TDGF1 is a novel predictive marker for metachronous metastasis of colorectal cancer

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Abstract. Teratocarcinoma-derived growth factor 1 (*TDGF1*) is a member of the epidermal growth factor-cripto *FRL1* cryptic protein family and is involved in the activation of several different signaling pathways during embryonic development and cellular transformation. Previous reports show that *TDGF1* regulates the activation of several signaling pathways and controls cellular transformation in embryonic status, whereas its significance in colorectal cancer (CRC) is not yet fully understood. The present study comprised 55 patients who underwent surgery for CRC, as well as two cell lines derived from human CRC. The correlation of gene expression with clinical parameters in patients was assessed. The biological significance of *TDGF1* expression was evaluated by knock-down experiments in the cell lines. Seventeen of 55 (30.9%) cases exhibited a higher *TDGF1* expression in cancerous regions than in marginal non-cancerous regions. Patients with high *TDGF1* expression were statistically susceptible to a recurrence of the disease, and showed poorer disease-free survival than those with low expression. The assessment of *TDGF1* knock-down in the 2 cell lines demonstrated that the siRNA inhibition resulted in a statistically significant reduction in cell growth and invasion. In conclusion, the present data strongly suggest the usefulness of *TDGF1* as a predictive marker for metachronous metastasis in CRC patients.

Introduction

Cancer is a prominent malignancy in many developed countries, including the United States and Japan (1,2). The incidence of colorectal cancer (CRC) has increased significantly in recent years in concert with the changing lifestyle (3). The major cause of death from CRC is liver metastases (4). Although treatment has recently improved, it fails in approximately one-third of patients, who require an alternative strategy (2). Thus, useful predictive markers are needed for CRC patients.

Tumor-promoting oncogenes and tumor suppressors control cell proliferation through CRC cell cycle arrest (1,5,6). Identifying additional genes responsible for the development and progression of CRC, as well as understanding their clinical significance would improve diagnosis and treatment of the disease. The characterization of key molecules is particularly promising for the development of novel approaches to treat gastrointestinal tumors.

The human teratocarcinoma-derived growth factor 1 (*TDGF1*) gene is a member of the epidermal growth factor-cripto *FRL1* cryptic gene family and was initially isolated from human teratocarcinoma (7). *TDGF1* is expressed in several types of human tumors and has been detected by immuno-histochemistry in the breast, stomach, colon, pancreas, and lung (8-16). For gastric cancer, the combined expression of *TDGF1* and E-cadherin is reported as a prognostic factor (16).

We investigated the importance of the *TDGF1* gene by analyzing it in 55 consecutive paired cases of CRC and non-cancerous regions as well as in 2 CRC cell lines. We propose that *TDGF1* expression is important for prognostic evaluation and suggest that *TDGF1* could be a novel marker for CRC prognosis.

Materials and methods

Clinical tissue samples. The study comprised 55 consecutive patients who underwent surgery for CRC at Osaka University from 2003 to 2004. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after written, informed consent was confirmed in accordance with the institutional ethics guidelines. The surgical specimens were fixed in formalin, processed through graded ethanol, embedded in paraffin, and sectioned with hematoxylin and eosin. All specimens were frozen immediately in liquid nitrogen after resection and kept at -80˚C until RNA extractions. After surgery, patients were followed up with a blood examination including the tumor markers serum carcinoembryonic antigen (CEA) and cancer antigen (CA19-9), as well as imaging modalities, such as abdominal ultrasonography, computed

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tomography, and chest X-ray every 3-6 months. Clinicopathological factors were assessed according to the tumor-node-metastasis (TNM) criteria classification of the International Union Against Cancer (UICC) (17).

Cell lines and culture. Two cell lines derived from human CRC, HCT116 and LoVo, were used in this study (18,19). They were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum and antibiotics at 37˚C in a 5% humidified CO_2 atmosphere. For siRNA inhibition, double-stranded RNA duplexes targeting human *TDGF1*, (5'-AAGACUUUAGAAAUGGCCAUGAUCC-3'/5'-GGAUCAUGGCCAUUUCUAAAGUCUU-3', 5'-UUUACUGGUCAUGAAAUUUGCAUGA-3'/5'-UCAUGCAAAUUUCAUGACCAGUAAA-3', and 5'-UGGACGAGCAAAUUCCUGAUGGCCC-3'/5'-GGGCCAUCAGGAAUUUGCUCGUCCA-3'), as well as negative control siRNA (NC) were purchased in the Stealth RNAi kit (Invitrogen, Carlsbad, CA, USA). CRC cell lines were transfected with siRNA at a concentration of 20 μmol/l using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen), treated in accordance with the manufacturer's protocols, and analyzed by proliferation assay. All siRNA duplexes were used together as a triple transfection. The number of cell cultures was measured by counting cells with a CellTac kit (Nihon Koden, Tokyo, Japan). siRNA knockdowns were performed in the two CRC cell lines to evaluate proliferation and invasion under *TDGF1* suppression. Each cell line with siRNA was compared to the wild-type and a negative control. Values were expressed as the mean ± standard error of mean (SEM) from five independent experiments.

RNA preparation and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was prepared using TRIzol reagent and a PureLink RNA Mini kit (Invitrogen) in accordance with the manufacturer's protocols. RNA was reverse transcribed with SuperScriptIII (Invitrogen), and a 119-bp *TDGF1* fragment was amplified. Two human *TDGF1* oligonucleotide primers for the PCR reaction were designed as follows: 5'-AGATGGCCCGCTTCTCTTAC-3' (forward), 5'-CAGGTATCCCCGAGATGGAC-3' (reverse). The forward primer is located in exon 1 and the reverse primer is located in exon 2. PCR was performed with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The *GAPDH* primers 5'-TTGGTATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCATCATATTGGCAGGTT-3' (reverse) produced a 270-bp amplicon. cDNA from the human reference total RNA (Clontech, Palo Alto, CA, USA) was used as a source of positive controls. Real-time PCR monitoring was performed using the Light Cycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for *TDGF1* and *GAPDH* cDNA amplification. The amplification protocol consisted of 35 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, and elongation at 72˚C for 10 sec. The products were then subjected to a temperature gradient from 55 to 95˚C at 0.1˚C s^{-1} with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Proliferation and invasion assays. To assess the cell proliferation after 48 h of siRNA transfection, they were grown for another 48 h. The cell viability was determined utilizing Cell Counting kit consisted of WST-8 (Dojin, Tokyo, Japan). WST-8 (10 μl) was added to the 100 μl medium containing each supplement above, and the absorbance was read at 450 nm using Microplate Reader (Model 680XR, Bio-Rad Laboratories, CA). All the experiments were performed at 50-80% cell confluence, and the results were confirmed in five independent experiments. The values were expressed as a ratio/control (every parental cell).

Cell invasion were assessed with CytoSelect Cell Invasion Assay according to the protocol of the manufacture (Cell Biolabs, San Diego, CA) after 48 h of the transfection. Cells ($1.0x10^5$) in DMEM were placed on each 8.0-μm pore size membrane insert in 96-well plates. DMEM with 10% FBS was placed in the bottom wells. After 24 h, cells that did not invade were removed from the top side of the membrane chamber and completely dislodge the cells from the underside of the membrane by tilting the membrane chamber in the Cell Detachment Solution (Cell Biolabs). Lysis Buffer/CyQuant GR dye solution (Cell Biolabs) were added to each well, the fluorescence of the mixture was read with a fluorescence plate reader at 480/520 nm. The values were expressed as a ratio/control (every parental cell).

Statistical analysis. The variable data are expressed as mean ± SEM. The relationship between *TDGF1* expression and clinicopathological factors was analyzed with the χ^2 test. Kaplan-Meier survival curves were plotted and compared with the generalized log-rank test. Univariate and multivariate analyses were performed to identify prognostic factors using a Cox proportional hazard regression model. The Wilcoxon rank test was used to compare differences in *TDGF1* siRNA among the cell lines. All tests were analyzed with JMP software (SAS Institute, Cary, NC, USA). Differences with $p<0.05$ were considered statistically significant.

Results

Expression of TDGF1 in clinical tissue specimens and clinicopathological characteristics. We performed quantitative real-time RT-PCR with paired primary and adjacent non-cancerous CRC regions. RT-PCR on 55 paired clinical samples showed that 17 of these cases (30.9%) exhibited higher levels of *TDGF1* mRNA in tumors than in paired normal tissues. *TDGF1* expression was calculated by dividing *TDGF1*/*GAPDH* expression. For clinicopathological evaluation the experimental samples were divided into 2 groups according to expression status. Patients with values more than the median *TDGF1* expression value (median, 1,960) were assigned to the high-expression group and the others were assigned to the low-expression group. Clinicopathological factors related to the *TDGF1* expression status of the 55 patients are summarized in Table I. The results indicated that *TDGF1* expression was correlated with lymphatic invasion (p=0.041), venous invasion (p=0.010), and metastasis (p=0.052). To examine the correlation with metastasis, which indicated a poor prognosis, the data were divided into monochronous and metachronous metastasis groups, and *TDGF1* expression was evaluated for

Table I. Clinicopathological factors and *TDGF1* mRNA expression in 55 colorectal cancers.

Factors	High expression n=27 (%)	Low expression n=28 (%)	P-value
Age (years)			
≥68	11 (40.7)	16 (57.1)	0.222
<68	16 (59.3)	12 (42.9)	
Gender			
Male	14 (51.8)	17 (60.7)	0.507
Female	13 (48.2)	11 (39.3)	
Histological grade			
Wel/Mod	23 (85.2)	25 (89.3)	0.648
Others	4 (14.8)	3 (10.7)	
Tumor size			
≥50 mm	10 (37.0)	17 (60.7)	0.079
<50 mm	17 (63.0)	11 (39.3)	
Tumor invasion			
Tis	1 (3.7)	6 (21.4)	0.051
T1	0 (0)	4 (14.3)	
T2	6 (22.2)	5 (17.9)	
T3	17 (63.0)	10 (35.7)	
T4	3 (11.1)	3 (10.7)	
Lymph node metastasis			
N0	17 (66.7)	20 (71.4)	0.702
N1-2	9 (33.3)	8 (28.6)	
Lymphatic invasion			
Absent	4 (14.8)	11 (39.3)	0.041
Present	23 (85.2)	17 (60.7)	
Venous invasion			
Absent	11 (40.7)	21 (75.0)	0.010
Present	16 (59.3)	7 (25.0)	
Metastasis			
M0	17 (63.0)	24 (85.7)	0.052
M1	10 (37.0)	4 (14.3)	
UICC stage			
0	1 (3.7)	6 (21.4)	0.133
I	4 (14.8)	8 (28.6)	
IIA	7 (25.9)	5 (17.8)	
IIB	2 (7.4)	1 (3.6)	
IIIA	1 (3.7)	0 (0)	
IIIB	2 (7.4)	2 (7.1)	
IIIC	0 (0)	2 (7.1)	
IV	10 (37.0)	4 (14.3)	

Statistically significant values are underlined. Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

Table II. Metastasis and *TDGF1* mRNA expression in the 55 patients.

Factors	High expression n=27 (%)	Low expression n=28 (%)	P-value
Monochronous metastasis			
Absent	24 (88.9)	25 (89.3)	0.052
Present	3 (11.1)	3 (10.7)	
Metachronous metastasis			
Absent	17 (70.8)	24 (96.0)	0.017
Present	7 (29.2)	1 (4.0)	

Underlined values indicate statistical significance.

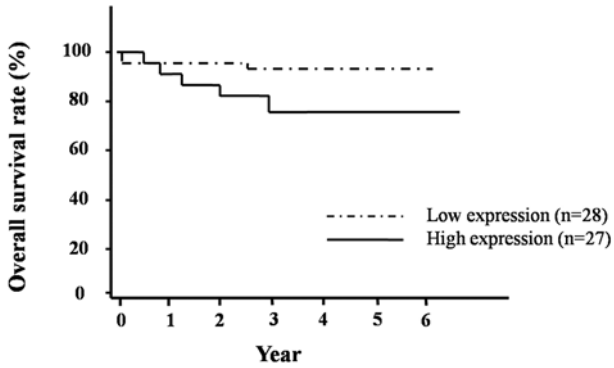

Figure 1. Overall survival rates of colorectal cancer patients based on *TDGF1* mRNA expression status. The overall survival rate was lower in the *TDGF1* high-expression group than the low-expression group (p=0.144).

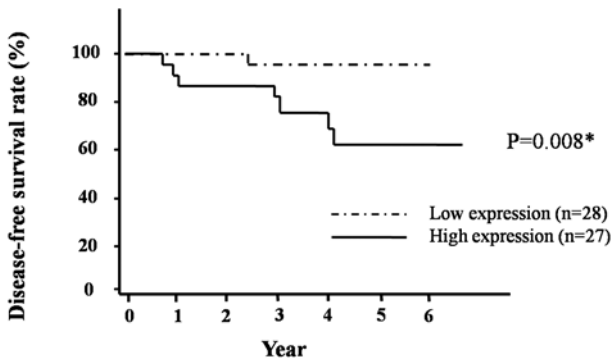

Figure 2. Disease-free survival rates of colorectal cancer patients, exclusive of monochronous metastasis, based on *TDGF1* mRNA expression status. The disease-free survival rate was significantly lower in patients whose samples highly expressed *TDGF1* mRNA than those with lower expression (p=0.008).

each factor (summarized in Table II). The results indicated that *TDGF1* expression was significantly correlated with metachronous metastasis (p=0.017).

Relationship between TDGF1 expression and prognosis. Postoperative overall survival was shorter in patients with elevated *TDGF1* expression (p=0.144) than in those with lower expression. The median follow-up was 4.16 years (Fig. 1). We also evaluated disease-free survival based on the relationship between *TDGF1* expression and metachronous metastasis after

Table III. Univariate and multivariate analyses for disease-free survival in 49 patients with curative surgery (Cox proportional hazards regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (years)						
≤68/>68	1.84	0.45-9.01	0.391			
Gender						
Male/female	2.17	0.62-18.62	0.192			
Histological grade						
Por-others/well-mod	713.31	-	0.241			
Tumor size (mm)						
≥50/<50	3.34	0.76-22.91	0.110			
Tumor invasion						
T3-4/Tis-2	3.02	0.69-20.70	0.145			
Lymph node metastasis						
N1-2/N0	4.21	0.99-17.85	0.051			
Lymphatic invasion						
Present/absent	-	-	<u>0.014</u>	-	-	0.067
Venous invasion						
Present/absent	2.53	0.59-10.72	0.196			
TDGF1 mRNA expression						
< Median/≥ median	10.42	1.84-195.08	<u>0.005</u>	7.78	1.37-146.02	<u>0.017</u>

Statistically significant values are underlined. RR, relative risk; CI, confidence interval; Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Por, poorly differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

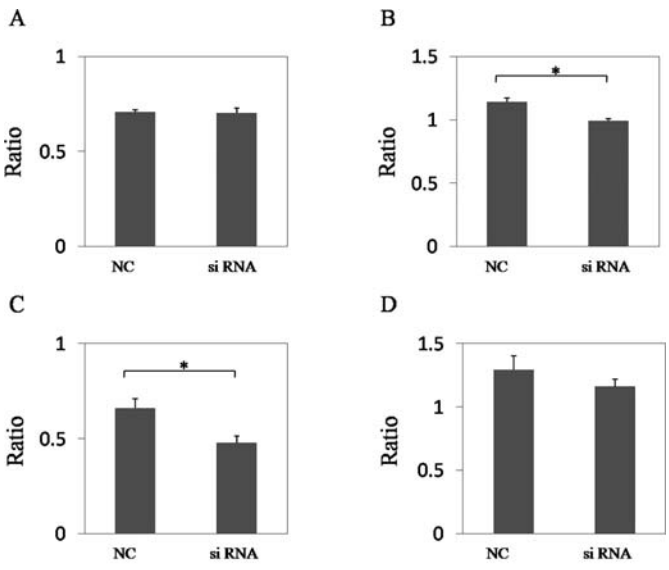

Figure 3. *In vitro* assays with siRNA inhibition in the two colorectal cancer cell lines. A proliferation assay was performed in two colorectal cancer cell lines (A, HCT116; B, LoVo). There were significant differences between NC and *TDGF1* siRNA in LoVo (n=5, *p=0.008). An invasion assay was performed in them (C, HCT116; D, LoVo). There were significant differences between NC and *TDGF1* siRNA in HCT116 (n=5, *p=0.009). *In vitro* assays showed differences in the ratio with control (untreated) cells. Values are means and SEM. NC, negative control.

curative surgery in 49 patients except stage IV at the time of primary operation. The disease-free survival rate was significantly lower in patients with elevated *TDGF1* expression (p=0.008; Fig. 2) than in those with lower expression. Table III shows the univariate and multivariate analyses of factors related to metastatic-free survival in 49 patients. The univariate analysis revealed that *TDGF1* expression (p=0.005) and lymphatic invasion (more than maximum repetition, p=0.014) were significantly correlated with post-operative metastasis. The multivariate regression analysis indicated that inclusion in the *TDGF1* high-expression group (relative risk, 7.78; 95% confidence interval, 1.37-146.02; p=0.017) was an independent predictor of metastatic-free survival.

In vitro assessment of TDGF1 expression knock-down. Two CRC cell lines were chosen for the proliferation and invasion study. A significant reduction in *TDGF1* by siRNA was also confirmed by quantitative real-time RT-PCR. The proliferation study was confirmed by seeding the cells ($1.0x10^5$) in 6-well dishes and culturing them for 48 h to determine proliferation. The results showed significant differences in HCT116 and LoVo cell numbers between NC and *TDGF1* siRNA (n=5, p<0.05, Fig. 3A and B). In the invasion study, the results showed significant differences in DLD-1 and LoVo between NC and *TDGF1* siRNA (n=5, p<0.05, Fig. 3C and D).

Discussion

TDGF1, also known as *CRYPTO*, Crypto-1, or CR-1, is expressed in various cancer tissues of different species (8-16,20-23). Previous *in vitro* and *in vivo* reports show that *TDGF1* regulates signaling pathways and cellular mechanisms as an oncogene (23-26). In mammary tumor, *TDGF1* is associated with molecular mechanisms that contribute to the loss of adherent junctions, referred to as epithelial-mesenchymal transition, which plays an important role in cancer invasiveness and metastasis and might cause a poor prognosis (25-28). The combined expression of *TDGF1* and E-cadherin by immunohistochemistry indicates a poor prognosis in gastric cancer (16).

The present study showed that *TDGF1* expression is an independent predictive factor for metachronous CRC metastasis, and the siRNA inhibition experiment demonstrated the functional relevance of expressed *TDGF1* in the CRC cell lines. To the best of our knowledge, this is the first report to show that *TDGF1* is a predictive marker for CRC metastasis, supported by the functional relevance to cell growth and invasion.

It can be useful to identify the necessity for intensive follow-up and adjuvant therapy by predicting CRC recurrence and metastases after curative surgical resection (29-31). Our clinicopathological analysis revealed that CRC patients with high *TDGF1* expression had a poorer prognosis for disease-free survival than the low-expression group. The results indicated that *TDGF1* is a good predictor for metachronous metastasis, and patients can be followed-up by curative surgical intervention. It is essential to prevent metachronous metastasis during gastrointestinal cancer therapy. Several adjuvant chemotherapies are helpful in particular disease stages, especially in CRC, and indicate the usefulness of a less invasive surgical approach for CRC (31-36). For these cases, a predictive informative marker for tumor recurrence, which is independent from traditional TNM classification and collectively contributes to diagnoses and treatments is very important. While improvement in preoperative and postoperative treatments such as chemotherapy and radiotherapy combined with surgery have contributed to a reduction in the recurrence and metastasis of CRC, half of the cases ultimately metastasize despite systemic chemotherapy followed by surgery (37). Adjuvant chemotherapy for CRC is desirable in highly suspicious metastatic cases. In these cases, an analysis of *TDGF1* may be useful to predict and treat patients with a poor prognosis.

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