Clinical significance of growth differentiation factor 11 in colorectal cancer

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Abstract. Growth differentiation factor 11 (GDF11), a member of the transforming growth factor-ß superfamily and bone morphogenetic protein (BMP) subfamily, plays a role in regulation of development and differentiation. Although some members of BMP subfamily have been reported to correlate with cancer, the significance of GDF11 has not been studied in a clinical oncology setting. The current study explored the clinicopathological significance of GDF11 expression in colorectal cancer. Quantitative real-time reverse transcription-PCR in colorectal cancer specimens obtained from 130 patients showed that GDF11 mRNA expression in cancer tissue was significantly higher than in normal tissue (p=0.001). Tumors were classified as high GDF11 expression (n=65) or low GDF11 expression (n=65). Patients whose tumors had high GDF11 expression showed a high frequency of lymph node metastasis (p=0.049) and had more cancer-related deaths (p=0.040). Furthermore, the patients with high GDF11 expression had significantly poorer overall survival than those with low expression (p=0.0334). Although multivariate analysis showed that GDF11 was not an independent prognostic factor, these findings suggest that GDF11 may be a novel diagnostic and prognostic biomarker in patients with colorectal cancer.

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Abbreviations: GDF, growth differentiation factor; BMP, bone morphogenetic protein; TGFß transforming growth factor-ß; PCR, polymerase chain reaction

Key words: growth differentiation factor 11, colorectal cancer, lymph node metastasis, prognosis, biomarker

Introduction

Colorectal cancer is a major cause of cancer-related death in Western countries. Mortality is increasing in Japan, where almost 40,000 people die annually of the disease. Identification of patients at high risk for recurrence or metastasis is important to improving the cure rate of colorectal cancer. Both the TNM staging system and Dukes' classification are useful to predict the overall course of disease, however, it can be difficult to predict prognosis for individual patients. Thus it would be valuable clinically to identify novel prediction factors for high-risk patients, especially factors that would be useful for individuals.

The cDNA microarray technique produces an expression profile of more than10,000 genes with one experiment, enabling us to identify genes that are differentially expressed between tumor and normal cells (1-6). Surgical samples used for the analysis have been the bulk specimens that contain both tumor and normal cells. Because the ratio of tumor to normal cells varies case by case, this can bias the data. To overcome this problem, laser microdissection has been used to obtain target cells, and use of the technique can produce precise information about target cells (7). Combining laser microdissection and cDNA microarray techniques, we compared the expression profiles of colorectal cancer cells with those of normal colorectal cells, identifying novel cancer-related genes (8). One of the genes overexpressed in colorectal cancer cells is growth differentiation factor 11 (GDF11), a member of the transforming growth factor-ß (TGFß) superfamily and bone morphogenetic protein (BMP) subfamily. BMP has been known as a factor that regulates cell proliferation, apoptosis and differentiation (9-11), and was reported to be correlated with various cancers such as esophageal cancer (12), prostate cancer (13) and breast cancer (14). GDF11 has been reported to act in normal tissues as a regulator of development and cell differentiation (15-19). However, to our knowledge, no report has yet shown a correlation between GDF11 and cancer. In this study, we analyzed expression of the GDF11 gene in colorectal cancer samples and clarified that this is a novel factor that correlates

with lymph node metastasis and prognosis. We herein report the data and discuss its significance in colorectal cancer.

Materials and methods

Patients and sample collection. The 130 tumor samples and the matched control samples taken from normal tissue located far from the tumor site of colorectal cancers were frozen in liquid nitrogen immediately after a surgical resection, and were kept at -80˚C until RNA extraction. The surgical samples were obtained at the Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan. Study design was approved by the institutional review board, and written informed consent was obtained from all patients. The diagnosis of colorectal cancer for all 130 patients was confirmed based on the clinicopathologic findings.

Total RNA extraction and cDNA synthesis. Frozen tissue specimens were homogenized in guanidinium thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion as described previously (20).

Semiquantitative real-time reverse transcription-PCR. The cDNA was synthesized from 8.0 μg of total RNA as described previously (21). The following primers were used to amplify the GDF11 gene: sense primer 5'-GATCCTGGACCTACAC GACTTC-3' and antisense primer 5'-GGCCTTCAGTACCT TTGTGAAC-3'. Glyceraldehyde-3-phosphate dehydrogenase (sense primer 5'-TTGGTATCGTGGAAGGACTCA-3' and antisense primer 5'-TGTCATCATATTTGGCAGGTTT-3') gene was used as an internal control. The reaction was done in a Light Cycler System (Roche Applied Science, Indianapolis, IN) using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Details of each reaction have been described elsewhere (22). Briefly, thermal cycling for all genes was initiated with a denaturation step of 95˚C for 10 min followed by 40 cycles at 95˚C for 10 sec, 65˚C (60˚C for glyceraldehyde-3-phosphate dehydrogenase) for 10 sec, and 72˚C for each optimal length (1 sec/25 bp). All calculated concentrations of target genes were divided by the amount of endogenous reference (glyceraldehyde-3-phosphate dehydrogenase) to obtain the normalized GDF11 expression values.

Immunohistochemistry. Immunohistochemical studies of GDF11 were done on colorectal cancer surgical specimens using the avidin-biotin-peroxidase method (Envision+ Dual Link/HRP, Dako Cytomation, Denmark) on formalin-fixed, paraffin-embedded tissues. All sections were counterstained with hematoxylin. The primary mouse polyclonal antibodies against GDF11 (Abnova, Taipei, Taiwan) were used at dilutions of 1:500.

Statistical analysis. Quantitative real-time reverse transcription-PCR data were calculated with JMP 5 for Windows software (SAS Institute Inc., Cary, NC). Differences between groups were estimated using the Student's t-test and the χ^2 test. Survival curves were estimated by the Kaplan-Meier method, and comparison between curves was made by the log-rank test. Relative risk was calculated using the Cox proportional hazard model. A probability level of <0.05 was chosen for statistical significance.

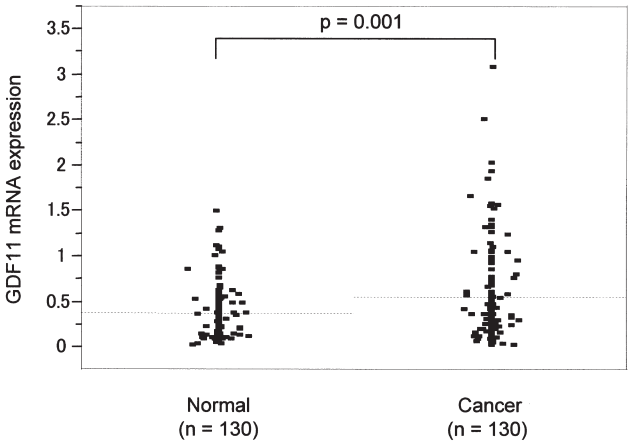

Figure 1. GDF11 mRNA expression in 130 paired samples of cancerous and normal colorectal tissue. Horizontal lines indicate the means. Cancer tissues had significantly higher GDF11 mRNA expression than normal tissues (p=0.001, Student's t-test).

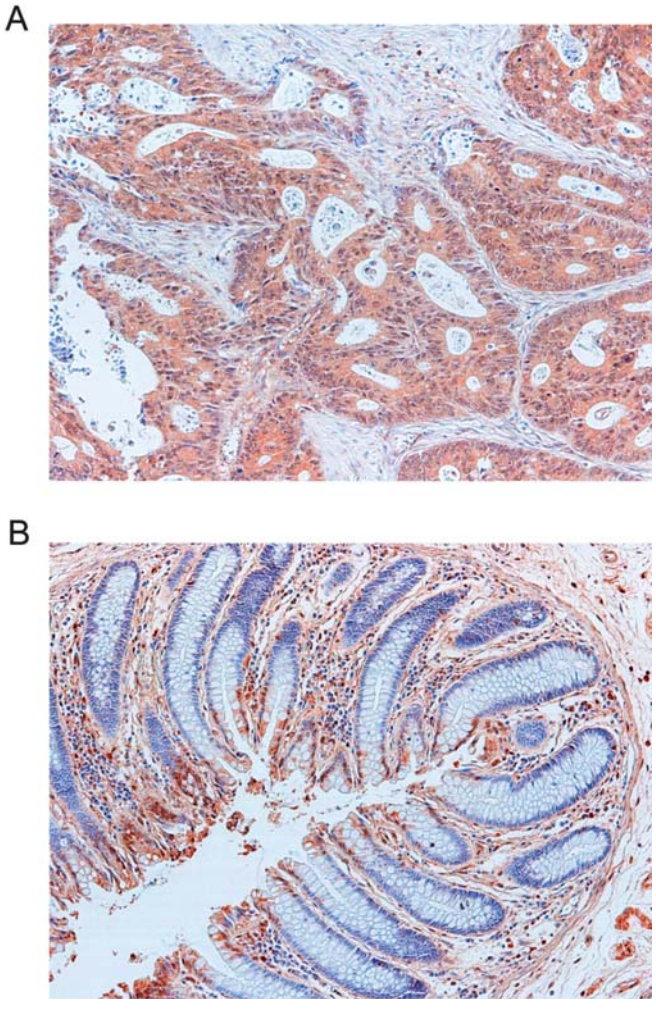

Figure 2. Immunohistchemical study of GDF11 expression in representative samples of colorectal cancer. A, GDF11 expression in cancer cells. B, GDF11 expression in normal epithelium.

Table I. Clinicopathological variables and GDF11 mRNA expession in 130 colorectal cancers.

Variables	Expression		P-value
	High (n=65)	Low (n=65)	
Age	64.3±13.3	68.7±9.37	0.030
Gender			
Male	37	41	0.474
Female	28	24	
Histological grade			
Well	25	26	0.857
Moderately and poorly	40	39	
Tumor site[a]			
Right colon	15	25	0.056
Left colon	50	40	
Serosal invasion			
Absent	44	48	0.440
Present	21	17	
Lymph node metastasis			
Absent	33	44	0.049
Present	32	21	
Lymphatic permeation			
Absent	39	45	0.271
Present	26	20	
Venous permeation			
Absent	46	54	0.094
Present	19	11	
Liver metastasis			
Absent	56	60	0.255
Present	9	5	
Peritoneal dissemination			
Absent	62	63	0.647
Present	3	2	
Clinical stage			
Stage 1 and 2	31	39	0.159
Stage 3 and 4	34	26	
Dukes classification			
A and B	31	42	0.051
C and D	34	23	
Cancer-related death			
Alive	44	54	0.040
Dead	21	11	

[a]Relative to splenic flexure.

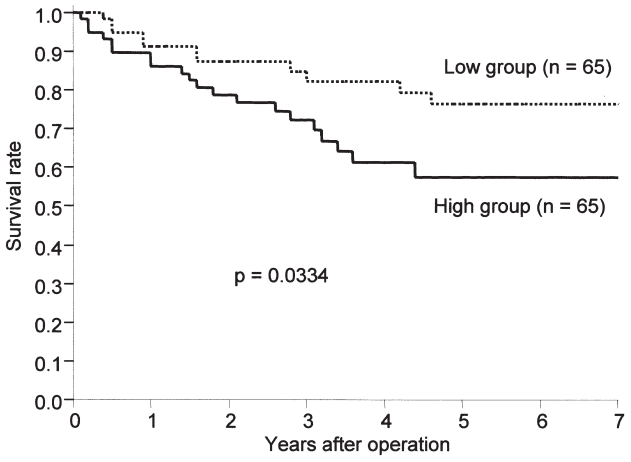

Figure 3. Kaplan-Meier survival curves for our 130 patients with colorectal cancer according to the status of GDF11 expression. Patients whose tumors showed high GDF11 mRNA expression (bold line) had a significantly poorer prognosis than those whose tumors showed low GDF11 mRNA expression (dotted line; p=0.0334, log-rank test).

Results

GDF11 expression in colorectal cancer samples and corresponding normal tissues. With regard to GDF11 mRNA expression in paired cancer and normal samples, 65 of 130 patients (50%) showed higher expression of GDF11 mRNA in cancerous tissues than in noncancerous tissues by quantitative real-time reverse transcription-PCR. The mean expression level of GDF11 mRNA in tumor tissues, 0.562±0.542 (mean ± SD), was significantly higher than the 0.379±0.315 in the corresponding normal tissues (p=0.001, Fig. 1). Median expression levels of GDF11 mRNA in tumor tissues and normal tissues were 0.371 and 0.277, respectively. Immunohistochemical analysis revealed that GDF11 was predominantly expressed in cancer cells (Fig. 2).

Clinical significance of GDF11 expression in colorectal cancer. In the current study, patients with values less than the median expression level of 0.371 in tumor tissues were assigned to the low expression group (n=65), whereas those with values ≥0.371 were assigned to the high expression group (n=65). Table I shows clinicopathological variables and GDF11 mRNA expression in tumor specimens from the 130 colorectal cancer patients. The incidence of lymph node metastasis was significantly higher (p=0.049) in the high expression group (32 of 65, 49.2%) than in the low expression group (21 of 65, 32.3%), and the incidence of cancer-related death was significantly higher (p=0.040) in the high expression group (21 of 65, 32.3%) than in the low expression group (11 of 65, 16.9%).

Survival analysis. The 5-year actuarial overall survival rates for patients with high GDF11 mRNA levels and for patients with low mRNA levels were 58 and 77%, respectively. The survival difference between groups was statistically significant (p=0.0334; Fig. 3). Table II shows the results of risk ratio (RR) and the 95% confidence interval (95% CI) assessment with the Cox proportional hazards model. Lymph node

Table II. Univariate and multivariate analysis for prognosis.

Variable	Univariate		Multivariate	
	Risk ratio (95% CI)	P-value	Risk ratio (95% CI)	P-value
Lymph node metastasis (present)	2.59 (1.75-4.11)	<0.0001	1.79 (1.17-2.92)	0.007
Liver metastasis (present)	2.53 (1.73-3.62)	<0.0001	2.02 (1.32-3.06)	0.002
Serosal invasion (present)	2.26 (1.59-3.28)	<0.0001	1.64 (1.12-2.46)	0.012
Lymphatic permeation (present)	2.07 (1.45-3.01)	<0.0001	1.56 (1.04-2.41)	0.031
High GDF11	1.47 (1.03-2.16)	0.033	1.32 (0.90-1.99)	0.156
Venous permeation (present)	1.31 (0.89-1.86)	0.166	–	
Gender (female)	0.92 (0.63-1.31)	0.645	–	
Histological grade (moderately and poorly)	1.08 (0.76-1.57)	0.678	–	
Age at surgery (>70 years)	0.93 (0.62-1.35)	0.715	–	
Tumor site (left colon)[a]	0.94 (0.65-1.43)	0.769		

[a]Relative to splenic flexure.

metastasis (RR, 2.59; 95% CI, 1.75-4.11; p<0.0001), liver metastasis (RR, 2.53; 95% CI, 1.73-3.62; p<0.0001), depth of invasion (RR, 2.26; 95% CI, 1.59-3.28; p<0.0001), lymphatic permeation (RR, 2.07; 95% CI, 1.45-3.01; p<0.0001), and high GDF11 expression (RR, 1.47; 95% CI, 1.03-2.16; p=0.033) were statistically significant. In multivariate analysis, the variable of high GDF11 expression was not an independent prognostic predictor for the patients with colorectal cancer (RR, 1.32; 95% CI, 0.90-1.99; p=0.156).

Discussion

This study demonstrated that GDF11, a member of the TGFß superfamily and the BMP subfamily, was significantly overexpressed in the tumor tissue compared with the corresponding normal tissue by the reverse transcription-PCR analysis of 130 cases of colorectal cancer. GDF11 was originally identified as a gene that related to development of mice (23,24). Its roles in normal tissue are as a regulator of axial skeleton patterning in developing vertebrates (15), renal development (16), inhibition of olfactory neural progenitor cell proliferation (18), control of the number of retinal ganglionic cells by affecting the competence of progenitor cells but not their proliferation (19), and promotion of dental pulp stem cell differentiation (17). The pathophysiological role of GDF11 in colon cancer, however, remains unknown. GDF11 may contribute to regulation of cancer stem cell proliferation or differentiation. The hypothesis needs further study.

The TGFß superfamily is divided into the TGFß/Nodal/Activin subfamily and the BMP/GDF subfamily (25). GDF11 belongs to the latter group and is also known as bone morphogenetic protein 11. Several clinical and experimental studies including the current one have demonstrated that some BMP members are overexpressed in colon cancer. For example, the BMP4 expression increased accordingly to the ß-catenin expression in colon cancer (26), and another study showed overexpression of BMP4 in colon cancer tissue with microarray analysis of 36 cases (5). In addition, either BMP5 or 6 was overexpressed in colon cancer with bone metamorphogenesis (27). On the other hand, a few experimental studies demonstrated conflicting results suggesting a colon tumor suppressive function for some BMP members, such as BMP 2, 7 (28) and 4 (29). Thus, further studies are recommended to disclose more details about the functions of BMP members, especially GDF11, in the development and progression of colon cancer. These studies may clarify the basis for discrepant results of physiological functions of BMP members including GDF11.

The current clinical study disclosed that patients whose tumors had high GDF11 expression showed a significantly more frequent lymph node metastasis than patients whose tumors had low expression. A similar finding regarding metastasis was recognized in prostate cancer, where patients whose tumors had high BMP6 expression developed bone metastasis more frequently than patients whose tumors had low expression (13). In esophageal cancer, GDF1, one of the ligands of activin receptor type IIb (30) like GDF11 (31), was overexpressed in cases with lymph node metastasis based on analysis with a cDNA microarray (32). With regard to prognosis, the current study revealed that the prognosis of the patients with high GDF11 expression tumors was significantly poorer than that of patients with low GDF11 expression tumors, although multivariate analysis demonstrated that GDF11 was not an independent prognostic factor. GDF11 may relate to poor prognosis through correlation with lymph node metastasis. A similar finding was recognized in patients with esophageal cancer; patients whose tumors had high BMP6 expression had a poorer prognosis than patients whose tumors had low expression (12). These clinical results demonstrated that higher expression of some BMP members probably correlates with tumor progression, prognosis, or both.

In conclusion, we found that GDF11 mRNA was overexpressed in colorectal cancer tissues compared with corresponding normal tissues. Further, patients with high GDF11 expression tumors had lymph node metastasis

significantly more frequently than patients with low expression tumors, and had a poorer prognosis. These findings suggest that GDF11 may be a novel diagnostic and prognostic biomarker in patients with colorectal cancer.

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