Near-Infrared Fluorescence Imaging Using a Protease-Specific Probe for the Detection of Colon Tumors

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Background/Aims: Early tumor detection is crucial for the prevention of colon cancer. Near-infrared fluorescence (NIRF) imaging using a target-activatable probe may permit earlier disease detection. Matrix metalloproteinases (MMPs) participate in tumorigenesis and tumor growth. The aim of this study was to determine whether NIRF imaging using an MMP-activatable probe can detect colon tumors at early stages.

Methods: We utilized two murine colon cancer models: a sporadic colon cancer model induced by azoxymethane (AOM), and a colitis-associated cancer model induced by a combination of AOM and dextran sodium sulfate (DSS). Colonic lesions were analyzed by histologic examination, Western blotting, immunohistochemical staining, and NIRF imaging using an MMP-activatable probe. **Results:** Multiple variable-sized tumors developed in both models and progressed from adenomas to adenocarcinomas over time. At the early stage of the AOM/DSS model, diffuse inflammation was observed within the tumors. MMP expression increased progressively through normal, inflammation, adenoma, and adenocarcinoma stages. NIRF signal intensities were strongly correlated with each tumor stage from adenoma to adenocarcinoma. NIRF imaging also distinguished tumors from inflamed mucosa. **Conclusions:** NIRF imaging using a protease-activatable probe may be a useful tool for early tumor detection. This approach could translate to improve the endoscopic detection of colon tumors, especially in patients with inflammatory

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Key Words: Colon cancer; Inflammatory bowel disease; Near-infrared fluorescence; Matrix metalloproteinases

INTRODUCTION

Colon cancer is the second leading cause of cancer-related deaths in the United States¹ and is increasing rapidly in many other countries.^{2,3} As most colon cancers develop via an adenoma-to-carcinoma sequence,⁴ cancer development can be prevented by removal at the earlier, adenoma stage.⁵ Therefore, the development of methods to detect such early-stage lesions is essential to prevent and/or cure this disease. Although recently developed endoscopic techniques, including chromoendoscopy, magnifying endoscopy, and narrow-band imaging, have proven useful,⁶⁻⁹ even these techniques may miss precancerous lesions. Furthermore, the ability of these approaches to clearly discriminate malignant polyps from benign lesions is limited because diagnosis is based on morphological characteristics only.^{10,11} Moreover, the diagnosis of chronic inflammation-associated colon tumors, colitis-associated cancer, is especially challenging because these lesions are often blended with gross inflammatory abnormalities.^{12,13}

A recently developed molecular imaging technique, de-

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detecting specific molecular targets, has yielded promising results.^{14,15} Molecular imaging not only detects tumors, but can show the expression and activity of specific molecules (e.g., proteases and protein kinases).¹⁵ One such technique, near-infrared fluorescence (NIRF) imaging, combined with an NIRF optical molecular probe activated by proteases, has several advantages when compared with white-light imaging, including much deeper tissue penetration and less nonspecific tissue autofluorescence, thus improving target-to-background ratios (TBRs).^{14,16,17} Several proteases, including cysteine proteases (e.g., cathepsin B), aspartic proteases (e.g., cathepsin D), serine proteases (e.g., urokinase-type plasminogen activator), and matrix metalloproteinases (MMPs), are known to play roles in tumorigenesis and tumor progression.¹⁸⁻²⁰ The roles of MMPs in colon cancer have been extensively and actively studied.²¹⁻²⁶ MMPs participate in the early stages of colon tumorigenesis, showing increased expression levels during tumor progression along the adenoma-to-carcinoma sequence.^{23,24} Thus, MMPs may be valuable target molecules for early tumor detection and to monitor biological changes taking place during colon carcinogenesis.

The ability of NIRF imaging, using an activatable probe, to detect colon tumors has been assessed in several animal colon tumor models, such as the *Apc^{min/+}* mouse and mouse xenografts.^{16,17} However, these animal models do not mimic the process occurring during human colon tumorigenesis. The models do not feature the adenoma-to-carcinoma sequence seen in sporadic colon cancer or the colitis-associated cancer arising from chronic inflammation. To date, the effectiveness of NIRF imaging in colon cancer models resembling human colon tumorigenesis has not been evaluated, nor has NIRF imaging using an MMP-activatable probe been assessed in a colon cancer model. We therefore determined whether NIRF imaging using an MMP-activatable probe could detect colon tumors in clinically relevant mouse colon cancer models, including a sporadic cancer model involving the adenoma-to-carcinoma sequence and a colitis-associated cancer model in which tumors arise from chronic inflammation.

MATERIALS AND METHODS

1. Animals and chemicals

Five week-old male A/J mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and 5 week-old male BALB/c mice were sourced from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The colonic carcinogen azoxymethane (AOM) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and dextran sodium sulfate (DSS) of molecular weight 36,000-50,000 Da was

the product of MP Biochemicals, LLC (Aurora, OH, USA), and was dissolved in distilled water at a concentration of 2% (w/v). All animal experiments and procedures were in compliance with the Principles of Laboratory Animal Care formulated by the Institutional Animal Care and Use Committee (IACUC) of the Asan Institute for Life Sciences, Asan Medical Center. The Committee abides by the Institute of Laboratory Animal Resources (ILAR) guide.

2. Colon tumor models

1) AOM model

Twenty-one male A/J mice were acclimatized for 7 days on tap water and a basal diet ad libitum. Eighteen mice were injected intraperitoneally with AOM (10 mg/kg body weight in normal saline) once per week for 6 weeks and maintained on a basal diet and tap water. The remaining three untreated control mice received a basal diet and tap water throughout the experiment. Seven mice (six AOM-treated animals and one control) were sacrificed at each of 12, 16, and 20 weeks after the final injection of AOM. At sacrifice, mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and killed by cervical dislocation.

2) AOM/DSS model

Twenty-one male BALB/c mice were acclimatized for 7 days to tap water and a basal diet ad libitum. Eighteen mice received a single intraperitoneal injection of AOM (10 mg/kg body weight) and, commencing one week later, these animals were given drinking water containing 2% (w/v) DSS for 7 days, followed by maintenance on a basal diet and tap water. The remaining three mice received the basal diet and tap water throughout the experiment. Seven mice (six AOM/DSS-treated animals and one control) were sacrificed at each of 6, 12, and 16 weeks after the end of DSS administration. At sacrifice, mice were anesthetized by inhalation of isoflurane and killed by cervical dislocation.

3. Histologic analysis

For polyp examination, colons were removed, flushed with saline, dissected longitudinally, and fixed in 10% (v/v) formalin. Polyps were examined under a dissection microscope at 30-60 x magnification. Paraffin-embedded colon sections were prepared using routine procedures, stained with hematoxylin-eosin, and examined by an experienced pathologist at the Asan Medical Center. Colon tumors were histologically assessed as recommended.²⁷

4. NIRF Imaging

1) NIRF-activatable probe

MMPsenseTM680 (VisEn Medical Inc., Woburn, MA, USA) is a protease-activatable fluorescent *in vivo* imaging agent that can be activated by MMPs including MMP-2, -3, -9, and -13.²⁸ MMPsenseTM680 is optically silent in the inactivated state but becomes highly fluorescent following protease-mediated activation. The probe has a peak absorption at approximately 680 nm and a peak emission at 700 nm.

2) Imaging procedure

At each designated timepoint, six A/J mice (five animals treated with AOM and one control) and six Balb/c mice (five treated with AOM/DSS and one control) were each injected intravenously via the tail vein with 150 μ L of MMPsenseTM680 (2 nmol of fluorochrome Cy5.5 per mouse). The remaining mouse in each treatment group was injected with an equivalent volume of saline solution. Two hours later, mice were sacrificed, and the colons were surgically excised and examined using two distinct fluorescence optical imaging systems.

3) Imaging station

Exposed colons were imaged employing the eXplore Optix system (ART Advanced Research Technologies Inc., Montreal, Canada) and Kodak Image Station 4000MM (Eastman Kodak Co., New Haven, CT, USA). Regions of interest were identified for each tumor as well as in adjacent size-matched intestinal mucosa. NIRF signal intensities were calculated as described,²⁹ as were the TBRs of lesion intensity compared with adjacent normal mucosa.¹⁶

5. SDS-PAGE and immunoblot analysis

Mouse tissues were homogenized in lysis buffer (50 mmol Tris-HCl [pH 7.4], 100 mmol NaCl, 10 mmol CaCl₂ containing 0.25% [v/v] Triton X-100, and a protein inhibitor cocktail). Total protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples (50 μ g protein) were loaded onto polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulfate, electrophoretically separated, and transferred to PVDF membranes. Membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline for one hour and incubated with polyclonal anti-MMP-2 (1:1,000; Cell Signaling, Danvers, MA, USA), monoclonal anti-MMP-3 (1:1,000; Abcam plc., Cambridge, UK), polyclonal anti-MMP-9 (1:1,000; Abcam), or monoclonal an-

ti-mouse MMP-13 (1:400; Calbiochem, San Diego, CA, USA) antibodies overnight at 4°C. Membranes were washed and incubated with the appropriate horseradish peroxidase-linked anti-IgG secondary antibody (Abcam) at room temperature for 1 hour. Chemiluminescence detection was used to identify bands (ECL system; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). As a loading control, membranes were incubated with a β -actin monoclonal antibody (Sigma-Aldrich). The positive controls were rhMMP-2 (Chemicon International Inc., Temecula, CA, USA), rhMMP-3 (R&D Systems, Minneapolis, MN, USA), rhMMP-9 (Abcam), and MMP-13 (50 μ g mouse colon adenocarcinoma CT-26 cell lysate).

6. Immunohistochemistry

Normal tissues and tumors were excised, fixed for 24 hours in 10% (w/v) phosphate buffered formalin (pH 7.4), embedded in paraffin, and sectioned into 4 μ m slices. Sections were dewaxed in xylene and rehydrated in alcohol. Antigen retrieval was performed using an electronic pressure cooker (Cell Marque Co., Rocklin, CA, USA) for 15 minutes in Trilogy buffer (Cell Marque). Slides were blocked with 3% (w/v) BSA in Tris-buffered saline for one hour, incubated with a polyclonal anti-MMP-9 (1:100) primary antibody for 40 minutes at room temperature, and next incubated with Polink-1 horseradish peroxidase-labeled rabbit anti-rabbit immunoglobulin (Golden Bridge International Inc., Mukilteo, WA, USA) for 15 minutes at room temperature, according to the manufacturer's instructions. After three additional washes, peroxidase activity was developed using diaminobenzidine at room temperature, and sections were counterstained with Harris hematoxylin.

7. Statistical analysis

Data are presented as means $\pm$ standard deviations of the means. Groups were compared using the chi-squared test, the McNemar test, the Kruskal-Wallis test, and the Mann-Whitney U test including the Bonferroni correction. p-values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

1. Macroscopic findings

Multiple, variable-sized tumors were detected in the distal half of the colon of mice treated with either AOM alone or with a combination of AOM and DSS (Fig. 1). The average total numbers of tumors per mouse were

9.6±2.1 and 8.2±2.3, respectively (Table 1). Both the number and size of tumors increased with time (Table 1,

Table 1. Number of Colon Tumors Developing over Time

A/J mice treated with AOM alone		BALB/c mice treated with AOM/DSS	
Time, wk	No. of tumors/mouse	Time, wk	No. of tumors/mouse
12	8.0±1.7	6	6.0±1.8
16	9.5±1.3	12	8.3±0.6
20	11.0±2.3	16	10.3±1.3
Average	9.6±2.1	Average	8.2±2.3

AOM, azoxymethane; DSS, dextran sodium sulfate.

Fig. 1). No untreated control mouse developed any colon tumor.

2. Histologic findings

All tumors in both animal models were either adenomas or adenocarcinomas. At earlier stages, adenomas predominated, with the number of adenocarcinomas increasing over time, resulting in a significantly higher adenocarcinoma-to-adenoma ratio at later stages in both the AOM ($p=0.045$) and AOM/DSS ($p<0.001$) models (Fig. 2). There was no evidence of distant metastasis in any animal. Inflammation of varying severity was observed around the tumors of AOM/DSS-treated mice at 6 weeks after cessation of treatment.

Fig. 1. Gross morphology of excised colons according to progressive stages. (A) Colons from A/J mice treated with AOM alone at weeks 12 (top), 16 (middle), and 20 (bottom). (B) Colons from BALB/c mice treated with AOM/DSS at weeks 6 (top), 12 (middle), and 16 (bottom).

AOM, azoxymethane; DSS, dextran sodium sulfate.

Fig. 2. Histologic analysis of colon tumor development. (A) A/J mice treated with AOM alone. (B) BALB/c mice treated with AOM/DSS. White and black columns represent adenoma and adenocarcinoma levels, respectively. The stated levels of significance (i.e., p -value) are comparisons at three different time points of histologic differences between developed tumors.

AOM, azoxymethane; DSS, dextran sodium sulfate.

Fig. 3. NIRF images of mouse colons. (A, B) Representative images of colons from A/J mice treated with AOM alone after injecting an MMP-activatable probe (A) or normal saline (B) (Kodak imaging system). (C, D) Representative images of colons from BALB/c mice treated with AOM/DSS after injecting an MMP-activatable probe (C) or normal saline (D) (eXplore Optix imaging system). Open and solid arrows indicate representative adenomas and adenocarcinomas, respectively. NIRF, near-infrared fluorescence; AOM, azoxymethane; DSS, dextran sodium sulfate; MMP, matrix metalloproteinase.

Fig. 4. Signal intensities of NIRF imaging according to colon histology. (A) A/J mice treated with AOM alone. (B) BALB/c mice treated with AOM/DSS. Signal intensities were analyzed using the eXplore Optix imaging system. The stated levels of significance (i.e., p-values) are for comparisons of signal intensities between groups after Bonferroni correction. NIRF, near-infrared fluorescence; AOM, azoxymethane; DSS, dextran sodium sulfate.

3. NIRF imaging

NIRF imaging using a protease-activatable probe showed that all colon tumors displayed high signal in-

tensities compared with adjacent mucosa, and that even tumors less than one millimeter in diameter could be specifically detected (Fig. 3A and C). In contrast, mice injected with saline showed a similar signal intensity of tu-

mors and adjacent mucosa (Fig. 3B and D). NIRF signal intensity increased as tumors progressed from the adenoma to adenocarcinoma stage ($p < 0.001$ in both models) (Fig. 4). The TBRs of adenomas and adenocarcinomas, relative to adjacent normal mucosa, were 7.9 ± 5.7 and 19.0 ± 11.7 , respectively, in the AOM model ($p = 0.022$) and 5.7 ± 2.3 and 19.8 ± 6.9 , respectively, in the AOM/DSS model ($p < 0.001$) (Table 2). Although inflammatory lesions in AOM/DSS-treated mice showed a

signal intensity higher than that of normal mucosa, the intensity was lower than that of adenomas (Table 2, Fig. 4). The sensitivities of NIRF and white-light imaging in detecting tumors were 85% and 76%, respectively, in the AOM model ($p = 0.115$) and 82% and 79%, respectively, in the AOM/DSS model ($p = 0.503$). The specificities were 93% and 64%, respectively, in the AOM model ($p = 0.008$) and 95% and 68%, respectively, in the AOM/DSS model ($p = 0.002$).

Table 2. Histology and NIRF Findings in BALB/c Mice Treated with AOM/DSS

	Normal	Inflammation	Adenoma	Adenocarcinoma	p-value
Histology* (H&E stain)
IHC*
NIRF image [†]
Signal intensity [†] (total photon count/mm ²)	181.3 $\pm$ 58.0	451.0 $\pm$ 80.1	1,039.2 $\pm$ 408.0	3,585.0 $\pm$ 1,251.6	<0.001
TBR		2.5 $\pm$ 0.4	5.7 $\pm$ 2.3	19.8 $\pm$ 6.9	<0.001

AOM, azoxymethane; DSS, dextran sodium sulfate; IHC, immunohistochemical stain; NIRF, near-infrared fluorescence; TBR, target-to-background ratio.

*Original magnification of histology and immunohistochemical stain, $\times 400$; [†]NIRF images were obtained using an eXplore Optix system (ART Advanced Research Technologies Inc.); [†]Signal intensities were quantified using Analysis Workstation Software (ART Advanced Research Technologies Inc.).

Fig. 5. MMP-9 expression. Colon protein extracts were prepared and analyzed for MMP-9 expression by Western blotting. (A) A/J mice treated with AOM alone. (B) BALB/c mice treated with AOM/DSS.

MMP, matrix metalloproteinase; AOM, azoxymethane; DSS, dextran sodium sulfate; P, positive control (recombinant human [rh] MMP-9); N, normal tissue; INFL, inflammation; A, adenoma; AC, adenocarcinoma.

Fig. 6. Expression of MMP protein in isolated colons. Colons were excised and protein extracts analyzed for MMP expression by Western blotting. (A) A/J mice treated with AOM alone. (B) BALB/c mice treated with AOM/DSS. MMP, matrix metalloproteinase; AOM, azoxymethane; DSS, dextran sodium sulfate; P, positive controls (rhMMP-2, rhMMP-3, MMP-13, and 50 μ g of mouse colon adenocarcinoma CT-26 cell lysate); N, normal tissue; A, adenoma; AC, adenocarcinoma.

4. Expression of MMPs

By Western blotting, we found that MMP-13 was expressed in normal tissue adjacent to tumors, whereas none of MMP-2, -3, and -9, were so expressed (Figs. 5 and 6). Inflamed mucosa in AOM/DSS-treated mice showed increased levels of MMP-9, but the concentration was lower than that of tumors. MMP-9 expression rose upon progression from adenoma to adenocarcinoma (Fig. 5). In contrast, MMP-13 level did not alter during tumor progression, whereas neither MMP-2 nor -3 were detected in tumors (Fig. 6). Immunohistochemical assays also showed that MMP-9 expression was higher in tumors and stromal cells than in normal mucosa, and increased during tumor progression (Table 2).

DISCUSSION

NIRF imaging techniques, based on the noninvasive visualization of molecular processes *in vivo*, can overcome the limitations of morphological and invasive diagnoses. Moreover, the specificity and sensitivity of such approaches are higher when used in combination with an activatable probe targeting a specific peptide.^{14,16} Activatable peptide-based probes generate an amplified fluorescence signal by means of target molecule-induced activation. Fluorophores in the quenched state are released from a peptide backbone by enzymes overexpressed in tumors.³⁰ The quenching effect of such probes results in a reduced background signal and higher target-to-back-

ground ratios. NIRF imaging with enzyme-activatable probes has shown high specificity and sensitivity in tumor detection.^{16,17}

The enzymes currently targeted for NIRF imaging in cancer diagnosis include cathepsins B and D, prostate-specific antigen, and the MMPs.^{14,16,17,31,32} MMPs, which we chose as targets of our activatable probe, are a group of zinc-dependent matrix-degrading enzymes that play an important role in the pathogenesis of various diseases, including rheumatoid arthritis, atherosclerosis, cancer, and inflammatory bowel disease.^{20,23} NIRF imaging using an MMP-activatable probe has been introduced in several disease models, including those for myocardial infarction and breast cancer, but not yet in colon cancer models.^{28,32} Evidence from *in vitro* and *in vivo* experimental systems has confirmed the importance of MMPs in the pathogenesis of colorectal cancer.²¹⁻²³ In addition, direct associations have been observed between increased MMP expression and progressive tumor staging, tumor invasiveness, development of metastasis, and shortened survival.²¹⁻²⁶ Several MMPs, specifically MMP-1, -2, -3, -7, -9, and -11, are overexpressed in human colorectal tumors.^{21,22}

We analyzed expression of MMP-2, -3, -9, and -13 in our mouse colon cancer models because these MMPs have been reported to react with our MMP-activatable probe.²⁸ We found that MMP-2 and -3 were undetectable, or present in negligible amounts, in the colon, in both normal tissue and tumors, whereas MMP-13 was consistently expressed at all disease stages. In contrast, the

level of MMP-9 was higher in neoplastic lesions than in the surrounding normal mucosa, and MMP-9 expression increased during tumor progression from the adenoma to adenocarcinoma stage. These findings suggest that MMP-9 may play a major role in activating the optically detectable probe in our animal models. MMP-9 expression has been found to be higher in advanced adenomas and adenocarcinomas than in non-advanced adenomas and normal tissue,^{24,25} and greater MMP-9 expression has been associated with cancer metastasis and decreased survival.²⁶ Quantification of MMP-9 expression by NIRF imaging may help predict cancer behavior and assist in therapeutic planning.

Although several studies have assessed NIRF imaging using an activatable probe in colon cancer models, the cited studies were performed using *Apc*^{min/+} and mouse xenograft models, both of which are of limited clinical applicability to humans.^{16,17} Most tumors in *Apc*^{min/+} mice are adenomas, and the majority are located in the small intestine.³³ Similarly, tumors in the xenograft model are all cancers and may be affected by the surrounding micro-environment because the cancers do not originate from the mice.³⁴ We therefore utilized two animal models that better mimic human colon tumorigenesis, a sporadic colon cancer (AOM) model and a colitis-associated cancer (AOM/DSS) model.^{35,36} The mouse strains chosen (A/J for the AOM model and BALB/c for the AOM/DSS model) have been reported to be highly sensitive to the respective disease-inducing compounds.^{36,37} Sequential examination of colons from mice sacrificed from week 5 to week 24 showed that the earliest timepoint at which both adenomas and carcinomas were present was week 12 in the AOM model and week 6 in the AOM/DSS model. The earlier tumor development in AOM/DSS-treated compared with AOM-treated mice may be caused by an acceleration of tumorigenesis as a result of the inflammation of the former model.^{35,37} Interestingly, tumors in AOM/DSS-treated mice became more agglomerated as they increased in size over time. Thus, at late stages, the AOM/DSS model might be inadequate for the acquisition of obvious tumor images showing the adenoma-to-carcinoma stage at a time. At earlier stages, however, this model is valuable for research on colitis-associated cancer, in that the model permits the coexistence of tumors and surrounding inflammation.

Patients with inflammatory bowel diseases (IBDs), primarily with ulcerative colitis or Crohn's disease, are at higher risk of developing colon cancer than are subjects in the general population.^{38,39} In clinical practice, therefore, regular colonoscopic surveillance is generally recommended for IBD patients with long-standing extensive

disease. Furthermore, in addition to biopsy of suspicious lesions, two-to-four random biopsies should be taken every 10 cm along the colon.^{12,13} However, a dysplastic lesion, or a dysplasia-associated lesion or mass, can easily blend with the gross inflammatory abnormalities commonly encountered in colons with IBD, making endoscopic detection of such lesions difficult even for experienced practitioners.¹³ In addition, in patients at high risk of bleeding, and when lesions are very friable, invasive techniques such as biopsies give cause for concern, emphasizing the need for more sensitive and specific endoscopic detection methods.

As in a previous study,¹⁶ we found that NIRF imaging could detect tumors with higher specificity and a similar sensitivity to white-light imaging. The good detection rate using white light may have been attributable to the relatively large size and more conspicuous appearance of tumors in the present study, compared with those of other tumor models such as the *Apc*^{min/+} mouse. However, the sensitivity of NIRF imaging in detecting tumors <2 mm in size was significantly superior to that of white-light imaging in both models (83% vs 57%, *p*=0.039 in AOM model; 80% vs 53%, *p*=0.039 in AOM/DSS model). No strong correlation was found between fluorescence signal intensity and tumor size. We performed NIRF imaging on excised colons because of strong interference from surrounding organs, including the bladder and kidneys, and the limitations of fluorescence transmission. In addition, imaging was performed just after sacrifice to minimize the difference between *in vivo* and *ex vivo* systems. These limitations may be overcome by direct observation of the lesion using fluorescence endoscopy in combination with conventional colonoscopy.

Using mouse cancer models showing features of human colon cancer, we found that protease-specific NIRF imaging could discriminate among normal, inflammatory, and malignant colonic lesions. We also found that imaging not only reflected the targeted protease activity, but may also estimate the malignant potential of the lesion. These results suggest that NIRF imaging with protease-activatable probes, as a complement to conventional colonoscopy, may be a useful tool in tumor detection. In particular, the technique may be helpful in monitoring of cancer development in patients with IBD.

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