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Molecular Imaging in Gastrointestinal Endoscopy:

Imaging and Advanced Technology Section

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Abstract

Molecular imaging is a rapidly growing new discipline in gastrointestinal endoscopy. It uses the molecular signature of cells for minimally-invasive, targeted imaging of gastrointestinal pathologies. Molecular imaging comprises wide field techniques for the detection of lesions and microscopic techniques for in vivo characterization. Exogenous fluorescent agents serve as molecular beacons and include labeled peptides and antibodies, and probes with tumor-specific activation. Most applications have aimed at improving the detection of gastrointestinal neoplasia with either prototype fluorescence endoscopy or confocal endomicroscopy, and first studies have translated encouraging results from rodent and tissue models to endoscopy in humans. Even with the limitations of the currently used approaches, molecular imaging has the potential to greatly impact on future endoscopy in gastroenterology.

Keywords

Molecular imaging; endoscopy; confocal endomicroscopy; autofluorescence imaging; cancer; EGFR

Introduction

Molecular imaging has raised increasing interest in the field of gastrointestinal (GI) endoscopy with the potential to significantly impact on our current diagnostic and therapeutic algorithms and biomedical research. Molecular imaging encompasses modalities that enable minimally-invasive visualization of disease-specific morphologic or functional tissue alterations based on the specific molecular signature of single cells or whole tissue. This discipline has been strongly driven by recent developments to provide individualized molecularly targeted therapies in the field of oncology and – to a lesser extent – inflammatory diseases. At the same time, technological and scientific advancements in endoscopy have provided us with new imaging devices to enhance detection and characterization of early neoplastic lesions, such as chromoendoscopy and virtual chromoendoscopy techniques, surface enhancement modalities in conjunction with high-resolution endoscopes and ultrahigh magnification during endoscopy. While subsequent endoscopic therapy usually relies on the detection of lesions at an early stage, recent studies have still reported a significant miss rate throughout the entire GI tract. Molecular imaging in GI endoscopy therefore aims at identification and characterization of lesions based on their molecular fingerprint rather than their morphology and ultimately at increasing the efficiency of endoscopic screening and surveillance. This usually requires detection of

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biomarkers with a device compatible with use in humans. On the basis of insights gathered from animal experiments most pre-clinical and clinical trials have utilized fluorescent detection of biomarkers. Ideally, molecular endoscopy combines wide-field macroscopic imaging, providing red flag detection of areas of interest within the large surface of the GI mucosa and a modality to provide targeted microscopic characterization of such a lesion.

Optical contrast /Contrast agents for molecular imaging

Optical contrast can arise from endogenous fluorophores or exogenously administered contrast agents. In autofluorescence imaging (AFI), tissue excitation with light of a short wavelength results in emission of a longer wavelength. Alterations in the autofluorescence pattern of neoplastic tissue have been attributed to altered metabolic activity, such as FAD, NADH, and porphyrins as well as hemoglobin content and a breakdown of collagen fiber cross-links. This results in a shift towards the red spectrum when such tissue is excited with blue light. In addition, typical morphologic signs of malignancy such as increased nuclear-to-cytoplasmic ratio influence the propagation of light. The altered autofluorescence signal is translated into false colored images, usually depicting neoplasia in purple against a green background of healthy mucosa. Many of these tissue alterations are not specific for neoplasia, and the resultant AFI image is a combination of multiple molecular alterations. Therefore, AFI suffers from a low specificity and a high false-positive rate but benefits from the fact that no contrast agent has to be applied during endoscopy. On the other hand, the intrinsic signal can be enhanced by the application of precursor molecules that are metabolized to photodynamically active substances. 5-aminolevulinic acid (5-ALA) is the most widely used agent. Similar to AFI, inflammation negatively impacts on the specificity.

Induced fluorescence is several orders of magnitudes more intense than autofluorescence. Exogenous molecular probes usually target a disease-specific biomarker(1). Such probes include antibodies, antibody fragments, peptides, nanoparticles and “smart” activatable probes (Fig. 1). Several studies have used fluorescently labeled antibodies against epitopes that are commonly overexpressed in most gastrointestinal cancers, such as vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) (2, 3). Antibodies bind to their target structure in a highly selective manner, thereby optimizing the signal-to-background ratio. In addition, the biologic relevance of their targets is often well established and exploited therapeutically even today, such as by Cetuximab or Panitumumab (against EGFR) or Bevacizumab (against VEGF). Imaging of tumors after a first labeled test dose could potentially predict response to targeted chemotherapy. On the other hand, antibodies may confer allergic reactions after systemic application, and their diffusion across epithelial borders and delivery to target structures is slow due to their high molecular weight. Peptides are low molecular weight molecules that consist of a few amino acids in length and face fewer of these limitations(4). The challenge in developing these peptides is to select unique sequences that have high specificity and affinity towards the target structures. Antibody fragments could serve as an alternative. Nanoparticles such as quantum dots and metallic nanoparticles can be coated with significantly stronger fluorophores. In animal and cell culture studies, they allow targeting of even minute amounts of target structures, and can be loaded with ligands to multiple biomarkers. Pharmacotoxic considerations have so far precluded widespread clinical testing.

The contrast agents described above all rely on direct binding to their target site. Depending on their affinity and the biodistribution of the target they may show a significant background signal of unbound or non-specifically bound agent. In contrast, “smart” probes are activated by specific biomarkers that are selectively upregulated in the tissue of interest. Imaging probes have been designed to be activatable by proteases overexpressed in tumors(5). In their native state, the fluorescent activity is quenched by energy resonance transfer among

fluorophores or by a molecular quencher. After cleavage by tumor-associated proteases, these probes show a significant increase of fluorescence intensity in the tumor. pH-activatable probes have also been developed that can be linked to target specific structures on the tumor cell surface that are to be internalized after probe binding(6). After subsequent integration into the acidic lysosomes, the pH-sensing fluorophore is activated. Both quenching in the quiescent state and tumor-specific activation optimize the signal-to-background ratio for fluorescent imaging.

In order to obtain a specific signal, the molecular probe has to gain access to the region of interest. Eventually the choice for either route of application will be determined by the distribution and accessibility of the target structure. Systemic application may be preferred if even distribution throughout the body is sought. Potential side effects of intravenous application may be higher than with topical application, and the timing of imaging after contrast application has to be well standardized to ensure optimal binding to the target while at the same time minimizing background signal. With topical application, e.g. via a spraying catheter during colonoscopy(4), a region of interest has to be identified a priori, and specific binding of the molecular probe to the target has to occur within a time frame that is compatible with the endoscopy procedure, usually within a few minutes. Targets have to be on the luminal surface of the tissue, or the fluorescent probe has to permeate rapidly towards the target structure to ensure binding.

None of the currently tested molecular probes has yet undergone extensive pharmacodynamic and safety assessments. However, in radiology and nuclear medicine molecular imaging with radioactively labeled probes has become part of the routine workup for certain indications. Similarly, protocols for the application of fluorescently labeled probes in GI endoscopy are now being tested.

Devices for molecular imaging

Devices for molecular imaging should be able to minimally-invasively detect and characterize molecular changes that are found in neoplasia or inflammation. This requires a form factor that is compatible with the use in conventional diagnostic and therapeutic endoscopy and sufficient sensitivity to detect the molecular probes at the relevant concentration that bind to the target structures.

In a typical endoscope for AFI, the mucosa is sequentially illuminated with red, green, and blue light for tissue excitation (blue light) and reflectance imaging (green and red light). The sequential images are overlaid and displayed as a pseudo-colored image. These endoscopes have recently been combined with high definition white light imaging and narrow-band imaging (NBI) to provide endoscopic tri-modal imaging. AFI has been studied in Barrett's esophagus, in screening colonoscopy and in ulcerative colitis(7, 8). Endoscopes for wide-field detection of induced fluorescence have not yet been evaluated in larger clinical trials for molecular imaging. For confocal endomicroscopy, two devices are currently being marketed, an endoscope based type and a probe based type. In the first, the miniaturized components of a confocal laser scanner have been integrated into the distal tip of a flexible white light endoscope for dual imaging in clinical use or into a handheld probe for animal in vivo microscopy or human laparoscopy. At variable imaging depth from surface to 250 μm , serial optical sections are obtained at approximately one frame per second with high resolution of 1024x1024 pixels by using a single optical fiber functioning as both the illumination and detection pinholes.

A different approach is used in flexible probe based confocal microscopy. These confocal probes are available with diameters being compatible with the working channel of most endoscopes for clinical use in GI endoscopy and even in the bile duct. Image acquisition is

faster with this probe (12 frames/s), but resolution limited by the number of the fibers (~30000 pixels). Different probe types encompass different imaging planes, but for each single probe the imaging plane is fixed. Both endomicroscopic devices use blue laser excitation and fluorescence detection at > 505 nm. A large number of clinical trials in a multitude of indications have been performed using these endomicroscopes, mostly with fluorescein for non-specific fluorescent staining for morphological imaging, and they have been demonstrated to be safe (9).

Preclinical and clinical studies using molecular imaging in gastroenterology

The appropriate selection of targets for molecular imaging highly depends on the disease entity investigated. All targets have to be accessible (depending on the mode of application of the molecular probe), specific for the disease entity with an established biological significance for the tumor or inflammatory condition. In animal or ex vivo studies, cell surface, interstitial, vessel, intracellular, and even intranuclear markers have been studied.

Fluorescence imaging can be used for targeted detection(10). This method is being developed for guiding biopsy of high-grade dysplasia in Barrett's esophagus using labeled peptides (Fig. 2). An affinity peptide at a concentration of 10 M, selected with techniques of phage display, was topically administered over a region of intestinal metaplasia in the distal esophagus. After a 5 minute incubation period, the unbound peptides were rinsed off. In Fig. 1a, the white light endoscopic image shows a 4 cm length of salmon pink mucosa consistent with endoscopically apparent Barrett's esophagus. No distinct architectural features can be appreciated to guide the physician in finding the pre-malignant lesion. The molecular image, shown in Fig. 1b, reveals increased intensity at a site (arrow) that was subsequently biopsied and found to be high-grade dysplasia on histology. Binding of the peptide to the outer surface of the dysplastic crypts can be seen in the fluorescence microscopy image shown in Fig. 1c. The corresponding histology (H&E) is shown in Fig. 1d (20X magnification).

In animals, probes were studied that were activated to emit light in the near infrared (NIR) spectrum by cathepsin B, a cystein protease that is overexpressed by many murine and human colorectal neoplasias(5). 24 hours after intravenous application in mice, the cathepsin B activated beacon demonstrated a high specificity to detect neoplasia with a benchtop imaging device. Recently, this has been linked to fluorescent imaging with a prototype illumination fiber bundle for white light and NIR imaging in mouse colonoscopy which could be potentially fitted through most endoscopes (Fig. 3) (10). During colonoscopy after orthotopic tumor induction, white light and NIR images could be displayed simultaneously, and pseudocolored overlay images were constructed at real time to assist in the detection of small lesions. Such imaging has also been performed with capsule endoscopy(12). In a proof-of-principle approach, a commercially available capsule has been equipped with a filter device capable of NIR imaging in addition to white light imaging. Although the device still relied on external modifications it demonstrated the technical feasibility of molecular imaging by capsule endoscopy.

The fluorescent labeling of receptors or proteins overexpressed on tumors by specific antibodies has been performed in both animal studies and on excised human tissue, however mostly relying on bulky bench top devices not appropriate for use in humans. Hsu et al. targeted EGFR on human squamous cell cancer using labeled monoclonal antibody(13). Becker et al. effectively labeled octreotate with a conjugated peptide in a mouse xenograft model of neuroendocrine tumors(14). Both EGFR and octreotate are well established therapeutic targets in oncology. However, selective labeling of surface receptors may carry the risk of missing tumors that do not express the specific biomarker. Therefore, dual

labeling has been performed in a xenograft mouse model with an antibody cocktail targeting both EGFR1 and EGFR2 labeled with conjugates with different emission spectra(2). This approach effectively differentiated tumors in vivo based on their fluorescence patterns in a whole body imaging device.

All the above described approaches used molecular imaging as a wide field, red-flag technique to increase macroscopic detection of neoplasia, relying on conventional ex vivo histopathology for tumor characterization. In contrast, confocal endomicroscopy was used in two trials for microscopic molecular imaging. In the first trial in humans, a phage library was screened, and in multiple clearings on healthy and neoplastic tissue, a heptapeptide was isolated and conjugated with fluorescein for labeling of human colorectal neoplasia (Fig. 4) (4). This peptide was topically applied to 18 neoplastic lesions during colonoscopy in patients. Although the molecular target of this sequence has not yet been identified, probe based confocal endomicroscopy visualized preferential binding in vivo of the molecular beacon to neoplastic cells with a sensitivity and specificity of 81% and 82%, resp. In a second trial, human xenograft tumors in mice with high or low EGFR expression could be identified and correctly classified by the intensity of EGFR expression in confocal endomicroscopy in vivo after the injection of a fluorescently labeled antibody against EGFR (Fig. 5) (3). In human tissue specimens, molecular imaging with endoscope-integrated endomicroscopy was able to discriminate healthy and neoplastic mucosa based on the EGFR expression after topical administration of labeled antibody.

Future Challenges

A strong clinical need for “molecular chromoendoscopy” exists for cancer screening and surveillance, and includes but is not limited to diseases such as Barrett’s esophagus, gastric intestinal metaplasia, flat and depressed sporadic colonic adenomas, ulcerative colitis, hereditary polyposis syndromes, and indeterminant biliary strictures. In these, a large mucosal area is at risk for developing neoplasia, and current endoscopic strategies have been reported to miss a significant proportion of lesions. It is not yet established whether the results of the rather simplistic animal models can be directly translated to the genetically more diverse human neoplasias in vivo. In addition, many of the above diseases develop neoplasia in the setting of inflammation. Here, the specificity of molecular markers must be evaluated to differentiate between non-neoplastic mucosa and initial neoplasia that is not yet visible on white light endoscopy, but may be amenable to endoscopic therapy. Fluorescence must be strong enough to allow detection even of minute early neoplasia as small as only few cells (“needle in a haystack”), and fluorescence detection devices should be equipped with a modality to quantify the specific fluorescence of a lesion against the background noise.

NIR imaging permits deeper tissue imaging than blue laser excitation because of reduced hemoglobin absorption and less tissue scattering. In the future, non-linear imaging techniques such as multi-photon microscopy could even further enhance imaging plane depth without compromises in resolution. However, most neoplasia in gastroenterology arises from the epithelial layer and is technically detectable with the currently used instrument settings with either blue or red light. Indeed, first studies used a set up for molecular imaging that is technically transferable to human endoscopy today(3, 11) or have already been performed in humans(4). Multiple excitation and detection wavelengths in conjunction with multiple labels(2) may further enhance the fluorescence signal intensity and specificity of detection.

Most studies using systemic application of antibodies or activatable molecular beacons required injection 24 hours before imaging, whereas topical application of labeled peptides

or antibodies was performed within a time frame compatible with use in colonoscopy. Pharmacokinetic studies in patients are required to define the optimal imaging time point to achieve a sufficient tumor-to-background ratio as well as the rapid clearance of the dye after imaging. Another key issue for translation of molecular endoscopy into clinical use is a stringent demonstration of lack of toxicity of exogenously applied agents.

In GI oncology, recent advances have concentrated on efforts to provide an individualized therapy to provide high anti-tumor efficacy while at the same time minimizing adverse events and the financial burden on the health care system. In vivo molecular imaging has a high potential to assist in the selection of patients who are likely to benefit from such tailored therapies and to monitor response to therapy. At the same time, in vivo molecular imaging of live tissue may be less prone to bias from sampling error, tissue processing and staining than conventional immunohistochemistry. Similar therapeutic algorithms could be anticipated for imaging in inflammatory diseases prior to therapy with biologics, such as in inflammatory bowel diseases. Once integrated into current approaches of screening and surveillance, such multimodal molecular endoscopy will have to prove its diagnostic and predictive efficacy.

A future endoscope for molecular imaging should encompass three imaging modalities: High resolution video white light imaging is mandatory for accurate wide field overview, navigation of the instrument, and therapeutic interventions. A second monitor should simultaneously display macroscopic fluorescence at real time as a red flag technique providing “molecular chromoendoscopy” to identify lesions at risk. A third imaging modality should enable endomicroscopic resolution of such a lesion to confirm the suspected diagnosis and permit immediate decision on the endoscopic management. Even with the limitations of the currently used approaches, these imaging modalities are available. Huge progress has been achieved in recent years, and molecular imaging will most likely greatly impact on future diagnostic and therapeutic endoscopy in gastroenterology.

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Type	Peptide	Antibody	Activatable probe	Nanoparticle

Advantages	<ul style="list-style-type: none"> • Easy delivery to target structure • Low immunogenicity • Low cost 	<ul style="list-style-type: none"> • High specificity • Defined target • Defined and approved therapeutic ab may be labeled 	<ul style="list-style-type: none"> • Specific activation • Optimized signal-to-noise ratio 	<ul style="list-style-type: none"> • Loading with multiple proteins for multivalent targeting • Strong fluorescence
Disadvantages	<ul style="list-style-type: none"> • Variable affinity 	<ul style="list-style-type: none"> • Potential immunogenicity 	<ul style="list-style-type: none"> • Internalization frequently required for activation • Undefined safety profile 	<ul style="list-style-type: none"> • Potential toxicity of non-biocompatible core • Renal clearance

Fig. 1.
Comparison of different molecular probe classes

Fig. 2. Targeted detection of high-grade dysplasia in Barrett's esophagus with fluorescence imaging. a) White light endoscopic image of Barrett's esophagus shows absence of architectural features to guide biopsy for dysplasia. b) Molecular image after topical administration of labeled peptides shows increased fluorescence intensity at a site (arrow) of high-grade dysplasia. c) Binding of the peptide to the outer surface of the dysplastic crypts can be seen on fluorescence microscopy. d) Corresponding histology (H&E) at 20X magnification.

Fig. 3.

In living mice after orthotopic tumor implantation, white light and NIR images captured with a prototype microcatheter show a small, flat adenocarcinoma. The whitish lesion in a) demonstrates strong NIR signal in b), reflecting protease activity after tumor specific activation of a molecular probe, which is absent in the surrounding healthy mucosa. c) shows white light images with false color overlays for tumor location. (reproduced from(10) with permission of the publisher.)

Fig. 4. In vivo fluorescence image collected at border of a) colonic adenoma with confocal miniprobe demonstrates selective binding of a fluorescein-labeled peptides selected from a phage library(4) and shows specific affinity to b) dysplastic crypts (left) in comparison to adjacent normal mucosa (right). Scale bars, 20 μm .

Fig. 5.

a) Full body fluorescent imaging of a human colorectal cancer xenograft in a nude mouse identifies the EGFR positive tumor in the right groin after injection of labeled monoclonal antibodies. b) In vivo confocal endomicroscopy of the xenograft visualizes the binding of the fluorescently labeled antibodies. c) Ex vivo immunohistochemistry confirms the EGFR overexpression on cancer cells. Scale bars 30 μ m.