Recent Advances in Molecular Imaging of Colorectal Tumors

Takanori Kashihara Naoki Muguruma Shota Fujimoto Yoshihiko Miyamoto
Yasushi Sato Tetsuji Takayama

Department of Gastroenterology and Oncology, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan

Keywords

Colorectal cancer · Molecular imaging · Fluorescence endoscopy

Abstract

Background: Recent endoscopic studies have revealed that small colorectal tumors are often overlooked during colonoscopy, indicating that more sensitive detection methods are needed. **Summary:** Molecular imaging has received considerable attention as a new endoscopic technique with high sensitivity. It generally employs a fluorescence-labeled compound that specifically binds to a molecule on the tumor. Fluorescent probes for molecular imaging are largely classified as 2 types: a fluorescence-labeled antibody targeting a molecule specifically expressed on the tumor cell surface such as epidermal growth factor receptor or vascular endothelial growth factor (VEGF); and a fluorescence-labeled small molecule compound targeting a molecule specifically expressed in tumor cells including c-Met, glutathione S-transferase, γ -glutamyltranspeptidase, cathepsin, or endothelin A receptor. These probes successfully detected colorectal tumors in several animal studies. Moreover, 3 recent human clinical trials evaluating endoscopic molecular

imaging for colorectal tumors have been reported. In one study, a Cy5-labeled synthetic peptide against c-Met was developed, and fluorescent endoscopic observation with this probe detected a greater number of colorectal adenomas than with white light observation. Another trial used IR800-labeled anti-VEGF antibody, which sensitively detected human colorectal adenomas by fluorescent endoscopy. Last, a fluorescent probe with synthetic peptide against BRAF-positive cells was able to visualize sessile serrated lesions. The fluorescent probes accumulated at very high levels in colorectal tumor cells but at lower levels in surrounding non-neoplastic mucosa. **Key Messages:** We expect that molecular imaging techniques with fluorescent probes will soon lead to the establishment of a highly sensitive endoscopic method for colorectal tumor detection.

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Introduction

Colorectal cancer (CRC) is a major cause of cancer-related death worldwide, and the rate of mortality due to CRC has been increasing in Asia including Japan [1]. It is well accepted that the adenoma-carcinoma sequence is a

Fig. 1. Various targets for molecular imaging of colorectal tumors. VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; ET_AR, endothelin A receptor; GST, glutathione S-transferase; GGT, γ -glutamyltranspeptidase.

major pathway leading to CRC [2]. In addition, the serrated neoplasia sequence has been reported to be an alternative pathway to CRC. In patients with IBD, dysplasia-carcinoma sequence is recognized as an additional carcinogenesis pathway [3]. Therefore, it is very important to detect early-stage CRC and/or precancerous lesions such as adenoma, serrated polyps, and dysplasia in IBD patients through the use of colonoscopy. In general, white light (WL) colonoscopy is the gold standard for colonoscopy screening [4]. However, 15–32% of adenomas go undetected with conventional colonoscopy, which is especially limited for detecting adenomas less than 10 mm in diameter or flat lesions [5]. With recent advances in the molecular biological analysis of colorectal tumors, molecular imaging, and fluorescence endoscopy targeting tumor-specific molecules are rapidly developing technologies that are expected to enable highly sensitive endoscopic detection of early neoplastic lesions. In this review, we summarize recent studies on the molecular imaging of colorectal tumors employing various types of fluorescence-labeled probes and discuss the current status of optical molecular imaging with fluorescence endoscopy for clinical application in the near future.

Targets of Molecular Imaging in Colorectal Tumors

The most important feature of a molecular imaging target is the highly specific expression in tumor cells. In the molecular imaging of colorectal tumors, the target molecule should ideally not only be overexpressed in tumors but also not expressed in surrounding nonneoplastic mucosa. Representative target molecules in colorectal tumors are shown in Figure 1. Epidermal growth factor receptor (EGFR), a receptor-type tyrosine kinase, is highly expressed in CRC and colorectal adenoma [6]. When ligands such as TGF- α or EGF bind to EGFR on the tumor cell surface, the EGFR molecule dimerizes and subsequent autophosphorylation of tyrosine kinase promotes signal transduction for tumor cell proliferation. Vascular endothelial growth factor (VEGF) is also highly expressed in CRC and colorectal adenoma tissue [7]. These tumor cells secrete VEGF to promote angiogenesis to receive nutrients and oxygen needed for rapid cell growth. Endothelin A receptor (ET_AR) is one of the 2 receptor subtypes (A and B) of the endothelin family [8]. ET_AR and its ligand are highly expressed in tumor cells including colorectal tumors, and their interaction induces tumor growth and metastasis by promoting tumor cell survival, proliferation, and angiogenesis.

In addition, enzymes such as glutathione S-transferase-P1 (GST-P1) in the cytosol, γ -glutamyl transpeptidase (GGT) in cell membranes, and cathepsin in lysosomes are highly expressed in certain tumor cell types including colorectal tumors [9, 10]. Molecular imaging studies using a fluorogenic substrate probe, which is fluorescently quenched but activatable by enzyme activity only in the tumor cells, have been reported [11, 12].

Fluorescent Probes for Colorectal Tumors

Fluorescent probes are largely classified into 2 categories: employing an antibody against a tumor-specific molecule and small molecule compounds that specifically bind to target molecules in tumor cells.

Fluorescence-Labeled Tumor-Specific Antibody

Several antibodies, including anti-EGFR (cetuximab and panitumumab), anti-VEGF (bevacizumab), and anti-VEGF receptor (ramucirumab) antibodies, have been approved for the therapy of metastatic CRC worldwide [13, 14]. They are currently recommended for the treatment of CRC as a standard regimen. Advantages of fluorescence-labeled antibody probes are that they are relatively

Table 1. Endoscopic fluorescent molecular imaging for colorectal tumor in animals

Lesion	Target	Species	Antibody/compound used	Fluorescence substance	Author
CRC	EGFR	Mouse	Anti-EGFR-Ab (cetuximab)	AF488	Goetz et al. [17]
CRC	VEGFA	Mouse	Anti-VEGF-Ab (bevacizumab)	ICG	Suo et al. [18]
Adenoma	EGFR	Rat	Anti-EGFR-Ab	AF488	Miyamoto et al. [19]
Adenoma	c-Met	Mouse	Peptide ligand for c-Met	Cy5.5	Wu et al. [20]
ACF	GST	Mouse	Substrate for GST	FITC	Muguruma et al. [11]
Dysplasia	GGT	Mouse	Substrate for GGT	ProteoGREEN	Mitsunaga et al. [16]
Dysplasia	Cathepsin	Mouse	Substrate for cathepsin	Cy5	Gounaris et al. [12]
Dysplasia	ET _A R	Mouse	ET _A R antagonist	Cy5.5	Mücke et al. [22]

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; VEGFA, vascular endothelial growth factor A; ICG, indocyanine green; ACF, aberrant crypt foci; GST, glutathione-S-transferase; GGT, γ -glutamyltranspeptidase; FITC, fluorescein isothiocyanate; ET_AR, endothelin A receptor.

easy to apply in human clinical trials and that they show high specificity for the targeting molecule. However, disadvantages of them include a longer time (more than 24 h) to bind antigen sufficiently after intravenous administration, and immunogenicity.

Fluorescence-Labeled Small Molecular Compounds

Small molecular compounds, including enzyme substrates, synthetic peptides, and receptor antagonists, which bind to a specific target molecule in tumor cells, were labeled with fluorophore and used for molecular imaging. The fluorogenic substrate probe is a fluorescently quenched small molecule substrate designed to react with an enzyme in tumor cells to release a strong fluorophore. Fluorogenic probe reacting with GGT, GST, and cathepsin in tumor cells has been reported [12, 15, 16]. Recently, a synthetic peptide has been used for molecular imaging studies. The peptide probe is usually developed using the phage display method against a specific protein expressed in tumor cells. Advantages of peptide probes include generally lower toxicity, lower immunogenicity, increased permeability, and flexibility in chemical modulation. Disadvantages of peptide probes include the labor intensiveness of identifying a specific peptide sequence during peptide synthesis and the need to sufficiently wash out residual fluorescent probe during colonoscopy.

Molecular Imaging of Colorectal Tumors in Animals

Representative endoscopic molecular imaging studies for detection of CRC and polyps in animals are summarized in Table 1. A CRC molecular imaging study using

anti-EGFR antibody (cetuximab) labeled with Alexa Fluor 488 (AF488) was reported by Goetz and associates [17]. They inoculated 2 human CRC cell lines (HT 29 cells, which strongly express EGFR, and SW600 cells, which weakly express EGFR) into nude mice, administered AF488-labeled cetuximab intravenously, and observed the tumors using confocal laser endoscopy (CLE) after 48 h. Stronger fluorescence was observed in the xenograft tumors with HT29 cells than in SW600 cells. The HT29-xenografted mice showed significantly slower tumor growth and better survival than SW600-xenografted mice. Since cetuximab has strong anticancer activity against CRC cells, molecular imaging with fluorescence-labeled cetuximab is useful not only for tumor imaging but also for inhibiting tumor growth during the diagnostic examination.

Suo and colleagues labeled the anti-VEGFA antibody bevacizumab with indocyanine green (ICG) and administered the fluorescent probe intravenously in an orthotopic xenograft rat model of CRC [18]. A clear fluorescent image of the tumor was visualized using their self-made fluorescent endoscopy system for the near infrared spectral range.

Since adenoma-carcinoma is one of the major pathways of colorectal carcinogenesis, studies targeting adenomas have been reported. Miyamoto and associates performed molecular imaging of colorectal adenoma using AF488-labeled cetuximab in an azoxymethane (AOM)-induced chemical carcinogenesis rat model [19]. They observed small adenomas on the colorectal mucosa of AOM-treated rats using veterinary endoscopy with WL. They, then, sprayed AF488-labeled cetuximab and observed the colorectal mucosa with a fluorescence endoscope. Strong fluorescent signals were seen on the mu-

Fig. 2. Endoscopic and histological findings of colorectal polyps from AOM-treated rats. Representative endoscopic image of a colorectal polyp in an AOM-injected rat observed using a thin endoscope (**a, b**). A 3-mL aliquot of AF488-EGFR-Ab (20 μ g/mL) was given by enema with or without pretreatment with non-labeled EGFR-Ab (200 μ g/mL) (**c, d**), and colorectal polyps were

detected by fluorescent thin endoscopy (**e**). Histopathological findings of the resected tumor (**h, e**), EGFR immunohistochemical staining of the polyp (**f**). Figure 2 was adopted and modified from Fig. 5 in *Cancer Sci.* 2019;110:1921–30. AOM, azoxymethane; AF488, Alexa Fluor 488; EGFR, epidermal growth factor receptor.

Table 2. Human clinical trial on endoscopic fluorescent molecular imaging for colorectal tumor

Target lesion	Target	Antibody/compound	Fluorophore	Patients, <i>n</i>	Administration route	Author
Adenoma	c-Met	Peptide ligand for c-Met	Proprietary cyanine dye	17	Intravenous	Burggraaf et al. [23]
Adenoma/SSL	VEGFA	Anti-VEGFA-Ab (bevacizumab)	RDye 800CW	15	Intravenous	Hartmans et al. [24]
SSL	unknown	Peptide	FITC	25	Intrarectal	Joshi et al. [25]

SSL, sessile serrated lesion; FITC, fluorescein isothiocyanate; VEGFA, vascular endothelial growth factor A; ICG, indocyanine green. IRDye 800CW.

cosa at the same site (Fig. 2). All polyps found under WL, including small polyps with a diameter of 1–2 mm, were detectable by fluorescence imaging. Strong expression of EGFR was confirmed in the adenoma tissue.

Wu and associates developed a synthetic peptide ligand for c-Met using a phage library method and labeled it with Cy5.5 to synthesize a fluorescent peptide probe [20]. They administered it intrarectally in CPC-Apc mice, which spontaneously develop adenomatous polyps, and observed the colorectum. Fluorescence signals were observed in flat adenomas as well as in polypoid adenomas.

We found that GSTP1-1 is highly expressed in aberrant crypt foci (ACF) and colorectal adenoma as well as in CRC [9]. ACF is the earliest form of colorectal tumor lesion, which shows dense staining with methylene blue and is detectable by stereomicroscopy or magnifying endoscopy [21]. Employing a fluorogenic probe (DNAT-Me) activated by GSTP1-1, we performed molecular imaging of ACF, colorectal adenoma, and CRC in an AOM-induced rat carcinogenesis model and in human colorectum *ex vivo* [11]. DNAT-Me is a fluorescently quenched substrate but is activated by GSTP1-1 in tumor cells to release a strong fluorophore. We clearly visualized small ACF on the colorectal mucosa of AOM-treated rats. We also visualized human ACF on colorectal mucosa in surgically resected specimens from 7 CRC patients. After the operation, we sprayed DNAT-Me on the colorectal mucosa of resected tissue and successfully detected ACF using our fluorescence imaging system. Almost all ACF were detected with a strong signal, and the S/N ratio of images taken with DNAT-Me (1.82 ± 0.42) was significantly higher than that with methylene blue staining. We found that this technology may enable easy and sensitive detection of ACF as a surrogate marker for risk stratification and the evaluation of chemopreventive agents for CRC.

Dysplasia-carcinoma sequence is also a well-recognized pathway to CRC in IBD patients, and the molecular imaging studies for such colitis-associated cancers have been re-

ported. Mitsunaga and associates conducted an imaging study using the fluorogenic probe γ -glutamylhydroxymethyl rhodamine green, which is cleaved by GGT to release the fluorescent compound rhodamine green [15, 16]. The fluorogenic probe was administered by enema in mice treated with dextran sodium sulfate/AOM, and dysplastic lesions were observed using a veterinary fluorescence endoscopy. Several tumors on the colorectal mucosa were detectable by fluorescence endoscopy, and the histopathology of the dysplasia was confirmed by colonoscopic biopsy.

A fluorescently quenched substrate-based probe activated by cathepsin activity to release strongly fluorescent Cy5.5 was synthesized for molecular imaging of dysplastic lesions in ulcerative colitis based on previous findings showing that the lesions have enhanced cathepsin activity [12]. The fluorogenic probe was intravenously injected in IL-10-knockout mice, and fluorescent signals were visualized in the colorectal mucosa using a veterinary fluorescence endoscope. Histological examination revealed dysplastic lesions where cathepsin activity was increased.

Moreover, Mucke and associates conducted a study to visualize dysplasia and cancer targeting ET_AR in cell membranes [22]. Since ET_AR has been reported to be highly expressed in CAC tissues, they synthesized a fluorescent probe by labeling an ET_AR antagonist with Cy5.5 and administered it intravenously in a dextran sodium sulfate/AOM mouse model, followed by observation of the colorectal mucosa by fluorescence endoscopy. Tumors were easily detectable compared with the adjacent mucosa with significantly high contrast.

Human Clinical Trial for Endoscopic Detection of Colorectal Tumors

Three human clinical trials of endoscopic molecular imaging for colorectal tumors have been reported to date (Table 2). Burggraaf and associates developed a 26-amino

acid cyclic peptide that specifically binds human c-MET employing the phage display method [23]. They labeled the peptide with cyanine dye (Cy5) (GE-137), injected it in mice in a xenograft model, and detected specific fluorescent signals on the tumor. After completing toxicity experiments in rats and monkeys, they performed a phase I study in humans to assess toxicity and determine optimal dosing. They administered the peptide probe intravenously in 15 patients at high risk of colorectal neoplasia and observed the colorectal mucosa using prototype endoscopy, which can generate fluorescence and WL images simultaneously. They were able to visualize all neoplastic polyps using fluorescent imaging, including 9 additional polyps that were not visible with WL.

Hartmans and associates performed a pilot study in humans using IRDye 800CW-labeled anti-VEGFA antibody (bevacizumab) [24]. They administered IRDye 800CW-labeled bevacizumab intravenously in 17 patients with familial adenomatous polyposis (FAP), and observations were made 3 days later using both WL endoscopy and NIR-fluorescence endoscopy on the same monitor simultaneously. Even small adenomas less than 3 mm in diameter were detectable by fluorescent endoscopy with IRDye 800CW in colorectal mucosa.

Recently, Joshi and associates using a phage display method identified a peptide that binds specifically to sessile serrated lesions (SSLs) [25]. SSLs are precancerous lesions that predominantly develop in the proximal colon and are positive for BRAF V600E mutation. Although the target molecule of the peptide is unknown, they synthesized an FITC-labeled peptide probe and validated the specific binding of the labeled peptide with SSL cells in vitro experiments. After conducting a toxicity study of the fluorescent probe in rats, they performed a pilot study in humans to test for safety and imaging of SSL. They observed the proximal colon using colonoscopy with WL, sprayed FITC-labeled peptide on the colorectal mucosa, and then performed fluorescence observation. They observed 19 SSLs in 38 patients with WL and fluorescent images. The mean fluorescence intensity in the SSLs was 2.43-fold higher than in surrounding nonneoplastic mucosa, and SSLs were distinguishable from nonneoplastic mucosa with 89% sensitivity and 92% specificity.

Fluorescent Dyes

Near infrared light has a wavelength between 700 nm and 1 μ m. It is useful in terms of its higher tissue penetration and safety for human body than ultraviolet rays.

Moreover, there is little background noise in the human body, especially in the gastrointestinal tract under infrared light exposure [26]. The interest in the use of near-infrared fluorescence imaging has been steadily growing.

ICG has been used in humans for more than 50 years. ICG emits light at 807–832 nm upon excitation at around 770 nm [27]. Although the structure of ICG must be modified to enable covalent binding with an antibody or small molecule, it might be suitable as a molecular imaging agent due to its low toxicity. IRDye 800CW (IR800) is a water-soluble near-infrared dye with a peak excitation at 774 nm and emission at 803 nm [28]. IRDye 800CW has ideal quantum yields and a high extinction coefficient, in addition to being more photostable and brighter.

Fluorescein and its derivative FITC have been used in humans for more than 30 years. FITC has an excitation peak at 498 nm and an emission peak at 522 nm. Although FITC is inexpensive and widely used for molecular imaging, its disadvantages include lower tissue penetration due to the relative short wavelength (517 nm) and a short decay time.

Other fluorescent dyes, including Alexa Fluor and Cy5 are used for molecular imaging studies. For the clinical use of these dyes, characteristics including toxicity, stability, and fluorescent intensity in the human body need to be analyzed.

Future Areas of Investigation

We did not describe herein endoscopic devices for fluorescent observation in detail. There are currently no commercially available fluorescent endoscopy device with sufficient performance. Therefore, in the human studies of endoscopic molecular imaging described above, the authors and collaborative groups used self-made endoscopic devices or prototype endoscopic systems. CLE is an emerging technology which allows real-time in vivo imaging of cellular and subcellular details of the mucosa and vessels with high-resolution images. CLE could be applicable to endoscopic molecular imaging [17] because of the optical characteristics, including deeper observation depth range (0–250 μ m). However, this device is not currently suitable for screening endoscopy because of the limited field of view (475 $\times$ 475 μ m). Moreover, the laser scanning unit is limited to the excitation wavelength of 488 nm/660 nm, and more flexibility for various fluorescent agents such as IRDye and ICG is required. Thus, it is important to develop endoscopic devices with sufficiently high performance and the ability to observe various wavelengths.

Requirement for Clinical Application and Limitation of Molecular Imaging

To apply the molecular imaging technology to clinical endoscopic examination, a large-scale clinical trial to test the utility and safety of the fluorescent probe is needed. The detection rate with molecular imaging using the fluorescent probe should be compared to those with WL and image-enhanced endoscopy such as narrow banding image and Blue laser image. Moreover, as described above, a high-performance fluorescent endoscopic device should be developed.

There are currently some limitations of molecular imaging technology. First, it needs administration of fluorescent probe into human body via either intravenous administration or topical administration. Therefore, we have to confirm the safety of the fluorescent probe. Second, the molecular target is different among the precancerous lesions such as adenoma, serrated polyps, and dysplasia, and therefore, currently there is no fluorescent probe which is able to detect all the precancerous lesion types simultaneously.

Conclusion

Molecular imaging endoscopy is expected to play an important role in the diagnosis of colorectal tumors and in guiding decision-making regarding the choice of

treatment, as well as to provide a means for early-phase treatment of colorectal tumors. The development of this technology is in its early stages, and several challenges remain before it can be brought to fruition, including target identification, compound screening, pre-clinical testing, and phase 1–3 trials. If successful, this technology could lead to great progress in clinical oncology.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

The authors have no finding sources.

Author Contributions

T. Takayama designed the structure of the manuscript. T. Kashiwara mainly wrote the draft manuscript. N. Muguruma described the fluorescent probe section. S. Fujimoto described the animal experiment section. Y. Miyamoto described the fluorescent dye section. Y. Sato was responsible for figures and tables and references.

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