Review

Molecular endoscopic imaging in cancer

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Cancer is one of the major causes of death in both the USA and Europe. Molecular imaging is a novel field that is revolutionizing cancer management. It is based on the molecular signature of cells in order to study the human body both in normal and diseased conditions. The emergence of molecular imaging has been driven by the difficulties associated with cancer detection, particularly early-stage premalignant lesions which are often unnoticed as a result of minimal or no structural changes. Endoscopic surveillance is the standard method for early-stage cancer detection. In addition to recent major advancements in endoscopic instruments, significant progress has been achieved in the exploration of highly specific molecular probes and the combination of both will permit significant improvement of patient care. In this review, we provide an outline of the current status of endoscopic imaging and focus on recent applications of molecular imaging in gastrointestinal, hepatic and other cancers in the context of detection, targeted therapy and personalized medicine. As new imaging agents have the potential to broadly expand our cancer diagnostic capability, we will also present an overview of the main types of optical molecular probes with their pros and cons. We conclude by discussing the challenges and future prospects of the field.

Key words: antibody labeling, confocal endomicroscopy, endoscopy, ex-vivo study, molecular imaging

INTRODUCTION

Cancer causes a significant number of deaths in the USA and Europe second only to heart disease. However, cancer death rates are dropping because of improvements in early detection techniques and treatment. In this context, the most recently introduced molecular endoscopic imaging (MI) approach has the potential to positively change the trend and aftermath of patient care. Here, we review the current knowledge on MI, provide an extensive summary of the already published literature in this field and discuss future applications of this rapidly evolving technique.

Molecular imaging may be defined as the non-invasive, real-time monitoring of biochemical activity at the molecular and cellular level of living cells, tissues and intact subjects. With the help of specialized instrumentation and imaging agents, physicians are able to characterize tissue based on cellular markers (Fig. 1). Although anatomy plays a major role in diagnosis and treatment strategies of luminal gastrointestinal disorders, the significant growth of molecular imaging may contribute to improvements in specificity and quantitative analysis for early detection and specified treatment of cancer. As it offers a unique insight into the human body it has the potential to meet the growing demand among physicians, patients, and societies for individualized and personalized medical care.

The major impetus behind the application of MI is based on the weaknesses of current techniques which are: (i) incapable of analyzing the intact organism; (ii) hamper real physiological conditions as a result of removal of tissue; and (iii) unable to make longitudinal studies etc. As a tool for patient care, MI approaches have a number of advantages, such as real-time study of cellular processes, longitudinal data, investigation of intact tissue etc. As an example, the recent and the most useful strategy in breast cancer treatment is largely dependent on the molecular profiling of the tumor (e.g. human epidermal growth factor receptor 2 [HER2]/neu-targeted therapy works only for HER2-positive breast cancers).

During the diseased condition, physiological processes are changed in the affected cells. For example, cancerous cells grow, divide and differentiate abnormally and...
incompetently, turning the cells into benign and malignant tumors. Conventional imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI) etc. are used to detect these. Unfortunately, these techniques often detect cancer too late to achieve a cure as they are largely dependent on morphological changes in tissue. In contrast, MI is capable of visualizing the slightest modifications of cellular activity.

MOLECULAR ENDOSCOPIC IMAGING

Endoscopy is being widely used for in vivo imaging and for clinical care of cancer in the digestive tract and, in recent years, it has experienced an extensive advancement of optics and mechanics. A successful combination of advanced endoscopic instruments with a diverse group of molecular probes could significantly improve patient care through early detection of cancer followed by image-guided therapy. In MI, there is a range of different image modalities allowing for visualization of specific physiological processes. Conventional imaging modalities such as MRI, positron emission tomography (PET), single-photon emission computed tomography (SPECT), CT, and ultrasound (US) have distinct advantages and limitations. Whereas we will not discuss these in detail in the present review, they all have relatively low spatial resolution in comparison to optics, some risks as a result of the use of radioactive agents and are significantly expensive. In contrast, optical imaging offers several advantages and has the potential to be used together with molecular probes for specific detection of disease-specific alterations. This technique is non-ionizing, able to provide resolution on the micron-scale level and thus permits for subcellular visualization and characterization. Moreover, optical imaging can measure in real time, therefore allowing for ad hoc decisions regarding diagnosis and therapy.

During the last decades, several endoscopic imaging methods were developed. In the luminal gastrointestinal tract, white-light endoscopy (WLE) plays an important role in the detection of lesions. However, it is well accepted that WLE can miss a significant number of lesions including early cancers. To overcome these limitations, chroendoendoscopy is used to enhance mucosal surface and vascular pattern morphology. However, because of a variety of staining materials and possible concentrations, the process is time-consuming, sometimes inconclusive and cost-expensive. Therefore, the need for improvements in endoscopy technology, particularly for detecting early-stage cancers or flat lesions appears obvious.

Endomicroscopy is a procedure that is capable of analyzing both surface and cellular structures in vivo. As a result of microscopic investigation, the nature of a lesion (benign or malignant) can be determined in real time. Two systems are approved by the European Medicines
Agency (EMA) and the US Food and Drug Administration (USFDA): (i) confocal laser endoscopy based on the integration of a confocal microscope into the distal tip of a conventional endoscope (Pentax, Tokyo, Japan); and (ii) a flexible confocal miniprobe comprising a fiberoptic bundle with an integrated distal lens connecting to a laser scanning unit (Cellvizio; Mauna Kea Technologies, Paris, France). 27,28

Confocal laser endomicroscopy (CLE) is based on the flashing of a tissue with a low-power laser (blue light, wavelength 488 nm) and collecting the reflected fluorescent light from the tissue (Fig. 2). Spatial resolution is markedly increased because the scattered light from above and below the plane of interest is not able to pass through the pinhole of the confocal aperture. As CLE depends on tissue fluorescence, contrast agents are applied either topically or systemically. To date, CLE has been evaluated in a number of clinical studies showing the effectiveness of the technology for real-time in vivo diagnosis of luminal, gastrointestinal diseases. 29–35

One major limitation of all the discussed techniques is that the lesions cannot be detected unless morphological changes are occurring. However, the combination of CLE with fluorescent molecules having specificity toward a specific target can potentially address this limitation as it potentially allows for ‘in vivo immunohistochemistry’. In order to carry out endoscopic molecular imaging study, several prerequisites must be considered: 5,17

1 Identification of disease-specific potential cellular marker.
2 Selection of specific molecular probes. The major requirement for a useful probe is to have high affinity for the cellular marker. Moreover, it should be less immunogenic, readily available and have slight tissue penetration ability. Such probes include antibodies, antibody fragments, peptides, nanoparticles, activatable probes and lectins (Table 1).
3 A fluorescence entity (typically a dye) that needs to be conjugated with probes to produce a distinct fluorescent signal.
4 Equipment to visualize the indicator at high resolution in real time.

**MOLECULAR PROBES/MOLECULAR IMAGING AGENTS**

In principle, different classes of molecular probes could be used for molecular imaging studies. Table 1 shows some widely used exogenous targeting agents. 5,11,17,37,38

![Figure 2 Schematic of confocal laser endomicroscopy. 36](image)
The above-mentioned molecular probes can be labeled with a variety of fluorophores having different excitation and emission wavelengths. Most of the current imaging devices are restricted to the range of 480–520 nm, thus limiting the use of a variety of fluorophore options. Fluorescein isothiocyanate (FITC), AlexaFluor (Thermo Fisher Scientific, Massachusetts, USA), infrared (IR) dyes etc. can be used for fluorescence labeling.

### APPLICATIONS OF MOLECULAR IMAGING

Even though it is still not possible to use molecular endoscopic imaging in clinical routines, preliminary human studies as well as animal experiments have already shown the potential of the technique. Endoscopic molecular imaging can be used for cancer screening and surveillance, but it also provides important information for deciding on treatment strategies. Unlike the visible cancer state of a tissue, dysplastic lesions express lower levels of target molecules. Nanoparticles provide signal enhancements and have the potential to contribute to detect early-stage cancers. Therefore, several endoscopic studies were conducted to detect gastrointestinal cancers using different types of nanoparticles.

Esophageal adenocarcinoma (EAC) is one of the leading cancers. Barrett’s esophagus is known to be a premalignant condition of EAC. Most adenocarcinomas of the distal esophagus have been shown to arise in Barrett’s tissue. Barrett’s esophagus can be identified histologically by the presence of specialized columnar epithelium with goblet cells. In spite of improvements in the early detection and treatment methods and extensive efforts for prevention, the
prognosis is still very poor and the 5-year survival rate is below 20%.40 Current methods of surveillance have limitations because of the flat appearance of premalignant dysplasia.

More recently, using lectin-based NIR imaging, Neves et al. detected early neoplasia in Barrett’s esophagus through an ex vivo study where 29 endoscopic mucosal resection (EMR) specimens from 17 patients were assessed.41 Wheat germ agglutinin (WGA) conjugated with near-infrared (NIR) fluorophore (IR800CW, LI-COR Biotechnology, Lincoln, Nebraska, USA) was introduced topically before visualization with wide-field fluorescence imaging.

Strum et al. carried out an in vivo imaging study where FITC-labeled peptide with the sequence ASYNYDA was used to visualize Barrett’s esophagus through confocal laser endomicroscopy.42

An important in vivo study combining organic NP (nanoparticle) and CLE for highlighting esophageal cancer conditions was carried out where NP conjugated with peptides (ASYNYDA) specific for human and rat esophageal cancer cells and loaded with fluorescent dye [4-((dicyanomethylene)-2-methyl-6-(4-dimethylaminostyril)-4H-pyran] (DCM) were i.v. injected and fluorescence was observed in rats affected by esophageal cancer, whereas no signal was observed in control.43 The study confirmed that nanoparticles could serve as a potential cancer diagnostic tool.

Gastric cancer is the second leading cause of cancer mortality worldwide and the most common form of cancer in Asia.44 In a preclinical study,45 a xenograft mouse model expressing MG7, a tumor-associated antigen overexpressed in human gastric cancer was prepared. Alexa Fluor 488 (Thermo Fisher Scientific, Massachusetts, USA)-labeled antibody against MG7 was injected 48 h before imaging was carried out with fluorescence in vivo endomicroscopy. Mice injected with non-specific antibodies were considered as controls and non-tumor tissue showed no specific signal.

Ding et al. carried out a study in murine models to evaluate the utility of activatable molecular probes and infrared fluorescence (NIRF) imaging for the detection of gastric cancer in both in vivo and ex vivo trials.46 In that study, Smad4−/− mice with gastric neoplasia were compared with wild-type controls. Cathepsin-activatable and matrix metalloproteinase (MMP)-activatable molecular probes were injected 24 h and 6 h before quantitative tomographic NIRF imaging. The study suggested that the MMP probe is activated by gastric dysplasia and adenocarcinoma, whereas the cathepsin probe is activated by hyperplastic lesions as well as by dysplasia and adenocarcinoma.

Inflammatory bowel disease (IBD) represents a group of intestinal disorders that cause prolonged inflammation of the digestive tract. Both ulcerative colitis (UC) and Crohn’s disease (CD) are immunopathogenic complex diseases. Specifically, ulcerative colitis causes inflammation in the lining of the colon, or large intestine, whereas Crohn’s disease can cause inflammation in any part of the gastrointestinal tract, from mouth to anus. About 1.2 and 3.7 million people, respectively, in the USA and Europe suffer from IBD and its worldwide incidence is increasing over time.47,48

There are some well-known molecular targets for a variety of cancers, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and human epidermal growth factor receptor 2 (HER2/neu). Specific antibodies to these molecular targets have been established and successfully applied in the clinic for targeted cancer therapy. Alternatively, these antibodies can be used as targeted imaging probes.49–51

In order to monitor early colitis-associated colon cancer (CAC), a mouse model of CAC was used to develop a rapid fluorescent detection method using a topically applied enzymatically activatable probe (γ-glutamyl hydroxymethyl rhodamine green; gGlu-HMRG) which fluoresces in the presence of γ-glutamyltranspeptidase (GGT), an enzyme associated with cancer.52 Mice were examined with white light and fluorescence colonoscopy before and after topical gGlu-HMRG administration and the results addressed the value of the fluorescent probe for cancer screening. Similar studies were carried out with mouse and xenograft models to detect colonic neoplasms and dysplastic polyps where protease-activatable smart probes,53,54 MMP activatable probe,55 fluorescently labeled peptides56,57 etc. were used.

The first in vivo MI human study was carried out in 37 patients with colorectal carcinoma (CRC).58 After topical introduction of fluorescent-labeled molecular probe against EGFR (mouse anti-hEGFR antibody), CLE was used for imaging and EGFR-specific fluorescence intensity was measured. The study showed that 18 out of 19 patients with CRC gave a positive signal whereas 12 out of 18 colorectal adenoma patients showed EGFR-positive results. In contrast, normal mucosa showed no fluorescence. This study showed that molecular imaging in combination with CLE and a fluorescent-labeled antibody is able to diagnose colorectal cancers (Fig. 3).

A similar in vivo imaging study was carried out in 25 patients with Crohn’s disease.59 Imaging was done upon topical administration of the fluorescently labeled monoclonal antibody adalimumab against membrane-bound tumor necrosis factor (mTNF). Fluorescent antibody was
prepared in accordance with good manufacturing practices (GMP) guidelines for in vivo imaging in humans. Patients with high numbers of mTNF+ immune cells showed considerably higher response rates to adalimumab therapy as compared to patients with low numbers of mTNF+ cells.

The first trial with topical application of oligopeptides in the colon was carried out by Hsiung et al.\textsuperscript{60} In the study including 26 patients, a specific hepta-peptide sequence VRPMPLQ was identified through an M13 phage library screening and imaging of fluorescein-conjugated peptide by fluorescence confocal microendoscopy indicated its higher binding to dysplastic cells compared to its normal counterpart with 81% sensitivity and 78% specificity. Therefore, identification of dysplasia-targeting peptides and merging with confocal microendoscopy might improve diagnostic imaging of dysplastic lesions.

Recently an ex vivo pilot study was carried out for the detection of dysplasia associated with ulcerative colitis and the combination of CLE with fluorescent molecular probes was assessed.\textsuperscript{61} Eleven lesions from nine patients were examined by staining the specimens with fluorescent-labeled peptide and visualized by confocal imaging. The results indicated that a combination of CLE with molecular probe is feasible for cancer screening.

Chen et al. carried out a prospective study that included a combination of in vitro, ex vivo and in vivo analysis for the early detection of colorectal polyps and cancers where lectin-functionalized fluorescently labeled mesoporous silica nanoparticles (MSN) served as endoscopic contrast agent.\textsuperscript{62} The findings suggest that fluorescent MSN can be used to assess premalignant colonic lesions.

As gastrointestinal cancers are heterogeneous, they can overexpress several protein targets that might be simultaneously imaged by endoscopy using multiple molecular probes. A recent in vivo mouse study highlighted the application of multiple fluorescently labeled peptides to detect colonic dysplasia.\textsuperscript{63} Three specific-binding peptides were labeled with three different organic dyes having absorption peaks close to the excitation wavelengths of the three laser sources. A multispectral scanning fiber endoscope was used to collect wide-field fluorescence images in real time from multiple peptides. Therefore, the methodology has the potential to translate into human patients by simultaneously visualizing multiple gene targets that are overexpressed in cancers (Fig. 4).

The first in-human molecular imaging study using i.v. fluorescent agent was carried out by Burggraaf et al.\textsuperscript{64} The pilot study was conducted on 15 colorectal cancer patients with 101 lesions. As c-Met was highly expressed in CRC, it was chosen as a suitable marker for the study. GE-137, a small peptide labeled with NIR cyanine dye was injected i.v. to detect c-Met-rich premalignant lesions (Fig. 5).

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Molecular imaging of epidermal growth factor receptor (EGFR) in patients with colorectal cancer (CRC). (a–c) In vivo confocal laser endomicroscopy views of three CRC with different EGFR expression levels. (d–f) Corresponding ex vivo immunohistochemical staining. ROI, region of interest. (Reproduced from Liu et al., 2013\textsuperscript{58} with permission of Elsevier.)}
\end{figure}
Generally in vivo imaging is used as a translational vehicle between preclinical and early clinical studies. It has high throughput and is cost-effective compared to other imaging modalities. The following two studies addressed the value of whole-animal imaging for prediction of cancers.

In the first study, an in vivo imaging system (IVIS® spectrum, PerkinElmer, Massachusetts, USA) was applied to assess/discriminate between fibrotic and non-fibrotic liver in acute and chronic fibrosis murine models. CCl₄ (carbon tetrachloride) was used to induce acute liver injury in wild-type (male C57BL/6) and cRel⁻/⁻ (cRel knockout) mice. Bile duct ligation was used to model chronic fibrosis. Scars are mainly developed as a result of liver damage or injury and composed of hepatic myofibroblasts (HM) which acted as a characteristic target component. C1-3, an antibody fragment that binds to HM was fluorescently labeled with a NIR fluorophore and given to the mice. C1-3 recognizes an extracellular domain in synaptophysin, the expression of which is highly increased on HM and could be a useful tool for liver cancer study. As C1 also binds to human HM, it has the potential to be translated to clinical in vivo imaging applications and could be an appropriate means for proof-of-concept investigation.

![Image of colon sections incubated with FMSN-PEG and FMSN-UEA1](Reproduced from Chen et al., 2017 with permission of Elsevier.)

**Figure 4** Ex vivo binding specificity of FMSN-PEG-UEA1 (nanoplatform). Colon sections of azoxymethane/dextran sulfate sodium-treated mice were incubated either with (a,d,g) FMSN-PEG (fluorescently labeled mesoporous silica nanoparticles, surface modified by polyethylene glycol) or (b,e,h) FMSN-UEA1 (fluorescent nanoparticles coupled with lectin, *Ulex europaeus* agglutinin), counterstained with 4',6-diamidino-2-phenylindole (DAPI). No signal was observed in colon tissues incubated with (a) FMSN-PEG whereas (b) FMSN-UEA1-treated tissue gave a positive signal because of UEA1 binding to α-L-fucose overexpressed during tumorigenesis and neoplastic progression. (c,f,i) FITC-UEA1 (with higher concentration) was used as a positive control for staining. Fluorescence channels: FITC (a–c, green), DAPI (d–f, blue) and merge (g–i). FITC, fluorescein isothiocyanate. Scale bars, 50 μm. (Reproduced from Chen et al., 2017 with permission of Elsevier.)
The second study of in vivo animal imaging assessed the visualization of head and neck squamous cell carcinoma (HNSCC) by targeting transferrin receptor (TfR), specific for head and neck cancer using near-infrared fluorescent transferrin conjugate (Tf NIR). Solid tumor xenograft models were developed by s.c. inoculation. TfR antibody was labeled with NIR conjugate and injected into immunodeficient mice bearing HNSCC by the tail vein. Animals were imaged using the IVIS 200 Imaging System (PerkinElmer, Massachusetts, USA) every 10–30 min up to at least 6 h. Results indicated a potential use of the animal imaging system for non-invasive tumor imaging.

**FUTURE VISION**

In 2003, MI was listed in a technology review by Massachusetts Institute of Technology as one of 10 most promising emerging technologies to change the world. In spite of having immense potential, there are still significant areas where improvements can be made. The top priority could be to develop fluorescence-based new imaging agents. However, the process is not straightforward and requires a significant amount of time, effort, and money. Since the studies involve systemic or topical introduction of molecular probes, extensive toxicity assessments must be carried out before clinical use. Several early-stage cancers can be considered as functional disorders without any physiological alterations in the tissue and are therefore left undetected. This could be considered a limitation of the technique. Moreover, the implementation of MI requires a multidisciplinary team of chemists, biologists, pharmacists, and clinicians, whereas during the clinical trials the challenges are even higher (e.g. validation of standard operating procedure (SOP), image analysis etc.).

In the field of probe discovery, today, high-throughput screening is applied worldwide. Microfluidics/lab-on-a-chip technology can be used in this regard. This will offer several advantages including shorter chemical reaction time, increased product yields, higher degree of automation etc., which will eventually contribute to the in vivo imaging probe production. Moreover, there is also an urgent need to explore new markers heavily expressed in cancer diseases, such as cell surface receptors, to develop fluorochromes and to work on improvements of...
instrumentation. Application of NIR-active dyes such as phthalocyanines, cyanine and squaraine dyes have great scope in the biomedical and clinical fields, because of minimum autofluorescence, reduced light scattering and high tissue penetration. A preclinical study was carried out by using a near-infrared endoscopy device and 780 nm excitation wavelength.

To understand disease in the context of clinical diagnosis, therapy and management of patients, MI is considerably contributing to endoscopic technology. To overcome current limitations where large numbers of lesions are reported to be overlooked by conventional wide field endoscopy, there is a strong need for ‘molecular endoscopic imaging’ for cancer screening and surveillance for diseases such as Barrett’s esophagus, IBD, colorectal cancer, colon polyps etc. Recent technical advancements and use of multiple modalities will increase the ability of endoscopic instruments. As the molecular changes are very small and target concentrations is in the pico- to nanomolar range, various fluorescent peptides or a cocktail of antibodies with different optical characteristics could be useful to simultaneously detect different tumors expressing distinct surface receptors.

In order to include MI as a tool for cancer detection it must be robust enough to ensure reproducible and quantitative data and can only be implemented into cancer management if it helps decision-making in early diagnostics. However, it would be rather difficult to translate imaging results from a mouse model to the human in vivo environment because of its large genetic diversity. Therefore, clinical ex vivo studies and in vivo imaging with a large-size animal with comparable anatomy, physiology, metabolism, and genetics to humans are highly recommended for translational research. In addition, regulatory requirements and safety concerns should be strictly monitored.

CONFLICTS OF INTEREST

AUTHORS DECLARE NO conflicts of interest for this article.

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