Prokineticin 1 Expression in Gastrointestinal Tumors

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Abstract. *Aim: We studied prokineticin 1 (PROK1) expression in human gastrointestinal carcinomas by immunohistochemistry. Materials and Methods: PROK1 expression was examined in human gastrointestinal cancer cell lines and primary gastrointestinal lesions. In addition the relationship between the number of blood vessels and PROK1 expression in these primary lesions was examined. Results: PROK1 expression was observed in gastrointestinal cancer cell lines. PROK1 expression was not observed in healthy gastrointestinal mucosa, but was observed in the primary lesions in 23 out of 98 (31.6%) patients with colorectal cancer, 19 out of 55 (34.5%) patients with gastric cancer, and 5 of 10 (50%) patients with cancer of the small intestine. PROK1 expression was observed in many patients with advanced gastrointestinal cancer. The number of blood vessels in PROK1-positive primary gastrointestinal lesions was higher than that in PROK1-negative primary lesions. Conclusion: PROK1 expression might be related to the extent of malignancy in gastrointestinal cancer.*

Gastrointestinal cancer is ranked high among malignant tumors in terms of its prevalence and associated death rate (1, 2). To improve the survival rate of patients, it is important to control the infiltration and metastasis of cancer cells. In general, metastasis is believed to occur *via* detachment of cancer cells from the primary lesion, destruction of the basement membrane, penetration into the vascular channel, and transfer to various distant organs for survival (3-6). Although the underlying mechanism of cancer metastasis and possible treatments have been widely studied, a complete cure has yet to be attained. Therefore, further investigations are required.

Prokineticin 1 (PROK1) was first reported in 2001 as an angiogenic growth factor that is expressed only in endocrine cells, including the adrenal gland, ovary, and testis (7). Its structure is homologous to that of venom protein A (VPRA), a protein found in snake venom. Like other angiogenic growth factors, PROK1 promotes vascular endothelial growth under hypoxic conditions. However, unlike other vascular endothelial growth factor (VEGF) family members, PROK1 contains cysteines in its amino acid sequence (7). We previously reported that introduction of the *PROK1* gene into a colorectal cancer cell line strengthened its metastatic ability (8), and that *PROK1* mRNA was highly expressed in the primary lesions of some patients with colorectal cancer (9). More recently, we reported that PROK1 was related to the cell-infiltrating ability of a colorectal cancer cells through an autocrine mechanism (10). Other reports on malignant tumors showed that PROK1 is related to the metastasis of prostate cancer and neuroblastoma, and that it increases the malignancy of pancreatic duct cancer and thyroid cancer, indicating that PROK1 activity in malignant tumors is substantial (11-15). Nevertheless, as far as we are aware of no studies of PROK1 expression in malignant gastrointestinal tumors have been reported. Therefore, we examined PROK1 expression in such malignant tumors using a monoclonal antibody (mAb) independently developed in our Department.

Materials and Methods

Cell culture. The human colon and gastric cancer cell lines Colo320, SW620, DLD-1, MKN45, MKN72, and SNU-5 (obtained from the European Collection of Cell Cultures, Salisbury, UK, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan, or RIKEN BioResource Center, Tsukuba, Ibaraki, Japan) were cultured at 37˚C in a standard humidified incubator containing 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (16). Cells were plated at 30-40% confluency and allowed to attach to an 8-well chamber slide (BD Biosciences, San Jose, CA, USA). Two days later, the chamber slide was fixed in cold acetone for 10 min, and then immunohistostaining procedures were performed.

Patients. The study was conducted on 98 patients with colon cancer, 55 patients with gastric cancer, and 10 patients with cancer of the small intestine treated surgically at the First Department of Surgery, University of Fukui, Japan, between 1983 and 2012 (randomized selection). Cancers were graded using criteria recommended by the general rules of clinical and pathological studies according to the tumor node metastasis (TNM) classification system of the International Union Against Cancer (17).

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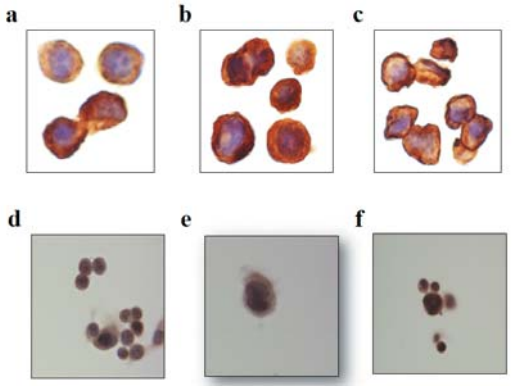


Figure 1. *Immunohistochemical staining of Prokineticin 1 (PROK1) protein in colorectal cancer cell lines. Expression of PROK1 protein was found in all studied cancer cell lines: a: Colo320, b: SW620, c: DLD-1, d: MKN45, e: MKN74, f: SNU-5.*

Surgical specimens. Specimens (most invasive lesions including normal adjacent mucosa) were fixed in 10% formalin and embedded in paraffin. Serial 2-μm-thick sections were examined by immunohistochemistry. De-paraffinized specimens were rinsed in phosphate-buffered saline (PBS; pH 7.4), followed by endogenous peroxidase blockade by a 10-min incubation with 0.3% hydrogen peroxide in methanol.

Antibodies. The following antibodies were used: anti-human PECAM-1 (CD31) (Abcam, Cambridge, UK) and PROK1 (established by our Department) (18).

Immunohistostaining. Specimens were then incubated at room temperature for 30 min with a primary antibody. Detection was carried out using the ChemMate method by the EnVision system (DAKO, Danmark) according to the manufacturer's instructions. The expression was interpreted as positive when the protein was expressed in more than 40% of all cancer cells.

Blood vessel area. The blood vessel area (%) per visual field was calculated using Morph® software (Molecular Devices Corporation, Tokyo, Japan), and then compared with PROK1 expression. The experiments were repeated four times.

Statistical considerations. Statistical analysis was performed by the Chi-square test or Mann-Whitney *U*-test. Data are given as the mean±SEM. Values of $p<0.05$ were considered as statistically significant.

Results

PROK1 expression in gastric and colorectal cancer cell lines. PROK1 expression was detected in the cytoplasm of all six gastric and colorectal cancer cell lines studied by immunohistochemistry (Figure 1).

PROK1 expression in healthy human gastrointestinal tissues. PROK1 expression was not detected in any healthy human gastrointestinal tissues (stomach, small intestine, and colorectal tissues) (Figure 2).

Table I. *Relationship between Prokineticin 1 (PROK1) expression and staging of gastrointestinal cancer.*

	Stage	No. of cases	PROK1-positive cases (%)	
Colorectal cancer	I-II	56	6 (10.7%)	
	III-IV	42	25 (59.5%)	$p<0.01$
Gastric cancer	I-II	19	2 (10.5%)	
	III-IV	36	17 (47.2%)	$p<0.01$

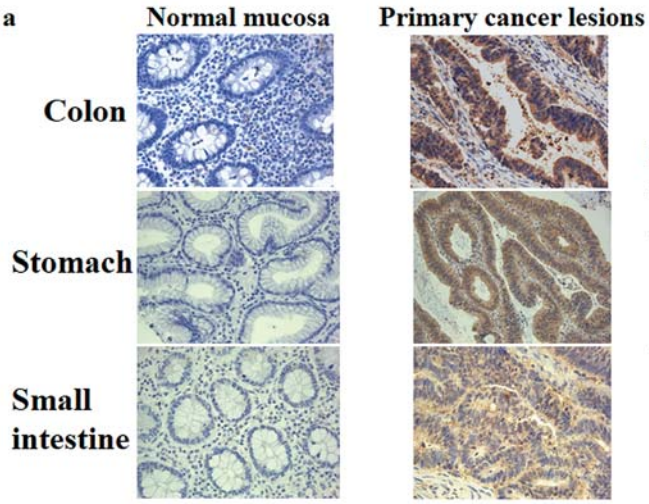
PROK1 expression in primary human gastrointestinal cancer lesions. Figure 2 shows the results of PROK1 immunohistochemistry in human gastrointestinal cancer tissues. PROK1 expression was detected in 31 out of 98 (31.6%) colorectal cancer lesions in 19 out of 55 (34.5%) gastric cancer lesions, in 5 out of 10 (50%) small intestine cancer lesions.

Relationship between PROK1 expression and staging of gastrointestinal cancer. Examination of PROK1 expression and the stage of each gastrointestinal cancer case showed that PROK1 was expressed in 6 out of 56 (10.7%) stage I-II *versus* 25 out of 42 (59.5%) stage III-IV colorectal cancer cases, and in two out of 19 (10.5%) stage I-II gastric cancer cases *versus* 17 out of 36 (47.2%) stage III-IV small intestinal cancer cases. This indicates that PROK1 was more frequently expressed in higher-stage gastrointestinal cancers (Table I).

Relationship between PROK1 expression and blood vessel area in gastrointestinal cancer. According to the immunohistochemistry results with an a monoclonal antibody against CD31, the blood vessel areas in PROK1-negative and PROK1-positive colorectal cancer cases were 2.87% and 5.32%, respectively. Those in PROK1-negative and PROK1-positive gastric cancer cases were 2.53% and 4.67%, respectively; and those in PROK1-negative and PROK1-positive small intestine cancer cases were 3.75% and 6.46%, respectively. These results indicate that angiogenesis was significantly higher in PROK1-positive cancer than in PROK1-negative cancer (Figure 3).

Discussion

In many countries, malignant tumors are among the most common causes of death. The prevalence of colorectal cancer is high in both Japan and the West, whereas gastric cancer is prevalent in Japan, Italy, and Chile, and esophageal cancer is prevalent in China and Eastern Africa (1, 2). For these malignancies, distant metastasis is the main life-threatening factor (1). One possible mechanism underlying metastasis is the increased expression of angiogenic growth factors in the primary lesion, leading to excessive angiogenesis, increased infiltration of cancer cells and invasion into the surrounding tissues and



b

	No of cases	PROK1 positive cases(%)
Colorectal cancer	98	31 (31.6%)
Gastric cancer	55	19 (34.5%)
Small intestinal cancer	10	5 (50.0%)

Figure 2. *Immunohistochemical staining of Prokineticin 1 (PROK1) protein in primary lesions of human gastrointestinal cancer. a: Left panel: PROK1-negative normal mucosa adjacent to tumor. Right panel: Positive staining for PROK1 in paraffin-fixed tumor tissue. b: The results of PROK1 immunohistochemistry in human gastrointestinal cancer tissues.*

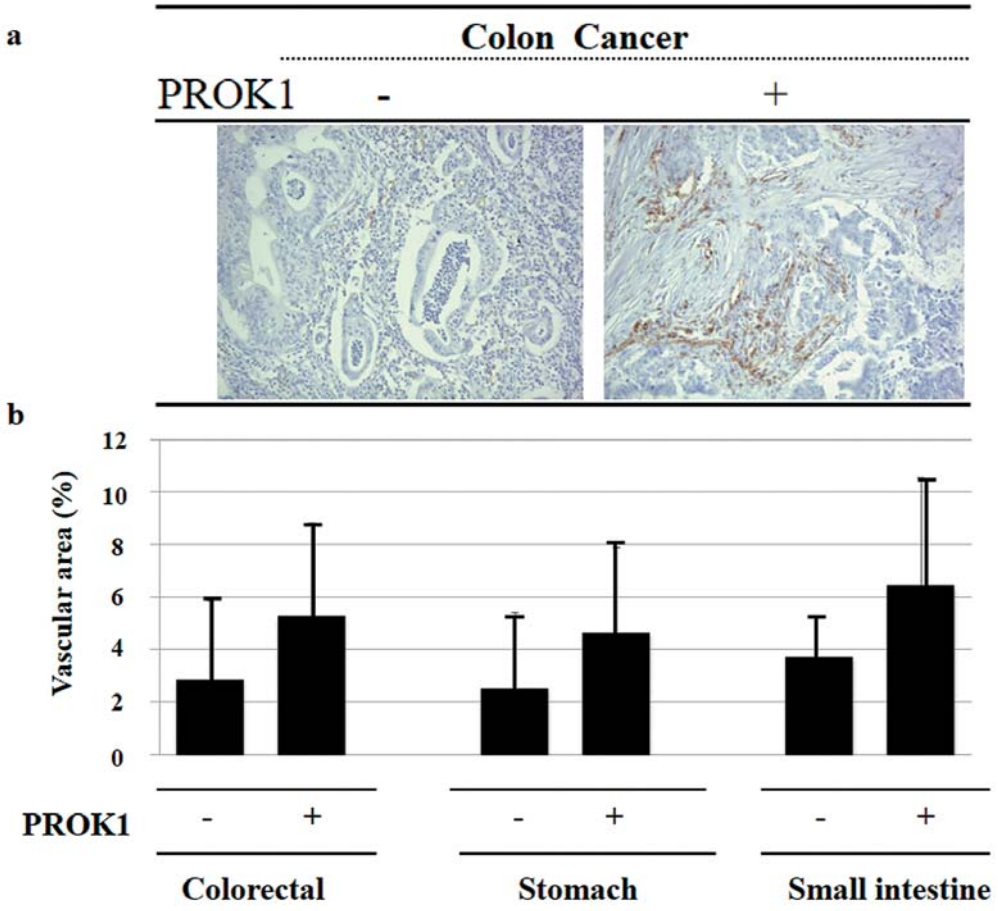


Figure 3. *Prokineticin 1 (PROK1) expression in gastrointestinal cancer. a: PECAM-1 (CD31) immunohistochemical staining in colonic carcinoma. b: Mean vascular area (%) per visual field by PROK1 expression. Data represent means±SEM (n=4). Mann-Whitney U-test, $p<0.03$.*

vessels, facilitating metastasis to other organs (3-6). Elucidation of this mechanism is key to the eradication of metastasis. VEGF is a well-known angiogenic growth factor, and its relationship with prognosis has been investigated (19-22). In recent years, VEGF has been a molecular drug target for advanced/recurrent colorectal cancer, which improves prognosis. In 2001 PROK1, an angiogenic growth factor expressed in healthy endocrine tissues was cloned (7). We previously found that PROK1 is related to angiogenesis and hematogenous metastasis in colorectal cancer cell lines (8). More recently, we determined that PROK1 is related to the infiltrative ability of colorectal cancer cells (10). We then attempted to examine, for the first time, PROK1 expression in gastrointestinal cancer. According to our findings, PROK1 was not expressed in the healthy mucosa of the stomach, small intestine, or large intestine. However, PROK1 expression was observed in approximately 20-40% of malignant tumors of each gastrointestinal organ, indicating that PROK1 might be expressed in response to a certain environment after canceration. Generally, when a cancer cell exceeds several millimeters in size, angiogenesis becomes necessary (23). Therefore, PROK1 expression might be required for growth of gastrointestinal cancer cells in certain cases.

Recent findings suggest that an appropriate environment is needed for the infiltration and metastasis of cancer cells, and that the surrounding mesenchymal cells, paraneoplastic macrophages, fibroblasts, various cytokines, extracellular matrix, and angiogenic factors are required for such an environment (5, 6, 24-27). Therefore, PROK1 is thought to be an important factor. PROK1 expression is frequently detected in advanced-stage colorectal, gastric, and small intestinal cancer. Because vessel formation and hematogenous metastasis are thought to be correlated, we examined the relationship between PROK1 expression and vessel area. Angiogenesis was significantly higher in PROK1-positive tumors than in PROK1-negative tumors, suggesting that PROK1 is an important factor for the infiltration and metastasis of gastrointestinal cancer. Our results indicate that PROK1 is a factor involved in the progression of gastrointestinal cancer.

Conflicts of Interest

The Authors do not have any significant financial interest in any company (or its competitor) producing any of the products discussed in the article. The Authors report no conflicts of interest.

Authors' Declaration

All the Authors have read the manuscript and have approved this submission.

We attest that the research was performed in accordance with the humane and ethical rules for human experimentation that are stated in the Declaration of Helsinki.

The article is original, is not under consideration by any other journal and has not been previously published.

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