Multiplexed endoscopic imaging of Barrett’s neoplasia using targeted fluorescent heptapeptides in a phase 1 proof-of-concept study

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MESSAGE
Improved methods for early cancer detection arising from Barrett’s oesophagus (BE) are still needed. Imaging molecular expression patterns in BE patients can target neoplasia. We demonstrate a multiplexed fluorescence imaging approach to detect premalignant lesions with two fluorescently labeled heptapeptides specific for EGFR and ErbB2. Twenty-two BE patients underwent endoscopic imaging with a multimodal scanning fiber endoscope (mmSFE). In this pilot study, 92% of neoplastic lesions could be imaged by comparison with pathology, with only 11% false positives. This first-in-human study demonstrates feasibility to concurrently detect multiple targets in vivo and potential for early detection of cancers that are molecularly heterogeneous.

INIEMDMORE DETAIL
Background
Oesophageal adenocarcinoma (EAC) is a deadly disease that has increased dramatically in incidence over the past several decades. Endoscopic screening with white light illumination and random biopsy is limited by sampling error. Dysplasia often presents with flat architecture and patchy distribution. EGFR and ErbB2 are transmembrane tyrosine kinase receptors that stimulate epithelial cell growth, proliferation and differentiation. Over-expression of these targets reflects a higher risk for cancer progression. Multiplexing imaging methods take advantage of the broad spectrum of light over the visible and near-infrared (NIR) range. We aim to demonstrate clinical feasibility to visualise EGFR and ErbB2 expression simultaneously in vivo to detect Barrett’s neoplasia.

Methods
Consecutive patients referred for either evaluation or therapy of Barrett’s neoplasia were recruited for the study (NCT03589443). An mmSFE was designed to collect multiplexed fluorescence images concurrently. Target/background (T/B) ratios were calculated for each fluorescence image. More details on the methods and the multiplexed imaging technology can be found in the online supplemental file.

Results
The peptide QRHKPRE specific for EGFR was labelled with Cy5 via a GGGSK linker, (figure 1A). KSPNPF, specific for ErbB2, was labelled with IRDye800 via a GGGSC linker (figure 1B). These fluorophores were chosen to minimise overlap between absorbance and emission spectra (figure 1C). The characteristics and stability of fluorescently labelled peptides were shown in online supplemental tables S1–S4. The pharmacology/toxicology study shows no acute adverse effects in animals (online supplemental tables S5 and S6). The phase 1 safety study was performed in n=25 human, and no abnormalities were identified in the laboratory results, urinalysis and ECG for either peptide, and no adverse events were found. The mmSFE was designed to collect multiplexed fluorescence images (figure 1D–H). Contrast agents were administered, and real-time images were collected from n=22 subjects, table 1 (online supplemental videos S1–S23). Representative white light images are shown for squamous (SQ) and nondysplastic BE (NDBE) (figure 2A,B). Minimal background was seen following peptide administration. Fluorescence images were collected in separate channels, and coregistered reflectance provided anatomic landmarks for image interpretation. A representative set of in vivo images for HGD and EAC is shown (figure 2C,D). Increased fluorescence intensities were seen from regions of HGD and EAC, and were confirmed by pathology. Immunohistochemistry (IHC) was performed to validate expression of EGFR and ErbB2 on excised specimens (online supplemental figure S1).

The T/B ratios using QRH*-Cy5 and KSP*-IRDye800 were measured from individual patients (figure 3A,B). For SQ (n=2) or NDBE (n=3), a mean (±SD) T/B ratio of 1.28±0.07 for QRH*-Cy5 and 1.33±0.15 for KSP*-IRDye800, respectively, was calculated. The T/B for (n=4) LGD was 1.23±0.05 and 1.18±0.10, respectively. For HGD (n=7) and EAC (n=6), a mean (±SD) T/B ratio of 1.61±0.21 and 1.68±0.24, respectively, was found. Leave-one-out cross-validation (LOOCV) was used to classify results (online supplemental table S7). Support vector machine (SVM) and logistic regression (LR) provided the highest classification accuracy of 91%. The imaging results revealed n=12, 1, 8 and 1 true positives, false positives, true negatives and false negatives, respectively, resulting in 92% sensitivity and 89% specificity. The decision
Figure 1  Fluorescently labelled peptides for multiplexed imaging. Biochemical structures are shown for (A) QRH*-Cy5 and (B) KSP*-IRDye800. (C) Peak absorbance of QRH*-Cy5 and KSP*-IRDye800 occurs at abs=648 and 776 nm, respectively. Peak fluorescence emits at em=675 and 812 nm, respectively. (D) Schematic diagram for the multimodal scanning fibre endoscope (mmSFE) is shown. Excitation at ex=638 and 785 nm is delivered through a single-mode fibre (SMF) that is scanned in a spiral pattern by a piezo tube actuator. The beam is focused onto the tissue surface (illumination plane) by a lens assembly. (E) Fluorescence is collected by a ring of large core multi-mode fibres (MMF) mounted around the instrument periphery. (F) The dimensions of the rigid tip are 9 mm in length and 2.4 mm in diameter. (G) This instrument passes forward through the 2.7 mm working channel of a standard medical endoscope (Olympus #GIF-HQ190). (H) The system is contained within a portable cart.

Adequate signal was collected by using large core, high numerical optical fibre using a prototype-generated system. Two laser excitation powers were administered topically in the distal oesophagus of n=22 BE patients. With conventional white light illumination, structural abnormalities associated with Barrett’s neoplasia appeared subtle. EAC, esophageal adenocarcinoma; EMR, endoscopic mucosal resection; HGD, high-grade dysplasia; LGD, low-grade dysplasia; NDBE, non-dysplastic Barrett’s oesophagus; SQ, squamous.

Table 1  Patient demographics

| Age  | Gender | Prague/stage | Tissue sampling | Pathology |
|------|--------|--------------|-----------------|-----------|
| 68   | M      | COM010       | ERM/biopsy     | SQ        |
| 57   | M      | COM010       | biopsy          | SQ        |
| 84   | F      | COM019       | biopsy          | NDBE      |
| 60   | M      | COM01        | ERM/biopsy     | NDBE      |
| 56   | M      | C1M3         | ERM/biopsy     | NDBE      |
| 57   | M      | C7M9         | biopsy*        | LGD       |
| 56   | F      | COM01        | biopsy*        | LGD       |
| 80   | F      | COM010       | biopsy*        | LGD       |
| 67   | M      | COM007       | ERM/biopsy     | LGD       |
| 79   | F      | COM02        | biopsy          | HGD       |
| 88   | M      | COM3         | ERM/biopsy     | HGD       |
| 79   | M      | COM11.5      | ERM/biopsy     | HGD       |
| 85   | M      | C12M13       | biopsy          | HGD       |
| 79   | M      | C4M5         | biopsy          | HGD       |
| 66   | M      | COM0          | biopsy          | HGD       |
| 60   | M      | C9M10        | biopsy          | HGD       |
| 75   | M      | T3M1         | biopsy          | EAC       |
| 73   | F      | COM002       | ERM/biopsy     | EAC       |
| 81   | M      | C10M0        | biopsy*        | EAC       |
| 71   | M      | C9M1213      | ERM/biopsy     | EAC       |
| 55   | F      | T1a          | biopsy          | EAC       |
| 64   | F      | COM01        | ERM/biopsy     | EAC       |

Multiplexed images were collected in vivo from the distal oesophagus of n=22 patients with a mean (±SD) age of 70.0±10.8 years. SQ, NDBE and LGD were identified in a total of n=7, 2, and 4 subjects, respectively. HGD and EAC were found in n=7 and 6 subjects, respectively. Modified Prague classification includes length in centimetres of circumferential Barrett’s oesophagus (C), maximal tongue (M) and any proximal island (I). These findings were confirmed by histopathology from either ERM or biopsy.

Comments

Here, we demonstrate feasibility to detect Barrett’s neoplasia endoscopically by imaging two targets concurrently in vivo. Fluorescently labelled peptides specific for epithelial growth factor receptor (EGFR) and epithelial growth factor receptor2 (ErbB2) were administered topically in the distal oesophagus of n=22 BE patients. With conventional white light illumination, structural abnormalities associated with Barrett’s neoplasia appeared subtle. By comparison, spatial patterns of target expression were visualised with high contrast using fluorescence. Two laser excitation wavelengths were delivered concurrently through a single flexible optical fibre using a prototype-wide-field endoscope accessory. Adequate signal was collected by using large core, high numerical aperture fibres. The regions imaged were compared with histopathology of specimens excised via either endoscopic mucosal resection or biopsy. IHC of these specimens confirmed heterogeneous expression of EGFR and ErbB2.

To our knowledge, this study first demonstrates clinical application of multiplexed fluorescence imaging during endoscopy. Many cancers, including EAC, are molecularly heterogeneous, thus detection of multiple targets is likely to be needed for accurate clinical diagnosis. Mucosal abnormalities with non-specific features, such as nodularity, ulceration and irregularities, may not be relied on to accurately locate Barrett’s neoplasia. Several medical societies recommend random 4-quadrant biopsies for EAC surveillance, but this sampling method is inefficient and has been poorly adopted by community physicians. Molecular biomarkers can be highly specific for disease, and are expressed well before neoplastic lesions become grossly apparent. Endoscopic imaging strategies for detecting these targets in vivo can be used to guide and prioritise high risk regions for resection, reduce surveillance frequency, and minimise over diagnosis.

Recently, detection of dysplasia and early EAC in BE patients was demonstrated using an antibody specific for vascular endothelial growth factor A. Bevacizumab was originally developed for cancer therapy, and was repurposed for diagnostic imaging by labelling with IRDye800. Compared with antibodies, peptides are smaller in size, have faster binding kinetics, and

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delivered to the mucosal surface in the distal oesophagus using topical administration of QRH*-Cy5 and KSP*-IRDye800. The merged images show high contrast regions-of-interest (ROI) where EGFR and ErbB2 (orange) are coexpressed. Coregistered reflectance images of interest (ROI) where EGFR and ErbB2 (orange) are coexpressed. Coregistered reflectance images provide anatomical landmarks to interpret the location of the ROI’s.

Figure 2  Barrett’s oesophagus. Representative in vivo images collected endoscopically are shown from patients with (A) squamous (SQ), (B) non-dysplastic Barrett’s oesophagus (NDBE), (C) high-grade dysplasia (HGD) and (D) oesophageal adenocarcinoma (EAC). The presence of NDBE is identified by the salmon red patches (arrows) in the white light images. Fluorescence images are collected after separate topical administration of QRH*-Cy5 and KSP*-IRDye800. The merged images show high contrast regions-of-interest (ROI) where EGFR and ErbB2 (orange) are coexpressed. Coregistered reflectance images provide anatomical landmarks to interpret the location of the ROI’s.

Figure 3  In vivo imaging performance. Scatter plot shows target/background (T/B) ratios measured for EGFR and ErbB2 expression in the fluorescence images collected in vivo from the distal oesophagus of n=22 patients. Decision boundaries show regions classified as either negative (blue) or positive (brown) for neoplasia using (A) support vector machine (SVM) and (B) logistic regression (LR) trained on all data. (C) ROC curves for classifying HGD/EAC from SQ/NDBE/LGD are shown using SVM and LR algorithms with leave-one-out cross-validation (LOOCV). (D) Average ROC curves from bootstrap using SVM (AUC=0.97) model trained on all data show that multiplexed detection provides improved performance than using either EGFR (AUC=0.95) or ErbB2 alone (AUC=0.94). AUC, area under curve; EGFR, epithelial growth factor receptor; ErbB2, epithelial growth factor receptor2; ROC, receiver-operator characteristic.

Table 1  Multimodal detection of neoplasia. Accuracy and reliability of the backscatter and fluorescence data are shown using (A) support vector machine (SVM) and (B) logistic regression (LR) trained on all data. Evaluated metrics include receiver-operator characteristic (ROC) area under curve (AUC), sensitivity, specificity, and accuracy. AUC, area under curve; EGFR, epithelial growth factor receptor; ErbB2, epithelial growth factor receptor2; ROC, receiver-operator characteristic.

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Figure 4  Inclusion of more non-neoplastic subjects would better reflect the prevalence of disease seen in the community. In conclusion, we demonstrated a proof-of-concept study for detecting multiple targets concurrently in patients with Barrett’s neoplasia and this strategy is promising for early detection of cancers in other hollow organs.

Figure 5  The clinical usefulness of this technology can be improved by addressing several study limitations. The peptides were administered separately to minimise potential binding interactions but can be combined to reduce time needed to reconstitute and prepare the peptides for delivery. After inserting the imaging accessory through the working channel, the fluorescence and HD-WLE images were not oriented. Accurate alignment would allow the fluorescence images to be more effective as a guide for tissue resection. This study was performed at a tertiary referral centre that specialises in treatment of patients with advanced BE, thus a cohort highly enriched with neoplasia was studied. Inclusion of more non-neoplastic subjects would better reflect the prevalence of disease seen in the community. In conclusion, we demonstrated a proof-of-concept study for detecting multiple targets concurrently in patients with Barrett’s neoplasia and this strategy is promising for early detection of cancers in other hollow organs.

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