Near-infrared Fluorescence Imaging Using a Protease-activatable Nanoprobe in Tumor Detection: Comparison with Narrow-band Imaging

Soon Man Yoon, In-Wha Kim, Miyeoun Song, Eun-Ju Do, Ju Hee Ryu, Kwangmeyung Kim, Ick Chan Kwon, Mi Jung Kim, Dae Hyuk Moon, Dong-Hoon Yang, Kyoung Jo Kim, Byong Duk Ye, Jeong-Sik Byeon, Suk-Kyun Yang, Jin-Ho Kim, Seung-Jae Myung

Department of Internal Medicine, Chungbuk National University College of Medicine, Cheongju, Asan Institute for Life Sciences, Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Departments of Pathology and Nuclear Medicine, Asan Medical Center, University of Ulsan College of Medicine, Department of Gastroenterology, Asan Digestive Disease Research Institute, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

Background/Aims: Advances in endoscopic technology seek to improve the accuracy of neoplastic tumor detection. Recently developed endoscopy devices such as narrow-band imaging (NBI) nevertheless have limitations in morphologic diagnosis. The purpose of this study was to investigate whether a novel imaging technique—near-infrared fluorescence (NIRF) imaging using a protease-activatable nanoprobe—could provide more accurate neoplastic tumor detection, compared to NBI.

Methods: Images of the intestines of ApcMin/+ mice were obtained by NIRF using a matrix metalloproteinase (MMP)-sensing probe, which was based on a nanoparticle platform. Immediately after imaging, endoscopy with NBI capability was performed on the same excised intestine. Macroscopic and microscopic findings in the intestines were assessed, and MMP expression was analyzed by Western blotting and real-time polymerase chain reaction.

Results: Numerous tiny polypoid lesions were present in the intestines of aged ApcMin/+ mice. These lesions included adenomas, lymphoid follicles, and protruding normal tissues. When using NIRF imaging with an MMP-activatable nanoprobe, adenomatous polyps showed higher fluorescence, compared to lymphoid follicles or adjacent normal tissues. The expression of MMP was higher in the adenomatous tissue than in the other tissues. The sensitivity and specificity for adenoma detection were 88.9% and 82.2%, respectively, when using NIRF imaging with a MMP-nanoprobe, compared to 77.8% and 66.7%, respectively, when using NBI (P<0.05).

Conclusions: Near-infrared fluorescence imaging with a protease-activatable nanoprobe could aid in the differentiation of tumor characteristics. Clinical application of this approach may improve the endoscopic detection of neoplastic tumors. (Intest Res 2013;11:268-275)

Key Words: Molecular imaging; Narrow band imaging; Neoplasms; Diagnosis
and to reduce the rate of missed adenomas.4

Narrow-band imaging (NBI) is a novel imaging technique that illuminates the target in the blue, red, and green colors of the spectrum by using narrow-band filters. Narrow-band imaging enhances the visualization of the surface architecture and the capillary pattern of the gastrointestinal mucosa.5 In early studies, NBI showed greater sensitivity and specificity than conventional colonoscopy for detecting colon tumors.6 However, NBI is limited by its inability to distinguish neoplastic lesions (e.g., adenomas) from non-neoplastic lesions (e.g., hyperplastic or inflammatory polyps) since these diagnoses are based on morphological characteristics alone.

In actuality, 10-30% of all colonic polyps are non-neoplastic and have no malignant potential.7 Therefore, a biopsy should be performed to characterize the polyp and avoid unnecessary polypectomy. However, a biopsy specimen may only indicate part of the pathology of a lesion. The preparation of pathological samples is moreover time-consuming and expensive, and the procedures for obtaining samples increase the risk of bleeding and perforation during a colonoscopy. Thus, there is a need for a more advanced imaging technology for the noninvasive and accurate discrimination of neoplastic polyps that should be removed.

Currently emerging molecular imaging techniques offer powerful tools and strategies for early tumor detection through noninvasive, real-time, and high-resolution modalities, and they provide information in regard to tumor biology.8,9 In particular, near-infrared fluorescence (NIRF) imaging in the wavelength range of 700-1,000 nm potentially has high spatial resolution and high sensitivity for tumor detection.10 To improve detection sensitivity and specificity, near-infrared (NIR) imaging utilizes NIR probes that specifically bind to tumor-associated molecules (e.g., tumor cell receptors, tumor extracellular matrix, and enzymes).

We recently developed a novel NIRF nanoprobe to target a tumor-associated protease, matrix metalloproteinase (MMP), and observed enhanced tumor detection in animal models of colon cancer.11 By using the NIRF imaging system with an MMP-activatable probe that is based on a polymeric nanoparticle platform, we found that all colon tumors that we examined showed more prominent fluorescent signaling, compared to the adjacent normal mucosa. This method moreover allowed the detection of very small tumors with a diameter of several millimeters.

The NIRF signal intensity is highly correlated with the level of MMP expression.11 To date, it has not been evaluated whether using NIRF imaging is beneficial, compared to using current mucosal-enhancing methods such as NBI. Therefore, by using a mouse model of colon cancer pathogenesis, NIRF imaging with an MMP-activatable nanoprobe was compared to NBI in Apc<sup>Min</sup> mice in this study to evaluate its efficacy in discriminating neoplastic lesions from non-neoplastic lesions.

METHODS

1. Animal Model

Fourteen 5-week-old Apc<sup>Min</sup> mice on a C57BL/6J background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were maintained on a basal diet and tap water. Apc<sup>Min</sup> mice are genetically engineered transgenic mice. They are a well-established animal model for studying familial adenomatous polyposis and sporadic CRC.12 All mice were sacrificed at approximately 15 weeks of age. All animal experiments and procedures were performed in compliance with the Principles of Laboratory Animal Care formulated by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Asan Medical Center (Seoul, Korea). This committee abides by the Institute of Laboratory Animal Resources guide.

2. Macroscopic and Histologic Analysis

The mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and sacrificed by cervical dislocation. The distal one-third of the small bowels and the total colon of each Apc<sup>Min</sup> mouse was removed, flushed with saline, and dissected longitudinally. The excised small bowels and colons were grossly examined. These tissues were then fixed in 10% phosphate-buffered formalin for 24 hours and cut vertically to horizontal axis at 1-2 mm intervals in a manner similar to the mapping procedure for the meticulous histologic evaluation of gastrointestinal endoscopic submucosal dissection specimens. Paraffin-embedded serial tissue sections were prepared by routine procedures, stained with hematoxylin-eosin dye, and analyzed by an experienced pathologist at the Asan Medical Center. The histology of each small bowel and colon tumor was assessed in accordance with the most recent consensus report and recommendations.13

3. Matrix Metalloproteinase-activatable Near-infrared Fluorescence Probe

The MMP-activatable nanoprobe was a polymeric nanoparticle-based activatable probe consisting of a self-assembled glycol chitosan nanoparticle and an activatable dark-quenched NIR fluorophore, Cy5.5-peptide substrate, which was quenched by the NIR dark quencher BHQ-3 and was primarily selective for MMP-7.11

4. Near-infrared Fluorescence Imaging and Narrow-band imaging

For NIRF imaging, each aged Apc<sup>Min</sup> mouse was injected 2 hours before NIRF imaging with either a MMP-activatable nanoprobe (n=6), a scramble control probe (MMP-inacti-
variable probe; n=4), or saline (n=4). The NIRF images were assessed by the eXplore Optix system (ART Advanced Research Technologies Inc., Montreal, Canada). Laser power and count time settings were optimized at 30 μW and at 0.3 second per point, respectively. To generate emission wavelength scans, the excitation and emission spots were raster-scanned in 1-mm steps over a selected region of interest (ROI). A 670-nm pulsed laser diode was used to excite the Cy5.5 molecules. The NIRF emission at 700 nm was collected and detected with a fast photomultiplier tube (Hamamatsu Photonics KK, Hamamatsu, Japan) and a time-correlated single photon counting system (Becker and H Hickl GmbH, Berlin, Germany). All data were calculated with the ROI function of the Analysis Workstation software package (ART Advanced Research Technologies Inc.). The target-to-background ratios (TBRs) of the intensity of each target to the TBRs of the adjacent normal mucosa were calculated, as previously described. The discrimination between an adenomatous polyp and a nonadenomatous polyp was evaluated by comparing the TBRs of each target, based on the cut-off value that was determined from the preliminary results.

Immediately after NIRF imaging, a single experienced endoscopist performed NBI with the same excised small bowels and colons of each mouse by using a commercially available high-definition colonoscope with NBI capability (CF-H180AL; Olympus America, Center Valley, PA, USA). The discrimination between adenomatous polyps and nonadenomatous polyps was based on the surface pattern and the microvascular features of polypoid lesions in comparison to the surrounding mucosa.

5. Western Blotting

Mouse tissues were homogenized in a lysis buffer (i.e., 50 mmol/L Tris-HCl; pH 7.4; 100 mmol/L NaCl; 10 mmol CaCl	extsubscript{2} containing 0.25% Triton X-100; and protein inhibitor cocktail). The total protein concentration was determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples (50 mg protein) were loaded onto polyacrylamide gels containing 0.1% sodium dodecyl sulfate. They were electrophoretically separated, and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked by incubation with 5% nonfat milk in Tris-buffered saline for 1 hour. They were then incubated overnight at 4°C with polyclonal anti-MMP-7 antibody (1:1,000; Abcam PLC, Cambridge, UK). The membranes were subsequently incubated with horseradish peroxide-linked anti-IgG secondary antibody (Abcam) at room temperature for 1 hour. Bands were identified by chemiluminescent detection (ECL; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). As a loading control, the membranes were incubated with a β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). The positive control was recombinant human MMP-7 (Abcam).

6. Real-time Polymerase Chain Reaction Analysis

Total RNA was extracted with the RNeasy mini kit (Qiagen, Carlsbad, CA, USA) and cDNA was synthesized with an ABI high-capacity cDNA reverse transcription reaction kit (Applied Biosystems Inc., Foster City, CA, USA) in accordance with the manufacturers’ instructions. Relative gene expression by real-time PCR was determined with the Lightcycler 480 SYBR Green I Master on a Lightcycler 480 II real-time PCR system (Roche Applied Science, Indianapolis, IN, USA). The primers for MMP-7, synthesized according to published sequences, were 5’-ACTTCAGACT TACCTCGGAT CG-3’ (sense) and 5’-TCCCCCAACT AACCCTCTTG A-3’ (antisense). The cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 70°C for 10 seconds.

To distinguish the specific amplification product from nonspecific products or primer dimers, a melting curve was constructed from the amplification reaction. This reaction was obtained by maintaining the temperature at 65°C for 1 minute, and then increasing the temperature to 95°C at a rate of 0.1°C per second. Relative gene expression, normalized to β-actin, was calculated by the 2\textsuperscript{−ΔΔCt} method, as previously described.

7. Statistical Analysis

Data are reported as the mean±the standard error of the mean. Between-group comparisons of continuous variables were assessed by the Mann-Whitney U test and the Kruskal-Wallis test with Bonferroni correction. A P-value less than 0.05 was considered statistically significant. The accuracy, sensitivity, specificity, and positive and negative predictive values for differentiating adenomatous from nonadenomatous lesions in the excised distal small bowels were assessed through comparing the histopathological results obtained by NIRF imaging and by NBI. The accuracy, sensitivity, and specificity of NIRF imaging and NBI were compared by the McNemar test for paired data. All statistical analyses were performed with the PASW Statistics 18.0 package (IBM Co., Armonk, NY, USA).

RESULTS

1. Macroscopic and Histologic Findings in Apc\textsuperscript{Min/+} Mice

Numerous sessile or flat polypoid lesions, approximately 2 mm in diameter, were detected in the excised distal third of the small bowels. Some sessile polypoid lesions were also detected in the colons. Polypoid lesions in the distal small bowels included adenomatous polyps, lymphoid follicles, and protruding normal tissue (Figs. 1, 2). Most colonic polyps were adenomatous polyps and some polyps were advanced...
adenomas with high-grade dysplasia.

2. Near-infrared Fluorescence Imaging and Narrow-band Imaging

Adenomatous polyps were highly fluorescent when imaging was performed with the MMP-activatable nanoprobe, but the polyps were not remarkably fluorescent when the scramble control probe or saline was used (Fig. 1). When imaging was performed with the MMP-activatable probe, with the scramble probe, and with saline, the fluorescent signals of the adenomas showed increasing intensity ($P<0.001$) (Fig. 2A). The signal intensity ratio of an adenomatous polyp to adjacent normal tissue obtained with the MMP-activatable probe (TBR, 6.53±0.43; n=77) was higher than the signal intensity ratio obtained with the scramble inactivatable probe (TBR, 3.26±0.44; n=56) or with saline (TBR, 2.50±0.14; n=37) ($P<0.001$) (Fig. 2B). The NBI finding for protruding lesions - including the sessile type - were distinctive, although it was not easy to discriminate morphologically between neoplastic lesions, adenomas, and non-neoplastic lesions (e.g., lymphoid follicles and elevated normal tissues) (Fig. 3A). By contrast, NIRF imaging showed more discriminate findings in detecting adenomas (Fig. 3B). Colon polyps with severe dysplasia showed a higher signal intensity than mildly dysplastic polyps in the small bowel (1,741±114 total photon count/mm$^2$ vs. 469±13 total photon count/mm$^2$) ($P<0.001$).

3. Diagnostic Yield of Near-infrared Fluorescence Imaging and Narrow-band Imaging

To ensure an effective and time-saving study, the diagnostic yield of the imaging study was evaluated only in the distal one-third of the small bowel because non-neoplastic polyps

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Fig. 1. Gross findings of the excised distal small bowels in aged Apc$^{Min/+}$ mice (upper rows) and the corresponding near-infrared fluorescence images (lower rows) obtained with (A) saline, (B) the scramble control (i.e., metalloproteinase [MMP]-inactivatable probe), and (C) the MMP-activatable probe. The small white boxes indicate adenomatous polyps in the small bowel (H&E stain, ×200).
Table 1. Comparison of Accuracy, Sensitivity, and Specificity by Near-infrared Fluorescent Imaging and by Narrow-band Imaging*

<table>
<thead>
<tr>
<th>Measure</th>
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<th>NBI</th>
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<th>P-value‡</th>
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<tr>
<td></td>
<td>Fraction (%)</td>
<td>95% CI</td>
<td>Fraction (%)</td>
<td>95% CI</td>
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<tr>
<td>Accuracy</td>
<td>138/162 (85.2)</td>
<td>0.797-0.907</td>
<td>116/162 (71.6)</td>
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<td>0.001</td>
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<td>Sensitivity</td>
<td>64/72 (88.9)</td>
<td>0.796-0.943</td>
<td>56/72 (77.8)</td>
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<tr>
<td>Specificity</td>
<td>74/90 (82.2)</td>
<td>0.731-0.888</td>
<td>60/90 (66.7)</td>
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<td>PPV</td>
<td>64/80 (80.0)</td>
<td>0.700-0.873</td>
<td>56/86 (65.1)</td>
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<td>NPV</td>
<td>74/82 (90.2)</td>
<td>0.819-0.950</td>
<td>60/76 (78.9)</td>
<td>0.685-0.866</td>
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*For differentiating neoplastic from non-neoplastic lesions in the small bowels of aged Apc<sup>Min/+</sup> mice.
†Resulted from all 162 lesions including 72 neoplastic and 90 non-neoplastic lesions.
‡Resulted from McNemar’s test for paired data.
NIRF, near-infrared fluorescence; NBI, narrow-band imaging; PPV, positive predictive value; NPV, negative predictive value.

Fig. 2. Comparison of (A) the signal intensities and (B) the target-to-background ratios obtained by near-infrared fluorescence imaging with saline, with the scramble control (metalloproteinase [MMP]-inactivatable) probe, and with the MMP-activatable probe in an adenomatous lesion from the small bowel of aged Apc<sup>Min/+</sup> mice. The P-values are obtained from the Kruskal-Wallis test with Bonferroni correction.

Fig. 3. The correlation between narrow-band imaging (NBI) or near-infrared fluorescence (NIRF) imaging with an matrix metalloproteinase-activatable probe and the histologic findings in the same small bowel of an aged Apc<sup>Min/+</sup> mouse. (A) The magnified NBI and (B) the corresponding NIRF image. Image (C) through image (E) show the histologic findings of each lesion in (B), which are marked by an arrow. Lesions are identified as (C) adenoma, (D) lymphoid follicle, and (E) protruding normal tissue (H&E stain, x400).
such as lymphoid follicles (e.g., Peyer’s patch) primarily exist at the distal portion of the small bowel. The sensitivity, specificity, and diagnostic accuracy of NIRF imaging using a MMP-activatable nanoprobe vs. NBI for distinguishing neoplastic adenomatous lesions from all neoplastic polypoid lesions (n=72) and non-neoplastic (n=90) polypoid lesions in the mice (n=6) were as follows: 88.9% vs. 77.8% for sensitivity (P=0.039); 82.2% vs. 66.7% for specificity (P=0.016); and 85.2% vs. 71.6% for diagnostic accuracy (P=0.001), respectively (Table 1).

4. Matrix Metalloproteinase Expression

The MMP-7 expression in the small bowel and colon tissues was analyzed by Western blotting and by real-time PCR analyses. Western blotting revealed a higher expression of MMP in adenomas than in normal tissues (Fig. 4A). This observation was confirmed by real-time PCR, which revealed a higher expression of MMP-7 in adenomas than in normal tissues (P=0.008) (Fig. 4B).

DISCUSSION

In this study, we found that NIRF imaging with a protease-activatable nanoprobe could more effectively discriminate between neoplastic lesions (e.g., adenomas) and non-neoplastic lesions (e.g., lymphoid hyperplasia and protruding normal tissue), compared to NBI. A possible reason for this finding is that NBI detects tumors through tumor surface morphology alone, whereas NIRF imaging can provide information on morphological changes and molecular events in the tumor. Using the NIRF imaging method could potentially reduce the need for biopsies (which are invasive and often unnecessary) in patients with gastrointestinal lesions of uncertain malignancy.

Patients with advanced adenoma (including severe dysplasia) are more likely to develop cancer than are patients with less severe forms of adenoma. In the current study, the colon polyps showed severe dysplasia and a higher signal intensity through NIRF imaging than the mildly dysplastic polyps in the small bowel. This result indicates that NIRF imaging can be used to classify the degree of dysplasia and to effectively detect cancerous lesions in real time without the need for an invasive biopsy. In a colon tumor model of A/J mice treated with azoxymethane, we previously reported that NIRF signals increase significantly as tumors progress from adenoma to adenocarcinoma.11,17

Virtual chromoendoscopy techniques such as NBI initially showed good results and enhanced the adenoma detection rates in high-risk patients.6,10 However, large follow-up studies of screening colonoscopy in patients with an average cancer risk could not reproduce the enhanced adenoma detection rate by using NBI in comparison to using high-definition endoscopy.19,20 Based on several recent meta-analyses, NBI colonoscopy was no better than white-light colonoscopy in detecting colorectal polyps.21,22 A recently developed endoscopic trimodal imaging technique that combines high-resolution endoscopy, autofluorescent imaging (AFI), and NBI did not show improved adenoma detection, compared to conventional endoscopy.23 We did not directly compare NIRF imaging to AFI; however, we found that NIRF imaging using only saline (which is similar to AFI based on the excitation of natural endogenous fluorophores) was less effective than NIRF imaging using an activatable probe.

For more than the last 20 years, NIRF imaging has been used in animal models for cancer detection. It has recently been used in human clinical trials.24,25 Unlike responses in the visible light spectrum (400-650 nm), light scattering is decreased and photon absorption by hemoglobin and water are diminished in the NIR region (650-900 nm). Thus, light is capable of deeper tissue penetration in the NIR region.

Satisfactory NIRF imaging requires excellent NIR probes that are highly tumor specific and have superior biochemical properties. In this study, we used a previously designed target-activatable NIRF imaging nanoprobe consisting of self-assembled chitosan nanoparticles and an activatable dark-quenched NIR fluorophore, Cy5.5-peptide substrate.11 We previously found that this probe enhanced tumor detection in several animal models of colon cancer.11 Numerous activatable probes have been documented in the literature.26
these probes, enzyme activation of the targeting NIR dyes is mediated primarily by tumor-associated proteases, including cathepsins, caspasas, and MMPs.

In designing nanoparticle-based activatable probes, we chose MMPs as the target proteases. Matrix metalloproteinases play a key role in the development and progression of human malignancies, especially colon and rectal carcinomas.\textsuperscript{27} In addition, the expression of MMPs increases during tumor progression (i.e., from normal mucosa to adenoma to carcinoma).\textsuperscript{28} Based on information in the literature, we chose MMP-7 as the tumor target enzyme because MMP-7 is highly overexpressed in tumors in ApoE\textsuperscript{−/−} mice.\textsuperscript{29}

Nanoprobes for human clinical applications must be nontoxic and have quick clearance through biodegradation (i.e., metabolism) in the liver or by excretion in the urine.\textsuperscript{30} The NIR fluorophores such as Cy5.5 that are used in many animal studies do not appear to be associated with acute toxicity.\textsuperscript{31} In addition, the toxicity of ICG, a commonly used and clinically approved fluorophore, has been well studied; this compound is safe at clinical doses.\textsuperscript{24} However, before NIR fluorophores can be used in human clinical trials, long term toxicity studies must be performed in appropriate animal models.

In this study, we could not perform in vivo imaging or endoscopy in the intestinal lumen because endoscopy with NIRF and NBI capabilities is unavailable for small animals such as mice. In addition, NBI did not allow clear visualization of the surface morphology (including the vascular pattern) because of the small size of the tumors and because of the lack of ongoing blood flow in the excised intestine. Because NBI uses special illumination through narrow-band filters, there was a more distinct appearance for polypoid lesions when using NBI than when using white-light imaging. In addition, some vascular patterns were visible on NBI because of residual blood content since we immediately performed NBI following NIRF imaging after the bowel excision. We believe that our current study may be worth a preliminary or pilot in vivo study. In the near future, we also expect to conduct follow-up studies by using advanced endoscopic instruments for small animals to verify the efficacy of NIRF imaging.

In conclusion, NIRF imaging using a protease-activatable nanoprobe could be helpful for discriminating neoplastic lesions from non-neoplastic lesions. By providing a high diagnostic yield and real-time display, NIRF imaging techniques could provide substantial advantages over current endoscopic imaging techniques. This advanced technology could play an important role in research and clinical endoscopic imaging and could potentially be used to lessen the burden of cancer in the future.

REFERENCES
19. Rex DK, Helbig CC. High yields of small and flat adenomas with
high-definition colonoscopes using either white light or narrow band imaging. Gastroenterology 2007;133:42-47.
22. Sabbagh LC, Reveiz L, Aponte D, de Aguiar S. Narrow-band imaging does not improve detection of colorectal polyps when compared to conventional colonoscopy: a randomized controlled trial and meta-analysis of published studies. BMC Gastroenterol 2011;11:100.