Additionally, TCGA data are mRNA level, which indicates that CTGF protein expression may be modified post-transcriptionally.

As a limitation, this analysis was performed using TMA, and a broad tissue area was not analyzed. However, we attempted to generate the TMA using a representative core, thus the result should be justified. Furthermore, we used automatic software in the analyses, and the data were quite objective and less biased.

In conclusion, CTGF expression in stromal cells affects prognosis, especially in diffuse-type GC. The development of a treatment to overcome peritoneal metastasis is strongly awaited. Our data indicates CTGF and its control by the Hippo pathway might be potential treatment targets in diffuse-type GC, and future studies will be necessary to elucidate this issue.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YM was responsible for conceptualization, data analysis and writing the manuscript. MYo, RM, HK, TF, TTa, MS, TTo, SL, MYa and KM were involved in data collection. MYa and KM supervised the study. YM and RM confirm the authenticity

of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Osaka Metropolitan University ethics committee on 2022/08/10 (approval no. 2022-077). Informed consent was obtained in the form of opt-out.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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