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Table 5. Incidence of adverse events and compliance

	Total $n = 179$	Placebo $n = 58$	Sulindac $n = 60$	Etodolac $n = 61$
Adverse events				
Abdominal pain	5 (3.0%)	2 (3.4%)	2 (3.3%)	1 (1.6%)
Heartburn	2 (1.1%)	1 (1.7%)	0 (0%)	1 (1.6%)
Diarrhea	2 (1.1%)	1 (1.7%)	1 (1.7%)	0 (0%)
Exanthema	1 (0.6%)	0 (0%)	1 (1.7%)	0 (0%)
Chest discomfort	1 (0.6%)	0 (0%)	0 (0%)	0 (0%)
Liver dysfunction[a]	2 (1.1%)	1 (1.7%)	0 (0%)	1 (1.6%)
Renal dysfunction[b]	2 (1.1%)	0 (0%)	1 (1.7%)	1 (1.6%)
Average compliance	92.7%	93.9%	91.7%	92.5%

[a]Liver dysfunction was defined as ALT level greater than the upper limit of normal.
[b]Renal dysfunction was defined as creatinine level greater than the upper limit of normal.

that in short-term treatment etodolac, which could not eradicate ACF, was ineffective in suppressing polyp development whereas sulindac was able to inhibit incidence of polyp 1 year after the initiation of treatment by eradicating ACF with short-term treatment.

Incidentally, intragroup analysis showed that in the placebo and etodolac groups, there was a slight tendency of a decrease in ACF number after 2 months although without statistical significance. At present, this cannot be explained but it can be speculated that subjects in these groups, as well as in the sulindac group, became very conscious of their dietary habits after enrollment in the study, which somehow influenced ACF occurrence. In this respect, the analysis among groups may be more reliable than intragroup analysis.

As to the relevance of histology of ACF (dysplastic and nondysplastic ACF) to their development into adenoma, no conclusive result was obtained in this study because 2 histologic types of ACF could not be analyzed separately because of the small proportion of dysplastic ACF in the total ACF population.

Explanation of the relevance of the effect of the drug on ACF to that on polyp development was another important task of the present study. Results showing in both analyses of the number and incidence of adenoma or total polyps either a significant or marked (marginal) reduction in the sulindac group strongly suggest not only the effectiveness of short-term treatment with sulindac in suppressing polyp occurrence but also the utility of ACF as precursor lesions for polyps although the possibility that the reduction in ACF was indirectly related to that of polyp occurrence cannot be completely denied. This notion was further supported by results of the analysis of responders versus nonresponders that showed significantly fewer polyps in the former than in the latter subjects in the sulindac group. Moreover, the average polyp size in the sulindac group was smaller than in the placebo group, although without statistical significance. Further, when the incidence of multiple adenomas was selectively analyzed, though statistically not significant because of the small number of patients, there was some tendency toward a decrease after sulindac treatment ($P = 0.25$). This also supports the notion that by suppressing ACF with sulindac, subsequent occurrence of adenoma may be reduced. In addition, the finding that the difference between the incidence of adenoma in polypectomized subjects in the sulindac group (29.2%) and that in the placebo group (50.0%) was almost the same as in previous studies in which aspirin or NSAIDs were administered over a long-term (7–9) suggests the possibility that 2 months of treatment may be as potent as 1 or 2 years in terms of adenoma prevention. Nonetheless, the suppressive effect of sulindac on adenoma in both the present study and in previous studies was not very substantial, that is, an up to 40% to 50% suppression rate. Thus, a future task is to develop a more effective drug, such as a specific inhibitor of GST-pi, which we showed in our previous reports to be quite potent in eradicating ACF (20, 32). Incidentally, the relatively high polyp recurrence rate after 1 year (43.9%), in agreement with that of a recent report (44.6%; ref. 6), may be because of advancements in endoscopic technology.

In conclusion, our results indicate that ACF may be more advantageous as surrogate lesions than adenomas for chemoprevention of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

A mechanism for abnormal angiogenesis in human radiation proctitis: analysis of expression profile for angiogenic factors

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Abstract
Background Radiation proctitis is an increasingly prevalent problem, with many patients being treated with radiotherapy for pelvic cancers. However, the mechanisms by which radiation proctitis develops in humans are not well understood. In this study, the expression profiles of angiogenic factors were analyzed to clarify their role in the etiology of radiation proctitis.
Methods Rectal biopsies were taken from 8 patients with radiation proctitis and 8 normal subjects. Protein lysates of the tissues were applied to an antibody array for angiogenesis-related factors. The mRNA level of each factor was evaluated by Taqman real-time PCR. Immunohistochemistry was performed using the labeled streptavidin biotin method.
Results Antibody array analysis revealed 2.12- to 7.31-fold higher expression levels of angiogenin, fibroblast growth factor 1 (FGF1), endoglin, matrix metalloproteinase (MMP)-8, urokinase-type plasminogen activator (uPA) and maspin in radiation proctitis tissues compared with normal rectal mucosa. The mRNA level of each factor in radiation proctitis tissue was significantly higher than in normal rectal mucosa, suggesting their transcriptional activation. Immunohistochemical staining showed strong expression of angiogenin and maspin in rectal epithelia, MMP-8 and uPA in infiltrating lymphocytes, FGF1 in fibroblasts and endoglin in endothelial cells. The expression of VEGF was not evident.
Conclusions Our results suggest that in radiation proctitis, MMP-8 and uPA cooperatively degrade the extracellular matrix and basement membrane to provide space for angiogenesis. Simultaneously, angiogenin and FGF1 promote endothelial cell proliferation, and endoglin induces vessel formation, culminating in angiogenesis. Inhibitors of angiogenic factors such as angiogenin and FGF1 may be effective for treating radiation proctitis.

Keywords Radiation proctitis · Angiogenesis · Angiogenic factor

Introduction

Radiation therapy is an important modality for cancer treatment as well as chemotherapy and surgery. It is a critical treatment method for intrapelvic malignancies such as cervical, prostatic, rectal and ovarian cancers. For cervical cancer, radiation therapy alone or a combination with chemotherapy (chemoradiation therapy), as well as surgery, is the first-line treatment for all clinical stages (stages I–IVa). For prostatic cancer, radiation therapy alone or a combination with endocrine therapy, as well as surgery, has been employed as first-line therapy for all cases with T1 to T4 without distant metastasis.

Although early radiation injury, which presents with local pain, mucus secretion and bloody stool, may occur

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during radiation treatment, most of these symptoms spontaneously disappear within a few months, and it rarely develops into a critical problem [1, 2]. In contrast, radiation proctitis is a form of late-radiation injury, occurring in 5–20% of cases treated with pelvic radiation, that typically emerges after 4 or more months post-irradiation and becomes the dose limiting toxicity for radiation therapy [2–4]. Radiation proctitis presents clinically with rectal bleeding, diarrhea, abdominal pain, fistulation and stenosis. The most common of these symptoms is rectal bleeding, which often impairs quality of life (QOL) and sometimes requires blood transfusion [5–7]. Radiation proctitis generally persists for life because there is no appropriate curative treatment.

Rectosigmoidal endoscopy in radiation proctitis shows proliferation of abnormal telangiectatic vessels and their bleeding, often accompanied by erosion and ulceration [8, 9]. Histopathologically, telangiectasia is seen with bleeding in the upper layer of the lamina propria and lymphocyte infiltration and fibrosis in the lamina propria [10, 11].

It has been reported that the main pathological findings in early radiation injury are acute inflammation and fibrosis caused by fibroblast proliferation, while in late radiation injury, abnormal angiogenesis is predominantly observed [10]. Liu et al. [12] investigated the expression of transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) at 1–3 months after irradiation of rat bowels and found that TGF-β was predominantly overexpressed at 1 and 2 months, causing fibrosis, and VEGF was expressed at 3 months, which induced angiogenesis. It was presumed that fibrosis and angiogenesis were induced by TGF-β and VEGF overexpression, respectively, in early radiation injury in animals.

Despite the significant clinical impact of late radiation injury, which is also characterized by abnormal angiogenesis, the role of angiogenic factors in this condition has not been investigated in animals or humans. Thus, the mechanism of onset of radiation proctitis remains to be elucidated. Should the mechanism be clarified, it may be possible to develop drugs to treat radiation proctitis with angiogenic factors as their target, and thus higher radiation doses could also be used, leading to improvement in cure rates.

In this study, the comprehensive expression profile of various angiogenic factors in tissues of patients with radiation proctitis was analyzed using an antibody array, and the mechanisms of onset were investigated. The analysis showed that the expression of VEGF, a major angiogenic factor expressed in animals with early radiation injury, was not evident, whereas angiogenin, fibroblast growth factor 1 (FGF1), maspin, matrix metalloproteinase-8 (MMP-8), endoglin and urokinase-type plasminogen activator (uPA) were overexpressed and supposed to accelerate abnormal angiogenesis in radiation proctitis.

Methods

Patients and samples

This study was approved by the Institutional Review Board at the University of Tokushima Graduate School. Written informed consent was obtained from all patients and healthy volunteers.

Eight patients with radiation proctitis (male/female, 6/2; average age, 67 ± 10.4 years) and 8 healthy volunteers (male/female, 5/3; average age, 65.3 ± 7.6 years) were enrolled in this study. Diagnosis of radiation proctitis was made according to the criteria of Cavcić et al. [13]. Briefly, the diagnosis requires a history of radiation therapy for an intrapelvic malignancy; characteristic colonoscopic findings such as erythema, edema, friability, ulcerations and telangiectasia; and histopathological exclusion of other diseases.

All 6 male patients with radiation proctitis received 70-Gy external irradiation for prostate cancer, and the 2 female patients received 50-Gy external irradiation and 20- or 27-Gy internal irradiation for uterine and vaginal cancers, respectively. All of the patients were referred to our department with the chief complaint of bloody stools. The mean period from the end of irradiation to the examination was 15.4 ± 10.2 months (range 7–38 months).

Biopsy samples were endoscopically taken from the telangiectatic lesions or non-telangiectatic lesions and snap frozen for antibody array analysis and Taqman real-time PCR, while the remaining samples were fixed in 10% formalin. The biopsy sites were treated by argon plasma coagulation. In the control group of healthy subjects, biopsy samples were taken at random from the rectal mucosa after total colonoscopy.

Antibody array

An antibody array (Proteome Profiler™ Array Human Angiogenesis Array, R&D Systems, Minneapolis, MN, USA), which could detect 55 angiogenic factors, was used according to the manufacturer's instructions. Briefly, fresh frozen samples that had been stored at −80°C were thawed, dissolved in lysis buffer [1% Triton-X-phosphate buffered saline (PBS)], and centrifuged to remove cellular debris. The samples were then incubated with biotin-labeled detection antibody for 1 h at room temperature. The sample–antibody mixtures were reacted with the membrane, on which capture antibodies for angiogenic factors were fixed, and incubated overnight at 4°C. The membrane was then washed and incubated with streptavidin-horseradish peroxidase (HRP) for 30 min. The membranes were developed using Chemiluminescent Detection Reagents (GE Healthcare, Buckinghamshire, UK) and visualized using a luminescent image analyzer (LAS3000-UVmini, Fujifilm, Tokyo, Japan).

Taqman real-time PCR

Total RNA was extracted using RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The extracted total RNA was reverse transcribed into complementary DNA (cDNA) with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The concentration and amount of total RNA were determined by UV spectrophotometry. Probes and primers were from TaqMan gene expression assay reagents (Applied Biosystems). The following TaqMan gene expression assays were used: angiogenin (Hs00265741_s1, Applied Biosystems), maspin (Hs00184728_m1), MMP-8 (Hs01029057_m1), endoglin (Hs00164438_m1), uPA (Hs00170182_m1), FGF1 (Hs00265254_m1) and GAPDH (Hs99999905_m1) as an internal control. Quantitative PCR was performed on a 7500 Real Time PCR System (Applied Biosystems). PCR amplification conditions were 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The measured value was calculated by comparative threshold-cycle number (Ct) methods, and GAPDH gene amplification was used as an internal control. In order to determine the efficiency of each Taqman gene expression assay, standard curves were generated by serial dilution of cDNA, and quantitative evaluations of target and internal control gene levels were obtained by measuring Ct. Statistical significance was determined by the Mann–Whitney U test. Differences were considered significant at $p < 0.05$.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method with labeled streptavidin-biotin (LSAB, Dako, Kyoto, Japan), according to the manufacturer's instructions. Briefly, 3-μm-thick sections were cut from formalin-fixed paraffin-embedded tissues and mounted on slides. Sections were deparaffinized in xylene and hydrated in graded ethanol solutions (100, 95, 80, 70%) and PBS. The endogenous peroxidase was inactivated by incubation with 0.3% H_2O_2-MeOH. Subsequently, the slides were heated in 0.01 M citrate buffer in a water bath at 95°C (pH 6.0) for 15 min. Primary antibodies used were mouse anti-human CD31 monoclonal antibody (Dako), rabbit anti-human maspin polyclonal antibody (Sigma, St Louis, MO, USA), rabbit anti-human MMP-8 polyclonal antibody (Bioworld, Minneapolis, MN, USA), rabbit anti-human uPA polyclonal antibody (Bioworld), rabbit anti-human endoglin polyclonal antibody (Thermo Fisher Scientific, Fremont, CA, USA), goat anti-human angiogenin polyclonal antibody (R&D systems) and goat anti-human FGF1 polyclonal antibody (R&D systems). The sections were incubated with primary antibodies, washed with PBS and incubated with secondary biotinylated antibody from an LSAB+ peroxidase kit (Dako). Subsequently, the sections were incubated with HRP and visualized with DAB chromogen (3′,3-diaminobenzidine, Dako). Finally, the sections were counterstained with Mayer's hematoxylin.

Results

Endoscopic appearance and histological features of radiation proctitis

A representative endoscopic view of radiation proctitis from patients enrolled in this study is shown in Fig. 1a. Abnormally dilated blood vessels, namely telangiectasia, were observed in the lower rectum. These lesions existed in the superficial layer of the rectal mucosa, and the remaining intervening normal mucosa looked slightly pale. Histological examination of a biopsied specimen revealed obvious telangiectasia in the upper layer of the lamina propria and lymphocyte infiltration and fibrosis in the lamina propria (Fig. 1b). Immunohistochemical staining for CD-31, an endothelial cell marker, confirmed that telangiectatic lesions indeed existed in the upper layer of the lamina propria (Fig. 1c).

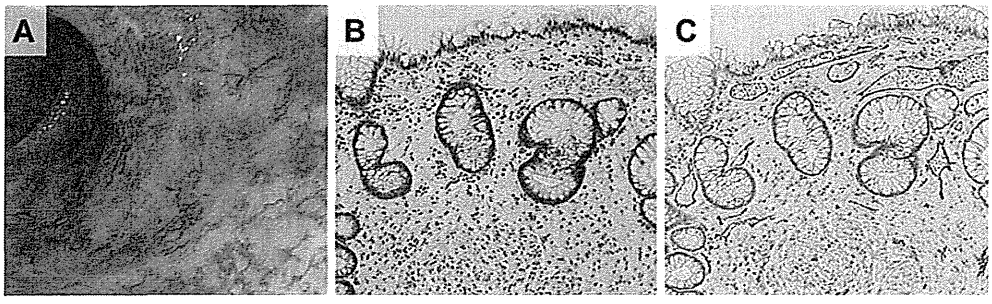
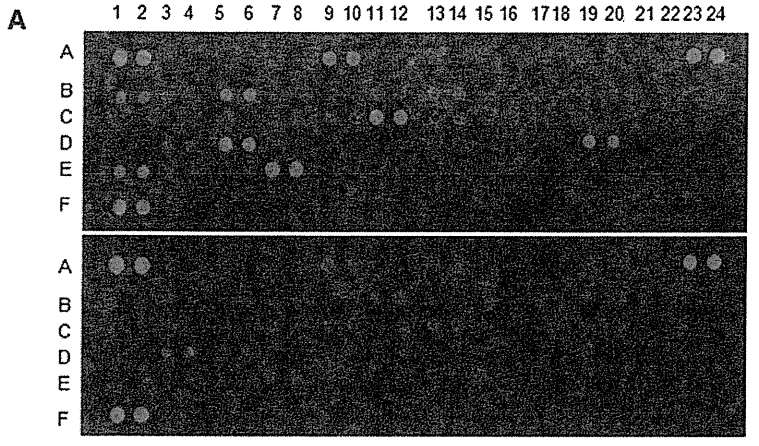


Fig. 1 Representative endoscopic and histological appearances of radiation proctitis. **a** Abnormally dilated blood vessels, a characteristic of radiation proctitis, were observed in rectal mucosa by endoscopy. **b** Telangiectasia and lymphocyte infiltration in the lamina propria were seen in H&E stained section (magnifications ×100). **c** Immunohistochemical staining for CD31, a marker for endothelial cells, confirmed that telangiectatic lesions existed mainly in the upper layer of the lamina propria



B

	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22	23,24
A	Positive Control		ActivinA	ADAMTS1	ANG	Ang1	Ang2	Angio-statin	AREG	Artemin		Positive Control
B	TF	CXCL16	DPPIV	EGF	EG-VEGF	Endoglin	Endostatin	ET1	FGF 1	FGF 2	FGF4	FGF7
C	GDNF	GM-CSF	HB-EGF	HGF	IGFBP1	IGFBP2	IGFBP3	IL1β	IL8	TGFβ1	Leptin	MCP1
D	MIP1α	MMP-8	MMP-9	NRG1β1	PTX3	PD-ECGF	PDGF-AA	PDGF-AB	Persephin	PF4	PLGF	Prolactin
E	Maspin	PAI1	PEDF	TIMP1	TIMP4	TSP1	TSP2	uPA	Vasohibin	VEGF	VEGF-C	
F	Positive Control											Negative Control

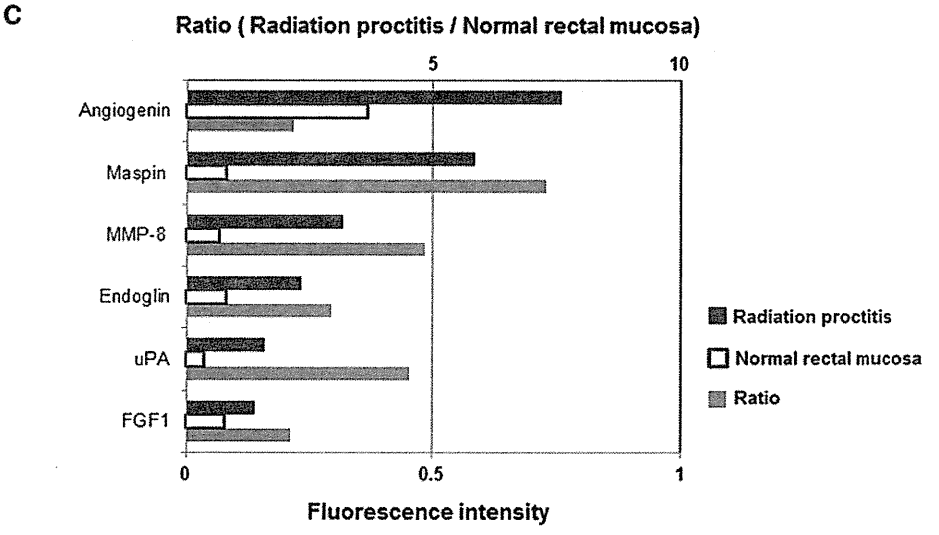


Fig. 2 The expression profile of angiogenic factors in radiation proctitis. **a** Expression of 55 angiogenic factors was detected by antibody array for radiation proctitis and normal rectal tissue. Three biopsy specimens from one patient were dissolved in the lysis buffer and applied to antibody analysis as described in "Methods." **b** The locations of antibodies for angiogenesis-related protein and positive and negative controls on the array are indicated on the chart as follows: *ADAMTS1* a disintegrin and metalloproteinase with thrombospondin motifs 1, *ANG* angiogenin, *Ang* angiopoietin, *ARGE* amphiregulin, *TF* coagulation factor III, *CXCL16* CXC chemokine ligand 16, *DPPIV* dipeptidyl-peptidase IV, *EGF* epidermal growth factor, *EG-VEGF* endocrine gland-derived vascular endothelial growth factor, *ET* endothelin, *GDNF* glial cell line-derived neurotrophic factor, *GM-CSF* granulocyte macrophage colony-stimulating factor, *HB-EGF* heparin-binding EGF-like growth factor, *HGF* hepatocyte growth factor, *IGFBP* insulin-like growth factor-binding protein, *TGF* transforming growth factor, *MCP* monocyte chemoattractant protein, *MIP* macrophage inhibitory protein, *MMP* matrix metallopeptidase, *NRG* neuregulin, *PTX* pentraxin, *PD-ECGF* platelet-derived endothelial cell growth factor, *PDGF* platelet-derived growth factor, *PF* platelet factor, *PLGF* placental growth factor, *PAI* plasminogen activator inhibitor, *PEDF* pigment epithelium-derived factor, *TIMP* tissue inhibitor of metalloproteinase, *TSP* thrombospondin, *uPA* urokinase-type plasminogen activator. **c** Expressions of angiogenin, FGF1, endoglin, maspin, MMP-8 and uPA were greater in radiation proctitis tissue than in normal rectal tissue

Identification of angiogenic factors in radiation proctitis by antibody array

To clarify the mechanism of abnormal angiogenesis, expression profiles of angiogenic factors in radiation proctitis were investigated in comparison with those in normal rectal tissue using the antibody array, which can detect 55 angiogenic factors. A representative result is shown in Fig. 2c. Angiogenin, FGF1, endoglin, maspin, MMP-8 and uPA were overexpressed by 2.12- to 7.31-fold in radiation proctitis as compared with normal rectal tissue. The same experiments repeated 3 times in 3 other patients also showed similar results in that all 6 angiogenic factors were overexpressed in radiation proctitis tissues. The major angiogenic factor, VEGF, was not overexpressed in radiation proctitis tissue compared with the normal rectal mucosa (0.91- to 1.32-fold). Antibody array analyses of rectal tissues from patients with active ulcerative colitis were also performed as a control applying the same method. Overexpression of angiostatin, angiopoietin-2, MMP-8, uPA, placental growth factor (PLGF) and interleukin-1β (IL-1β) were observed (Supplemental Fig. 1). Thus, the expression pattern of angiogenic factors in radiation proctitis was different from that in inflammatory bowel disease (ulcerative colitis).

mRNA levels of angiogenic factors in radiation proctitis tissue

The mRNA level was measured by Taqman real-time PCR for each angiogenic factor that showed protein overexpression in radiation proctitis tissue. The results showed that the mRNA levels of angiogenin, maspin, MMP-8, endoglin, uPA and FGF1 in the telangiectasia of patients

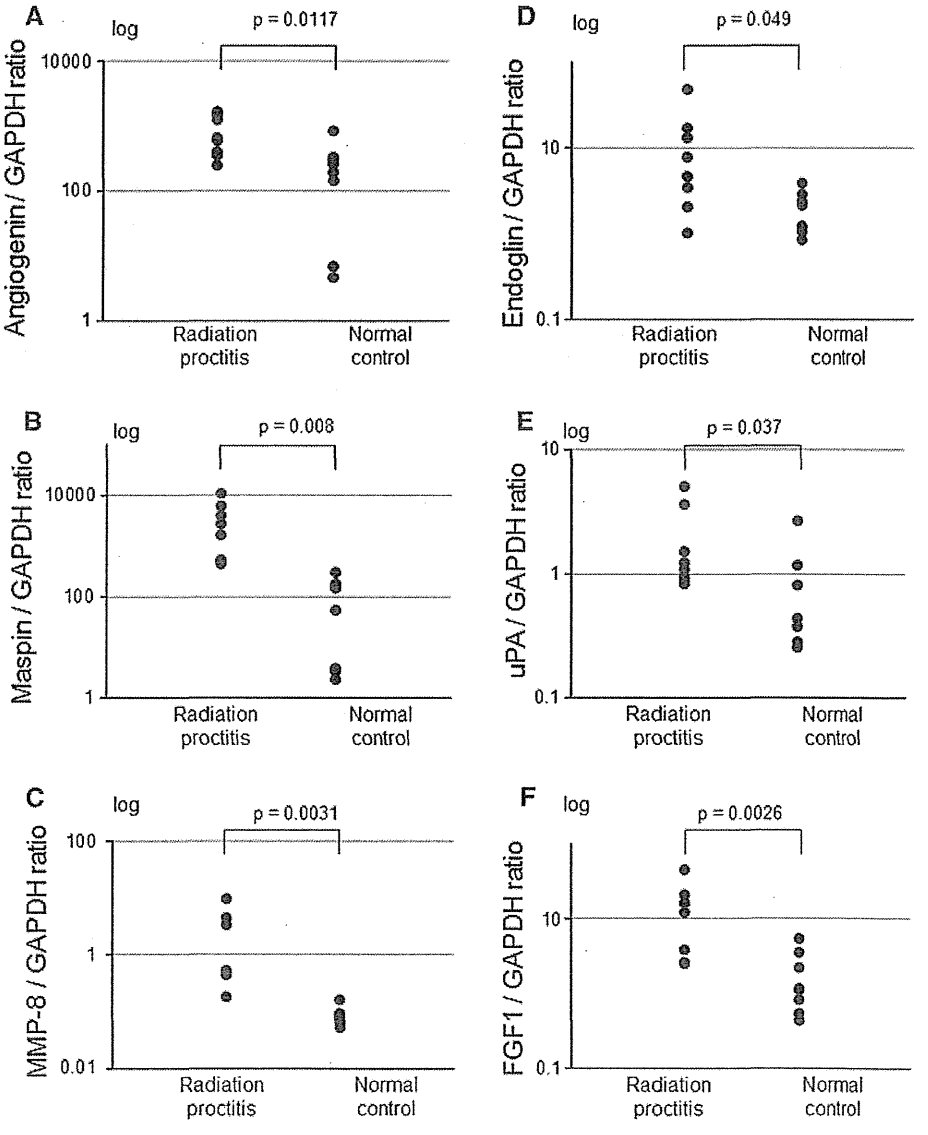


Fig. 3 Comparison of angiogenic factor mRNA levels between radiation proctitis and normal rectal mucosa. **a** Angiogenin; **b** Maspin; **c** MMP-8; **d** Endoglin; **e** uPA; **f** FGF1. The mRNA levels of all angiogenic factors in radiation proctitis tissue were significantly higher than those in normal rectal mucosa. Gene expression values were expressed as ratios between each angiogenic factor and an internal reference gene (GAPDH)

with radiation proctitis were significantly higher ($p < 0.05$) than those in normal rectal tissues (Fig. 3a–f, $p < 0.05$), indicating that these angiogenic factors were overexpressed by transcriptional activation.

The mRNA levels of angiogenic factors in the non-telangiectatic areas from patients with radiation proctitis were also analyzed (Supplemental Table 1). The mRNA levels of uPA, MMP-8 and FGF1 were significantly higher than in normal mucosa and were similar to those levels in telangiectatic areas. The mRNA values for angiogenin, maspin and endoglin were relatively higher than in normal mucosa, but without statistically significant differences.

The relationship between mRNA levels and the period after irradiation was also analyzed (Fig. 4). Interestingly, there were significant positive correlations between the mRNA levels of MMP-8, endoglin or FGF1 and the time after irradiation (from 7 to 38 months). However, no statistically significant correlations were observed between those values for angiogenin, maspin or uPA and the time after irradiation. Expression of the latter three factors remained at almost the same level and did not decline over time.

Immunohistochemical staining of angiogenic factors in radiation proctitis tissue

Since there are several kinds of stromal cells, including infiltrating lymphocytes, fibroblasts and endothelial cells, as well as epithelial cells in radiation proctitis tissues, immunohistochemical staining was performed to investigate the

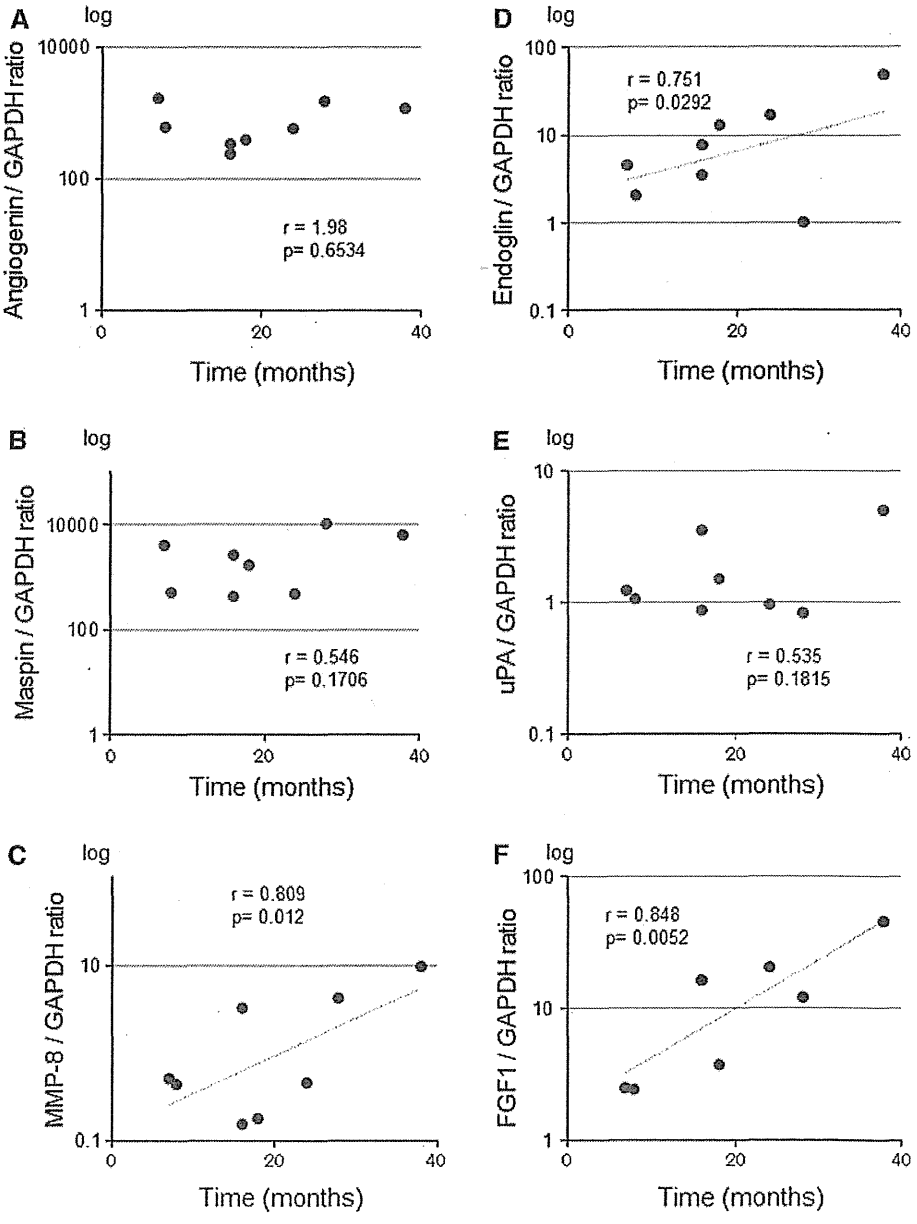


Fig. 4 The relationship between mRNA levels and the time after irradiation. **a** Angiogenin; **b** Maspin; **c** MMP-8; **d** Endoglin; **e** uPA; **f** FGF1. Significant correlations were observed between mRNA levels of MMP-8, endoglin or FGF1 and the time after irradiation (from 7 to 38 months). No significant correlations were observed between the mRNA levels of angiogenin, maspin or uPA and the time after irradiation

localization of each angiogenic factor. For each angiogenic factor, 5 different tissue specimens from radiation proctitis patients were stained; the representative staining patterns for each factor are shown in Fig. 5.

Angiogenin staining was sparsely positive in epithelial cells, particularly in the basal side of the epithelial cytoplasm (Fig. 5a). Although the reason for the sparse staining pattern was unclear, angiogenin was reported to be heterogeneously stained in prostatic neoplasms [14]. Maspin staining was strong in the cytoplasm of epithelial cells and weak in infiltrating lymphocytes (Fig. 5b). MMP-8 staining was evident in the cytoplasm of infiltrating lymphocytes and plasma cells. MMP-8 showed weak staining in epithelial cells (Fig. 5c). It was found that MMP-8 was positive in the CD20 (+) cells but not in the CD3 (+) cells by double immunofluorescence, indicating that MMP-8 was expressed in infiltrating B cells (Supplemental Fig. 2).

Staining for endoglin was intense in the cell membrane and cytoplasm of vascular endothelial cells of vessels (Fig. 5d). uPA staining was strong in infiltrating lymphocytes but was very faint in endothelial and epithelial cells (Fig. 5e). FGF1 staining was observed in fibroblasts, which often showed proliferation in radiation proctitis tissue, but not in epithelial cells or infiltrating lymphocytes (Fig. 5f).

Discussion

In this study, the protein expression profile of angiogenic factors was investigated for the first time in tissues of

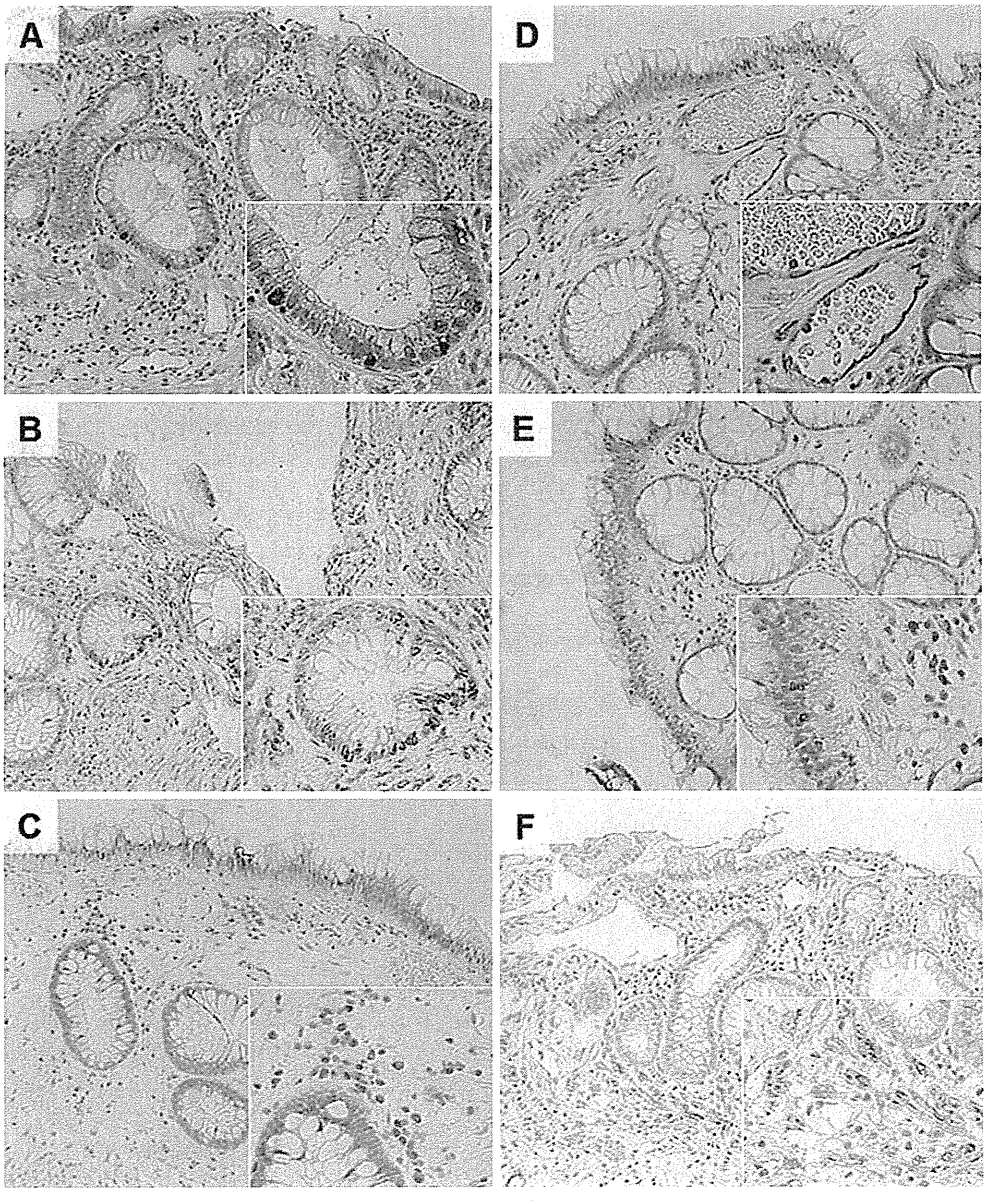


Fig. 5 Immunohistochemical staining for angiogenic factors in radiation proctitis. **a** Angiogenin showed sparse staining in epithelial cells. **b** Maspin showed strong staining in epithelial cells and weak staining in infiltrating lymphocytes. **c** MMP-8 was positive in infiltrating lymphocytes and epithelial cells. **d** Endoglin showed intense staining in vascular endothelial cells. **e** uPA showed strong staining in infiltrating lymphocytes but very faint staining in endothelial cells and epithelial cells. **f** FGF1 showed staining in fibroblasts. (magnification ×200, ×400)

humans with radiation proctitis, and it was demonstrated that angiogenin, FGF1, endoglin, uPA, MMP-8 and maspin were overexpressed in these lesions. It was also revealed that overexpression of these angiogenic factors was induced by transcriptional activation.

From the results of our experiments, the developmental mechanism of radiation proctitis in humans is envisioned as follows. First, MMP-8 and uPA, produced by infiltrating lymphocytes (B lymphocytes), cooperatively degrade the extracellular matrix (ECM) and basement membrane to provide space for angiogenesis. Simultaneously, angiogenin and FGF1, secreted from rectal epithelial cells and fibroblasts, stimulate proliferation of endothelial cells. Then, endoglin stimulates vessel formation by endothelial cells, leading to angiogenesis (Fig. 6). On the other hand, maspin is known to suppress migration and activation of endothelial cells. Therefore, maspin may inhibit angiogenesis in radiation proctitis. In the analysis of mRNA levels in relation to the time after irradiation, there were significant positive correlations between the mRNA levels of MMP-8, endoglin or FGF1 and the time after irradiation (7–38 months). However, the mRNA levels of angiogenin, maspin and uPA remained at almost the same level and did not decline over time. These results are consistent with the fact that radiation proctitis frequently develops within a few years and lasts for a long time of life.

It is well known that MMP induces the degradation of ECM and the basement membrane, whereas the tissue inhibitor of metalloproteinase (TIMP) inhibits MMP and promotes collagen formation in various organs. These two factors are physiologically well balanced in normal gastrointestinal mucosa as well as in other normal organs. However, in the patients with radiation proctitis, MMP was overexpressed, while TIMP was not, suggesting that ulceration and erosion are more likely to occur in these lesions. This is consistent with previous reports that radiation proctitis is often accompanied by rectal ulceration and erosion [15, 16].

Liu et al. [12] examined VEGF expression in rat rectal tissues at 1 to 3 months after 25 Gy (1 fraction) irradiation and found that it peaked at 3 months. Vujaskovic et al. [17] also reported that VEGF was overexpressed in rat lung tissues at 6 months after 28 Gy (1 fraction) irradiation. In the present study, at 7–38 months (15.4 ± 10.2 months) after irradiation the human radiation proctitis tissues did not express VEGF, but did express angiogenin and FGF1. We also confirmed by Taqman real-time PCR that there was no significant elevation of VEGF mRNA (data not shown). The difference between our data and those of Liu et al. or Vujaskovic et al. may be attributable to the difference in species studied or irradiation methodology.

It has been generally accepted that tissue hypoxemia induces angiogenic factors in late radiation injury. That is, irradiation impairs endothelial cells in the microvessels and causes hypoxemia. Subsequently, transcription factors such as hypoxia-inducible factor (HIF) are induced, and they then promote angiogenic factors such as VEGF, leading to angiogenesis [18, 19]. This postulation is based on in vitro experiments that showed that irradiation induced HIF in a cancer cell line and that HIF induced VEGF by binding to the promoter region of the VEGF gene [20–22]. In this study, however, there was no overexpression of VEGF. It is surmised that cancer cell lines inherently produce VEGF and therefore can easily increase VEGF expression

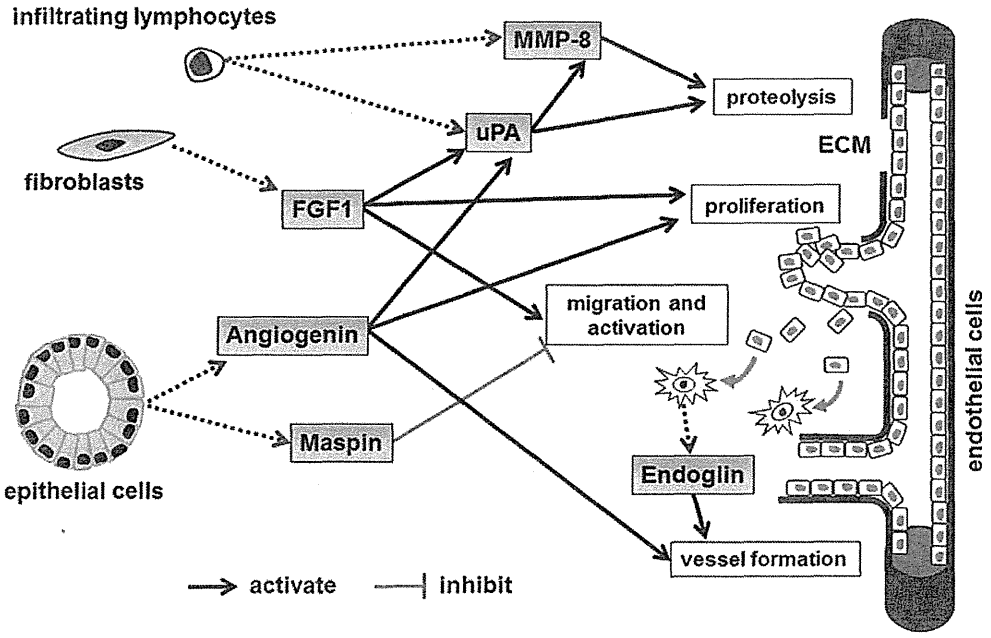


Fig. 6 The postulated mechanism of radiation proctitis development in humans. MMP-8 and uPA, produced by infiltrating lymphocytes, cooperatively degrade the extracellular matrix (*ECM*) and basement membrane to provide space for angiogenesis. Angiogenin and FGF1, secreted from rectal epithelial cells and fibroblasts, stimulate proliferation of endothelial cells. Endoglin stimulates vessel formation of endothelial cells, leading to angiogenesis

following irradiation. However, a non-cancerous tissue, that is, a normal rectal epithelial cell, does not inherently produce HIF and VEGF, and thus non-cancerous radiation proctitis tissue did not show VEGF expression.

In radiation proctitis, angiogenesis is induced abnormally in the superficial layer of the lamina propria but not in the submucosal layer, as would occur in normal tissues, and bleeding occurs easily from these abnormal vessels. However, the mechanism accounting for angiogenesis in the superficial mucosal layer remains unclear. It was reported that TGF-β overexpression promotes widespread fibrosis in the submucosal layer in acute radiation injury within 3 months after irradiation [23–25]. This fibrosis already present in the submucosal layer may cause abnormal angiogenesis in the superficial layer of the lamina propria in the late stages of radiation injury. The predominant environment of MMP rather than TIMP may also promote angiogenesis in the mucosal layer.

The mechanism responsible for the induction of angiogenin and FGF1 overexpression in this study remains elusive. Although there has been one report of angiogenin induction under hypoxic conditions [26], the precise mechanism is unclear. Moreover, the mechanism for induction of FGF1 remains to be elucidated. To clarify the mechanism by which these angiogenic factors are induced, further study is warranted.

Radiation proctitis is a refractory disease without effective treatment methods. However, the inhibitors of major angiogenic factors observed in this study, such as angiogenin and FGF1, may be effective for the treatment of radiation proctitis. Moreover, they also may be effective for other forms of radiation injury such as radiation pneumonitis, dermatitis, pleuritis, pericarditis and myelitis.

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ORIGINAL

Our experience of treatment of cribriform morular variant of papillary thyroid carcinoma; difference in clinicopathological features of FAP-associated and sporadic patients

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Abstract. Cribriform-morular variant (CMV) is a comparably rare histological subtype of papillary thyroid carcinoma (PTC). This can be associated with familial adenomatous polyposis (FAP) due to *APC* gene mutations. In this study, we investigated the difference in the biological characteristics between FAP-associated and sporadic CMV. Between 1991 and 2010, 32 patients with CMV were treated in Kuma Hospital. Thirty-one of these underwent initial surgery for CMV in Kuma Hospital. Twelve patients were FAP-associated and the remaining 19 were sporadic CMV. All patients were female. Tumors of FAP-associated CMV were more frequently multiple than those of sporadic CMV. Patient age and tumor size did not differ between the two groups. Of 12 FAP-associated CMV, 5 were detected by thyroid nodule (thyroid precedent group) and 7 were detected by FAP (polyposis precedent group) as an initial manifestation. Patient age was younger and tumor size was smaller in the polyposis group than in the thyroid nodule group. All patients lacked extrathyroid extension on intraoperative finding and were node-negative on pathological examination. To date, two patients with FAP-associated CMV who initially underwent hemithyroidectomy (one in Kuma Hospital and one in another hospital) showed recurrence to the remnant thyroid during follow-up. None of the patients showed recurrence to other regions or died of carcinoma. Taken together, CMV is considered an indolent disease in our series. FAP-associated CMV showed multiple tumors more frequently than sporadic CMV. Total thyroidectomy is recommended for FAP-associated CMV, but extensive lymph node dissection is not necessary.

Key words: Cribriform morular variant, Papillary thyroid carcinoma, FAP, Prognosis

PAPILLARY thyroid carcinoma (PTC) is the most common malignancy arising from thyroid follicular cells. There are many histological subtypes of PTC and some of them showed a different prognosis from conventional PTC. Cribriform-morular variant (CMV) is a rare subtype of PTC. Historically, in 1949, Crail first reported malignancies originating from the rectum, brain and the thyroid gland [1]. In 1968, the relation between familial adenomatous polyposis (FAP) and thyroid carcinoma was reported [2]. Furthermore, one study showed that young women with FAP had approximately 160 times more risk of thyroid carcinoma than healthy people [3]. In 1994, Harach *et al.* demonstrated unique histological features of FAP-associated PTC such as a cribriform pattern and solid areas with a spindle cell component [4]. The *APC* gene located in the 5q21 region is strongly associated with FAP and individuals with *APC* mutations have almost 100% risk of developing colorectal carcinoma [5, 6], allowing us to conclude that this variant is significantly associated with *APC* mutations. Other studies have also reported FAP-associated CMV [7, 8], but CMV can occur also sporadically without any relation to colonic polyposis [8-10].

In 2004, we reported 7 patients with CMV [11]. Three

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of these patients were FAP-associated CMV because they had FAP or a family history of colonic carcinoma involving *APC* gene mutations. The remaining 4 were sporadic and lacked *APC* gene mutations. However, primary lesions in all of these patients were detected by thyroid nodule as an initial manifestation. Recently, the relationship between CMV and FAP has been more widely understood and CMV has been detected during thyroid screening in FAP patients without complaint of thyroid nodule.

In this study, we investigated the biological characteristics of CMV in 32 patients. Thirty-one patients underwent initial surgery in Kuma Hospital. Twenty of these patients were FAP-associated and the remaining 19 were sporadic CMV. We investigated the difference in clinicopathological features between these two groups.

Patients and Methods

Between 1991 and 2010, 32 patients with PTC underwent surgery and pathologically diagnosed as CMV in Kuma Hospital, who were enrolled in this study. All patients were females and patient age ranged from 17 to 38 years (average 27 years). These 32 patients underwent ultrasound-guided fine needle aspiration biopsy (FNAB) and were cytologically diagnosed as having PTC. Sixteen (50%) were also diagnosed as or highly suspected of CMV based on cytological findings such as the presence of a morular component, peculiar nuclear clearing, absence of colloid in the background and immunocytochemistry finding such as nuclear and cytoplasmic localization of beta-catenin and high positivity of estrogen receptor (ER) [12, 13].

Thirty-one of these patients underwent initial surgery in Kuma Hospital. Twelve of these patients (31%) were FAP-associated CMV because they had one or more of the following three features; 1) colonic polyposis was detected on total colonoscopy before or after thyroid surgery; 2) presence of *APC* gene mutations; and 3) family history of colonic polyposis or colorectal carcinoma. CMV in 5 patients was detected by thyroid nodule based on patient complaints or findings on medical check-up as the initial event. These patients underwent total colonoscopy after thyroid surgery and were diagnosed as having polyposis. The remaining 7 underwent thyroid ultrasound for screening after the detection of FAP and were referred to our hospital because thyroid nodules were found. None of these patients had complained of thyroid nodule prior to its detection. Nineteen patients (69%) were diagnosed as having sporadic CMV without any of the 3 features, which includes 3 patients who had no family history of colonic polyposis or colorectal carcinoma and rejected examination of colonic polyposis.

The extent of thyroidectomy for FAP-associated CMV was total thyroidectomy in 11 patients and hemithyroidectomy in 1 patient. That for sporadic CMV was total thyroidectomy in 9 patients and hemithyroidectomy in 10 patients. Completion total thyroidectomy was performed as the second surgery in one patient who underwent initial surgery at another hospital. All patients underwent central node dissection. Three patients with FAP-associated CMV and 8 with sporadic CMV also underwent modified radical neck dissection (MND). All patients were diagnosed as having CMV on postoperative pathological examination based on morphological findings such as cribriform pattern, morular component, lack of colloid, peculiar nuclear clearing and immunohistochemical findings of beta-catenin, ER and progesterone receptor (PgR) [4, 8, 9, 14].

Our series included 7 patients reported in our previous study who underwent *APC* gene mutation analysis [11]. The *APC* gene mutation was not analyzed in other patients.

None of the patients in our series underwent radioactive iodine (RAI) ablation or RAI therapy. After surgery, all patients were followed by ultrasound at least once per year. The follow-up periods of these 31 patients ranged from 4 to 217 months (average 59 months).

Fisher's exact test and Mann-Whitney U test were adopted for comparing variables. p values less than 0.05 were considered significant.

Results

Of 32 patients with CMV, 31 underwent initial surgery in Kuma Hospital. Twelve of these patients (39%) were diagnosed as having FAP-associated CMV because they had one or more of the following three features as indicate above, and the remaining 19 (61%) demonstrated sporadic CMV. Nine patients with FAP-associated CMV (75%) and 7 with sporadic CMV (37%) were preoperatively diagnosed as having or highly suspected of CMV based on the cytological findings indicated above. Multinodular goiter and euthyroid Graves' disease were detected in 3 patients and 1 patient with sporadic CMV, respectively. No

Table 1 Comparison of multiplicity on ultrasound for FAP-associated and sporadic CMV

	Multilplicity (%)		
	Solitary	Multiple	Total
FAP-associated CMV	3 (25)	9 (75)	12
Sporadic CMV	19 (100)	0	19
Total	22	9	31

$p = 0.00001$

Table 2 Comparison of multiplicity on pathological examination for FAP-associated and sporadic CMV

	Multilplicity (%)		
	Solitary	Multiple	Total
FAP-associated CMV	2 (17)	10 (83)	12
Sporadic CMV	17 (89)	2 (11)	19
Total	19	12	31

$p = 0.0005$

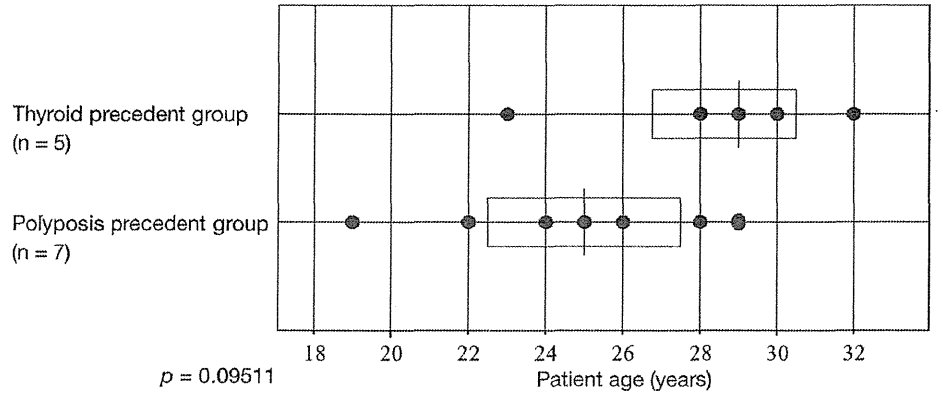

Fig. 1 Comparison of age of FAP- associated patients between the thyroid precedent group and polyposis precedent group.

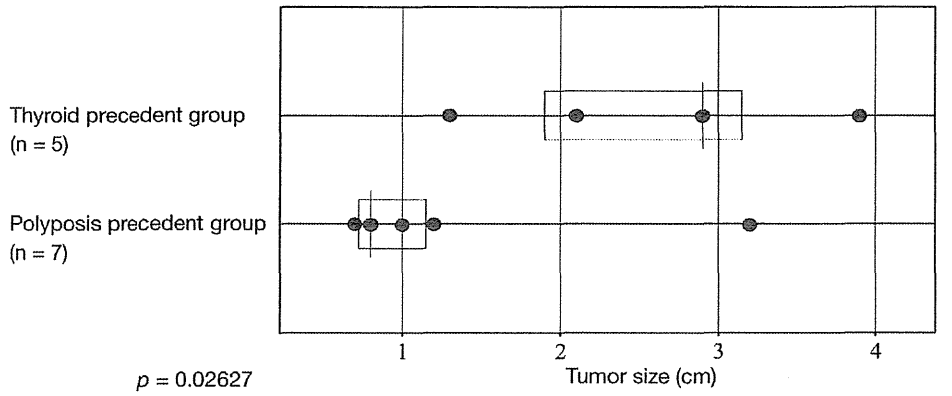

Fig. 2 Comparison of tumor size of FAP- associated patients between the thyroid precedent group and polyposis precedent group.

other thyroid comorbidities can be observed in FAP-associated CMV.

We compared the clinicopathological features between the two groups. Tumor size (1.79 + 1.15 cm *vs* 2.38 + 0.70 cm) and patient age (26.3 + 3.7 years *vs* 26.8 + 6.6 years) did not significantly differ between FAP-associated and sporadic CMV. Nine of 12 patients (75%) with FAP-associated CMV but none of the sporadic CMV were evaluated as multiple on preoperative ultrasound ($p = 0.00001$) (Table 1). On pathological examination, 10 patients with FAP-associated CMV (83%) and 2 with sporadic CMV (11%) showed multiplicity ($p = 0.00005$) (Table 2).

Diagnosis of CMV in 5 of 12 FAP-associated CMV patients (42%) was based on finding a thyroid nodule (thyroid precedent group) and that in 7 (58%) was based on FAP (polyposis precedent group) as the initial manifestation. Although not significantly different, patient age in the thyroid precedent group (28.4 + 3.4 years) tended to be older than that in the polyposis precedent group (24.7 + 3.5 years) ($p = 0.09511$) (Fig. 1). Tumor size in the thyroid precedent group (2.6 + 1.0 cm) was significantly larger than that in the polyposis precedent group (1.2 + 0.9 cm) ($p = 0.02627$) (Fig. 2).

None of the patients showed extrathyroid extension corresponding to T3 or T4 or lymph node metastasis corresponding to N1 in the UICC TNM classification [13] based on preoperative and intraoperative findings. On pathological examination, all patients were node-negative. To date, two patients with FAP-associated CMV, who underwent hemithyroidectomy at the initial surgery (one in Kuma hospital and one in another hospital) and were diagnosed as having polyposis thereafter, showed recurrence to the remnant thyroid 86 and 90 months after initial surgery, respectively. These patients underwent a second surgery and further recurrence has not been detected to date. Furthermore, one patient died of colorectal carcinoma 64 months after thyroid surgery and one died of lung suppuration unrelated to PTC or colorectal carcinoma 28 months after surgery. The other patients have survived without any evidence of PTC recurrence to date.

Discussion

There are two types of CMV etiology; FAP-associated and sporadic. Furthermore, there are two initial manifestations of FAP-associated CMV detection: thyroid nodule and FAP. We compared clinicopathological features between the two groups.

CMV can be diagnosed by preoperative cytological findings [12]. In our series, 75% of FAP-associated CMV and 37% of sporadic CMV were diagnosed as or highly suspected of CMV on cytology. The higher incidence in FAP-associated CMV is definitely because we ordered immunocytochemistry for beta-catenin and ER for patients who were known to have FAP.

Since it is well-known that CMV can be associated with FAP, screening of colonic polyposis by total colonoscopy should be recommended for patients who were diagnosed as having CMV. We demonstrated that FAP-associated CMV showed multiple tumors more frequently than sporadic CMV both on preoperative imaging studies and on pathological examination, indicating that the presence of FAP is highly suspected especially for patients with multiple CMV. In the subset of FAP-associated CMV, patients in the polyposis precedent group were younger and had smaller tumors than those in the thyroid precedent group. It is therefore suggested that thyroid screening for FAP patients contributes to early detection of CMV, even before patients become aware of thyroid nodule.

All patients in our series were node-negative and lacked extrathyroid extension. Furthermore, none of these patients died of carcinoma. These findings indicate that CMV normally shows an indolent character regardless of whether it is FAP-associated, which was not discrepant with previous studies [8, 9, 10, 16]. There is one report of CMV in an older (42 years) male patient who died of carcinoma only 17 months after surgery, but this seems atypical because neuroendocrine differentiation and a poorly differentiated component were present [17]. To date, however, 2 patients who underwent hemithyroidectomy for FAP-associated CMV recurred to the remnant thyroid during follow-up. Based on these findings, total thyroidectomy is recommended for CMV patients with multiple carcinoma lesions, a family history of colonic polyposis or colorectal carcinoma or diagnosed as having colonic polyposis before thyroid surgery. In contrast, none of the sporadic patients showed recurrence to the remnant thyroid, even though they underwent hemithyroidectomy, indicating that total thyroidectomy may not be mandatory for such patients. In our department, prophylactic MND has been performed for PTC patients having tumor larger than 3 cm, because these patients are likely to develop recurrence in regional lymph nodes [18]. However, such an extensive prophylactic node dissection is not needed for patients who were preoperatively diagnosed as having CMV.

Taken together, we showed that FAP-associated CMV showed multiple tumors more frequently than sporadic CMV. Total thyroidectomy is recommended at least for CMV patients associated with FAP, but extensive lymph node dissection is not necessary.

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Magnifying Narrowband Imaging Is More Accurate Than Conventional White-Light Imaging in Diagnosis of Gastric Mucosal Cancer

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BACKGROUND & AIMS: It is difficult to accurately diagnose patients with depressed gastric mucosal cancer based on conventional white-light imaging (C-WLI) endoscopy. We compared the real-time diagnostic yield of C-WLI for small, depressed gastric mucosal cancers with that of magnifying narrow-band imaging (M-NBI). **METHODS:** We performed a multicenter, prospective, randomized, controlled trial of patients with undiagnosed depressed lesions ≤10 mm in diameter identified by esophagogastroduodenoscopy. Patients were randomly assigned to groups that were analyzed by C-WLI (n = 176) or M-NBI (n = 177) immediately after detection; the C-WLI group received M-NBI after C-WLI. We compared the diagnostic accuracy, sensitivity, and specificity between C-WLI and M-NBI and assessed the diagnostic yield of M-NBI conducted in conjunction with C-WLI. **Results:** Overall, 40 gastric cancers (20 in each group) were identified. The median diagnostic values for M-NBI and C-WLI were as follows: accuracy, 90.4% and 64.8%; sensitivity, 60.0% and 40.0%; and specificity, 94.3% and 67.9%, respectively. The accuracy and specificity of M-NBI were greater than those of C-WLI ($P < .001$); the difference in sensitivity was not significant ($P = .34$). The combination of M-NBI with C-WLI significantly enhanced performance compared with C-WLI alone; accuracy increased from (median) 64.8% to 96.6% ($P < .001$), sensitivity increased from 40.0% to 95.0% ($P < .001$), and specificity increased from 67.9% to 96.8% ($P < .001$). **CONCLUSIONS: M-NBI, in conjunction with C-WLI, identifies small, depressed gastric mucosal cancers with 96.6% accuracy, 95.0% sensitivity, and 96.8% specificity. These values are better than for C-WLI or M-NBI alone.**

Keywords: Gastric Cancer; Early Detection; Benign; Malignant; Neoplasm; Biopsy.

Gastric cancer is the fourth most common malignancy and the second leading cause of death from cancer worldwide.[1] Early detection and curative treatment are the best strategies for improving patient survival. Esophagogastroduodenoscopy is the most sensitive method of early detection of gastric cancers. However, an accurate early diagnosis of gastric mucosal cancer is difficult with conventional white-light imaging (C-WLI) endoscopy; nevertheless, it remains the standard endoscopic examination modality worldwide.

Detection of mucosal cancers ≤20 mm in diameter is ideal, because they are curable using minimally invasive treatments such as endoscopic mucosal resection and endoscopic submucosal dissection.[2,3] Among the gastric mucosal cancers, the depressed type is the predominant morphology.[4–6] However, small depressed cancers (≤10 mm in diameter) are more difficult to distinguish from benign abnormalities (such as inflammation) compared with elevated cancers. Although chromoendoscopy using indigo carmine has contributed to an improvement in the diagnosis of gastric mucosal cancers,[7] there is no evidence of the superiority of chromoendoscopy over C-WLI. Therefore, C-WLI endoscopy remains the standard imaging modality for diagnosing gastric mucosal cancers.

Histologic evaluation of biopsy specimens from suspicious lesions is conventionally used to confirm a diagnosis. A highly accurate diagnosis without the need for a biopsy is the ultimate goal of endoscopists, because this would decrease the number of unnecessary biopsies, especially when confirming a negative biopsy of any suspicious cancerous lesion. This could reduce the risk of postbiopsy bleeding, costs associated with the procedure, and the workload on pathologists.

Magnifying narrow-band imaging (M-NBI), a recently developed advanced endoscopic imaging technology, was reported to be useful for the accurate diagnosis of gastric abnormalities such as cancers,[8–13] adenomas,[14] and intestinal metaplasia.[15] However, no randomized trials have been conducted to compare M-NBI with C-WLI. The present study was designed to assess and compare the real-time diagnostic yield of C-WLI for depressed gastric mucosal

Abbreviations used in this paper: CI, confidence interval; C-WLI, conventional white-light imaging; M-NBI, magnifying narrow-band imaging; NPV, negative predictive value; PPV, positive predictive value.

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cancers with that of M-NBI when performed by skilled endoscopists.

Patients and Methods

Study Design and Participants

This randomized, controlled, open-label, multicenter trial was conducted at 9 centers in Japan. This study was conducted according to the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) initiative[16] and the Declaration of Helsinki.

The frequency of synchronous or metachronous multiple gastric cancers was reported as 3 to 5 per 100 patient-years,[17-19] which is higher than the incidence of gastric cancer in the general population. In other words, patients with gastric cancer might constitute a cancer-enriched population, which may be a more suitable model for screening of potential gastric cancers than the general population. Therefore, we recruited patients aged 20 years or older with untreated gastric cancers and patients with a history of gastric cancer. Patients who had been treated with endoscopic mucosal resection or endoscopic submucosal dissection were included in the latter group, because their stomachs were preserved with minimum injury. We excluded patients who had been treated with surgical resection, because the stomach was either removed or was reduced in size. Other exclusion criteria were serious complications that could interfere with the examination protocol and the use of medication that might interfere with the collection of a biopsy specimen. Written informed consent was obtained, and the institutional review board of each participating hospital approved the study. The clinical trial number of this study was UMIN-CTR000001072.

To detect a target lesion, screening was performed using C-WLI endoscopy. Previously undetected lesions were considered ideal potential targets for evaluating the diagnostic yield without bias. Therefore, the target lesions for this study were "newly detected and undiagnosed" small, depressed gastric lesions ≤10 mm in diameter. We did not target lesions that had been analyzed histologically. Small, depressed lesions with apparent erosion or ulceration were also not evaluated, because it is difficult to visualize surface changes in these lesions. If the patient had multiple such lesions, only the first lesion detected was selected for examination. The diameter of each lesion was estimated by comparing it with the size of the biopsy forceps.

Randomization and Masking

When a target small, depressed lesion was detected by C-WLI screening, patients were immediately assigned randomly to undergo detailed examination using C-WLI or M-NBI at a 1:1 ratio. After the randomization, all endoscopists knew which imaging method would be used for the detailed examination when making a diagnosis of the target lesion. Randomization was performed promptly on-site using tables of random numbers stratified by hospital, and the results thereof were kept in sealed, numbered envelopes. The random allocation sequence was prepared at the data management center. Both the assignment result and the corresponding envelope number were recorded by the data management center. At each participating hospital, sealed envelopes were stored by a third party who was not involved in the study, and the envelopes were opened by an assistant physician in serial order only when randomization was performed. The assigned patient identification number, envelope number, and assignment result were recorded on-site and faxed to the data management center on the day of the examination.

Procedure and End Points

The study design and the protocol examination are outlined in Supplementary Figure 1 and Supplementary Materials and Methods. The diagnosis for the target lesion was made by one endoscopist according to predetermined diagnostic criteria for C-WLI and M-NBI without any consultation with other physicians, and an assistant physician immediately recorded the results using a case report form. For each modality, the interval between the start of the observation and the time at which an endoscopic diagnosis was made was measured using a stopwatch. For the C-WLI group, M-NBI examination was performed after completion of a diagnosis based on C-WLI. This procedure was used to evaluate the effect of using M-NBI in conjunction with C-WLI. After all records were compiled, at least one biopsy specimen was obtained from the target lesion.

The primary aim of the study was to compare the diagnostic accuracy between C-WLI and M-NBI. The secondary aim was to compare diagnostic sensitivity, specificity, and examination time between C-WLI and M-NBI and to evaluate the effects of an additional M-NBI study after the initial C-WLI in terms of diagnostic accuracy, sensitivity, specificity, and examination time. Histopathology diagnosis of obtained biopsy specimens was used as a gold standard for the diagnosis.

Endoscopy System

The NBI system is an innovative optical image-enhanced technology that involves a narrow-bandwidth NBI filter in the video endoscopy system. The central wavelengths of the NBI filters are 415 nm and 540 nm, and each has a bandwidth of 30 nm. Because 415-nm and 540-nm light are well absorbed by hemoglobin, the microvascular architecture of the mucosal surface can be visualized readily. Details of this system have been reported elsewhere.[20-22]

We used high-resolution magnifying endoscopy with a capability of 80-fold optical magnification (GIF-Q240Z, GIF-H260Z, and GIF-FQ260Z; Olympus Medical Systems, Tokyo, Japan) and a high-resolution liquid-crystal monitor (OEV191H; Olympus Medical Systems). We alternated between the 2 imaging modalities (C-WLI and M-NBI) by pushing a button on the endoscope (Evis Lucera Spectrum System; Olympus Medical Systems). We used a fixed structure enhancement setting and color tone for the video processor.

Participating Endoscopists

All examinations were performed by 31 endoscopic specialists accredited by the Japan Gastroenterological Endoscopy Society in 9 institutes. Before the onset of the study, all participating endoscopists were trained using images of small, depressed lesions to minimize diagnostic variation between them.

Diagnostic Criteria for C-WLI and M-NBI

Figure 1 shows a representative endoscopic image of a small, depressed gastric cancer and a small, depressed benign lesion. The diagnostic method based on endoscopic findings is outlined in Supplementary Materials and Methods.

The endoscopic diagnostic criteria for small, depressed gastric cancers using C-WLI were defined based on previous reports of C-WLI findings: an irregular margin and a spiny depressed area.[23] The observation of 2 findings (irregular margin and spiny

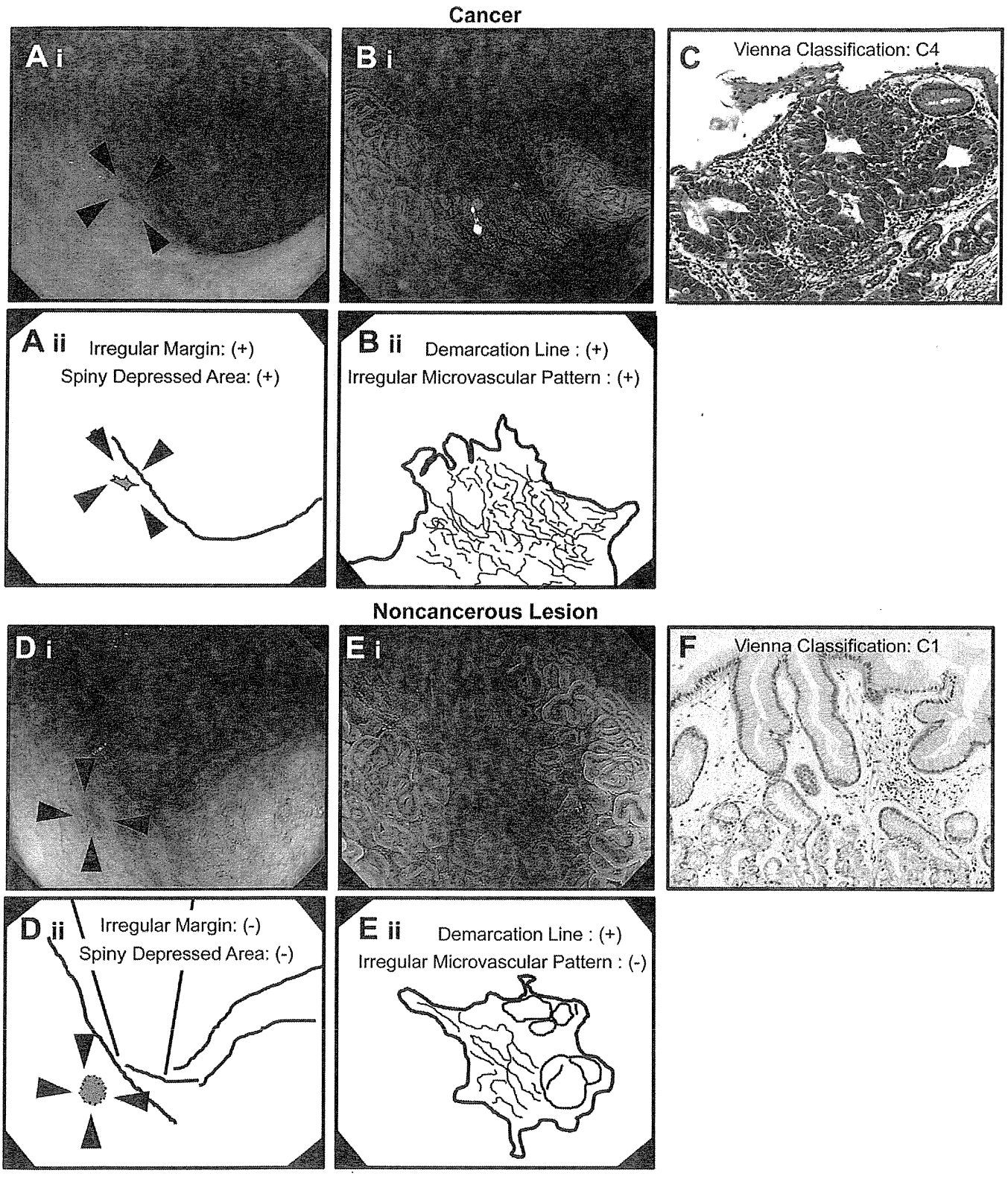

Figure 1. Representative endoscopic findings for gastric small, depressed lesions. *A–C* show a case of cancer, and *D–F* show a case of noncancerous lesions. *A* shows an endoscopic image obtained using C-WLI. A small, depressed lesion (*arrowheads*) is evident in the anterior wall of the lower part of the gastric body. This lesion was evaluated as having an irregular margin and a spiny depressed area. *B* shows an endoscopic image obtained using M-NBI, which enabled clear visualization of the demarcation line and an irregular microvascular pattern. *A′* and *B′* are schematic representations of the images shown in *A* and *B*, respectively. *C* shows a lesion that was histologically diagnosed as a differentiated adenocarcinoma, Vienna Classification C4. *D* shows an image obtained using C-WLI. A small reddish area (*arrowheads*) is evident in the anterior wall of the upper part of the gastric body. Because the depressed area was not "spiny" and because a definite margin was not apparent, this case was evaluated as not having a spiny depressed area or an irregular margin. *E* shows an image obtained using M-NBI, which enabled clear visualization of a demarcation line and the absence of an irregular microvascular pattern. *D′* and *E′* are schematic representations of the images shown in *D* and *E*, respectively. *F* shows a lesion that was histologically diagnosed as gastritis, Vienna Classification C1.

depressed area) in the target lesion was classified according to 3 categories: present, absent, or indeterminate.

The endoscopic diagnostic criteria for small, depressed gastric cancers using M-NBI were defined based on previous reports by Yao et al: a demarcation line between the depressed cancerous lesion and the surrounding noncancerous area and an irregular microvascular pattern inside the lesion.[24] Observations of 2 findings (demarcation line and irregular microvascular pattern) in the target lesion were also classified according to 3 categories: present, absent, or indeterminate.

Endoscopic diagnoses were determined according to the combined visibility of the 2 findings as follows (Supplementary Figure 2). (1) If both findings were present, the diagnosis was "cancer." (2) If either finding was indeterminate, the diagnosis was "inconclusive." (3) If either or both findings were absent, the diagnosis was "noncancerous."

For analyzing diagnostic accuracy, sensitivity, and specificity, lesions diagnosed as "inconclusive" were considered as endoscopic "noncancerous" lesions.

Pathology Diagnosis

The biopsy specimens were evaluated using H&E staining. The diagnostic pathology criteria were based on the revised Vienna classification.[25] C4 (mucosal high-grade neoplasia) or C5 (submucosal invasion by neoplasia) were diagnosed as cancer, and C1 (negative for neoplasia), C2 (indefinite for neoplasia), or C3 (mucosal low-grade neoplasia) were diagnosed as noncancerous lesions. In this study, we used a central system of consultation with a main expert pathologist. If an indeterminate lesion were to be encountered, it was scheduled to be reviewed by this consulting pathologist in making a final diagnosis.

Statistical Analysis

We assumed that the accuracy, sensitivity, and specificity of C-WLI and M-NBI compared with histologic diagnosis would be 60% and 85%, respectively. To set a probability for error of 0.05 and attain a power of 80% for testing the superiority of M-NBI, 108 patients including at least 43 cancerous lesions were needed. Next, we calculated how many patients would need to be screened. Because the frequency of small depressed lesions was reported to be 8.1% in the general population,[9] the required size of the screening sample was 1100 patients.

Statistical analysis was performed using SPSS software, version 17 (SPSS Inc, Chicago, IL). For diagnostic performance, accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are presented as percentages with 95% confidence intervals (CIs). Continuous variables are expressed as medians and interquartile ranges. Analyses of the difference in diagnostic performance between C-WLI and M-NBI were conducted using the population whose diagnoses had been confirmed by pathology using Pearson's χ^2 test. Analyses of the effect of additional M-NBI after the initial C-WLI on diagnostic performance were conducted using the population whose diagnoses had been confirmed by pathology and McNemar testing. Analysis of the examination duration was conducted using the population who completed protocol examination and the Mann–Whitney nonparametric test for comparisons between C-WLI and M-NBI, as well as the Wilcoxon signed rank test for comparisons between C-WLI and C-WLI plus M-NBI. All probability values calculated in this analysis were 2 sided, and $P < .05$ was considered significant.

Results

Between June 2008 and May 2010, 1365 patients were enrolled in the study. Eight patients refused to participate and 4 were registered twice; therefore, the remaining 1353 patients were registered correctly and underwent endoscopic screening. Screening was discontinued for 2 patients because of a large amount of residual digesta in the stomach and a severe vomiting reflex. Endoscopic screening was completed for the remaining 1351 patients.

Of the screened patients, 362 (26.8%) had newly detected and undiagnosed small, depressed lesions and were randomly assigned to one of 2 groups: (1) 180 patients were examined using C-WLI followed by M-NBI, and (2) 182 patients were examined using M-NBI alone. Four patients in the C-WLI group (one patient's lesion was >10 mm in diameter, one was discontinued from the examination because of Mallory–Weiss syndrome, and 2 had a missed biopsy) and 5 patients in the M-NBI group (one was examined with an unpermitted endoscope and 4 missed biopsy) were excluded. Data for 176 patients in the C-WLI group and 177 patients in the M-NBI group were used for the final analysis (Figure 2). The demographic and lesion characteristics of the 2 groups were balanced. In both groups, 13% of patients had newly diagnosed gastric cancer (20 per group; Table 1).

Table 2 shows endoscopic diagnoses for all lesions. Inconclusive diagnoses were obtained for 3 lesions (1.7%) using M-NBI, for 6 lesions (3.4%) using C-WLI, and for 2 lesions (1.3%) using C-WLI followed by M-NBI. These lesions were considered endoscopic "noncancerous" lesions for analysis.

The real-time diagnostic accuracy of M-NBI was significantly greater than that of C-WLI (90.4% [95% CI, 85.1%–94.3%] and 64.8% [95% CI, 57.2%–71.8%], respectively; $P < .001$; Table 3). Real-time M-NBI diagnosis had greater specificity than C-WLI diagnosis (94.3% [95% CI, 89.4%–97.3%] and 67.9% [95% CI, 60.0%–75.2%], respectively; $P < .001$; Table 3). The diagnostic sensitivities of M-NBI and C-WLI did not differ significantly (60.0% [95% CI, 36.1%–80.9%] and 40.0% [95% CI, 19.1%–63.9%], respectively; $P = .34$; Table 3). M-NBI in conjunction with C-WLI significantly enhanced the diagnostic performance of the latter; accuracy increased from 64.8% (95% CI, 57.2%–71.8%) to 96.6% (95% CI, 93.5%–99.1%; $P < .001$), sensitivity increased from 40.0% (95% CI, 19.1%–63.9%) to 95.0% (75.1%–99.9%; $P < .001$), and specificity increased from 67.9% (95% CI, 60.0%–75.2%) to 96.8% (92.7%–99.0%; $P < .001$; Table 3).

The median durations of the C-WLI and M-NBI procedures were 21 seconds (interquartile range, 12–40 seconds) and 55 seconds (interquartile range, 23–97 seconds), respectively, and this difference was highly significant ($P < .001$). The median total duration of C-WLI followed by M-NBI (72 seconds [interquartile range, 40–144 seconds]) was significantly longer than that of