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Targeted imaging of esophageal neoplasia with a fluorescently labeled peptide: First in-human results

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Abstract

Esophageal adenocarcinoma is rising rapidly in incidence, and usually develops from Barrett's esophagus, a precursor condition commonly found in patients with chronic acid reflux. Pre-malignant lesions are challenging to detect on conventional screening endoscopy because of their flat appearance. Molecular changes can be used to improve detection of early neoplasia. We have developed a peptide that binds specifically to high-grade dysplasia and adenocarcinoma. We first applied the peptide *ex vivo* to esophageal specimens from 17 patients to validate specific binding.

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Competing interests: SL and TDW are inventors on U. S. Patent No. 8,247,529 for ASY* peptide.

Data and materials availability: ASY* peptide can be made available for research purposes via a materials transfer agreement (MTA) with Stanford University (webmaster@otlmail.stanford.edu). Stability testing MS data over the course of 3 years are available upon request.

Supplementary Materials

Methods

Fig. S1 – Peptide selection using phage display.

Fig. S2 – Flow cytometry analysis of ASYNYDA peptide binding specificity to cells.

Fig. S3 – ASY*.FITC binding to cell lines on confocal microscopy.

Fig. S4 – Competitive inhibition of antibody binding to CypA.

Fig. S5 – Limitations of confocal imaging for ulcers and neo-squamous epithelium.

Table S1 – Peptide characterization on HPLC and mass spectrometry.

Table S2 – Pharmacology and toxicology studies in rodents.

Table S3 – Baseline and post-procedural labs.

Table S4 – Adverse events in clinical study reported to FDA.

Author contributions: Study concept and design: MBS, BPJ, SL, DKT, TDW. Peptide selection, design and characterization: SL, BPJ. Acquisition of clinical data: MBS, CP, BJE, RSK. Collection of *in vitro* data: MBS, BPJ, SK. Analysis and interpretation of data: MBS, BPJ, SL, SK, DGB, HA, DKT, TDW. Drafting of the manuscript: MBS, BPJ, TDW. Technical support: MBS, BPJ, SL, SK. Study supervision: DKT, TDW.

Next, we performed confocal endomicroscopy in vivo in 25 human subjects after topical peptide administration and found 3.8-fold greater fluorescence intensity for esophageal neoplasia compared with Barrett's esophagus and squamous epithelium with 75% sensitivity and 97% specificity. No toxicity was attributed to the peptide in either animal or patient studies. Therefore, our first-in-humans results show that this targeted imaging agent is safe, and may be useful for guiding tissue biopsy and for early detection of esophageal neoplasia and potentially other cancers of epithelial origin, such as bladder, colon, lung, pancreas, and stomach.

Introduction

There are ~480,000 new cases of esophageal cancer diagnosed each year worldwide, nearly 85% will result in death (1). Diagnosis usually occurs when the cancer is advanced in stage, resulting in an overall 5-year survival rate <15%. Over the past 3 decades, the incidence of esophageal adenocarcinoma (EAC) has increased >460% in men aged 65 or older (2), and this disease has the fastest rate of growth in incidence of all cancers (3). Barrett's esophagus (BE) is a replacement of normal squamous epithelium with intestinal metaplasia. This condition is found in 10–15% of patients undergoing routine endoscopy for chronic acid reflux. BE increases the risk of developing EAC by more than 30-fold (4), and presents a unique opportunity for early detection on imaging. BE is known to transform sequentially to low-grade dysplasia (LGD) and high-grade dysplasia (HGD) before developing into EAC. Detection of these pre-malignant lesions on endoscopy can therefore provide an opportunity for intervention before more advanced disease develops. Patients with BE and HGD progress onto adenocarcinoma ~3.5% and ~59% of the time, respectively (5). Early detection provides several curative options, including endoscopic mucosal resection (EMR), radiofrequency ablation, and surgery, that can lead to an improvement in patient outcomes.

Cancer prevention guidelines recommend imaging with white light endoscopy and random 4-quadrant tissue biopsies every 2 to 3 years (6). Imaging is critical to surveillance because these lesions are heterogeneous in shape, flat in architecture, and patchy in distribution. This surveillance strategy is labor-intensive, limited by sampling error, and not effective for predicting the presence of cancer in patients undergoing esophagectomy (7). Improved screening can be achieved by visualizing the expression and activity of molecules and cells that influence the behavior of diseased tissues. Endoscopic methods, including chromoendoscopy (8), narrowband (9), and autofluorescence imaging (10), have been evaluated clinically, but are limited by non-specific contrast mechanisms. Other optical biopsy techniques, including endoscopic polarized scanning spectroscopy (11), angle-resolved low coherence interferometry (12), Raman spectroscopy (13), optical coherence tomography (14), and elastic scattering spectroscopy (15), have shown promise. Here, we demonstrate potential for use of targeted contrast agents to visualize cellular function and follow molecular processes in vivo.

Molecular changes specific for Barrett's neoplasia can be visualized using immunofluorescence in vivo and can serve as an adjunct to conventional endoscopy. Antibodies have been studied as targeting agents for detection of colorectal cancer (16,17). However, widespread clinical use of antibodies for routine imaging has been limited by slow binding kinetics, immunogenicity, and production costs. Conversely, peptides can approach the binding affinity achieved by antibodies, are easy to label, and are inexpensive to produce in large quantities. Peptides that bind specifically to protein targets on cells and in tissues can be identified using phage display. Their small size (~1 kDa) allows for penetration through leaky cell junctions, as was demonstrated in dysplastic human colonic crypts (18).

Targeted imaging can visualize and quantify cellular and molecular processes in living tissues. Antibodies and peptides establish a biological basis for image contrast and can

achieve sufficient target-to-background (T/B) ratio for in vivo detection (19). We have previously identified a peptide SNFYMPL that was validated ex vivo and is being developed for clinical use in esophagus (20). Preclinical testing and rigorous validation of these imaging agents and methods are necessary for translation into the clinic. Here, we describe a regulatory strategy for a fluorescently labeled peptide that is specific for Barrett's neoplasia. This process starts with development of an imaging agent using Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP) (21). Validation of the purity, efficacy, safety, and non-toxicity of the agent is necessary to provide data required for a long and complex regulatory path to ultimately achieve FDA approval. After successfully completing these steps, we performed a first-in-humans clinical study. We demonstrate that the specific binding properties of this peptide can be visualized in vivo on endomicroscopy, which can be combined easily with conventional endoscopy for early detection of EAC.

RESULTS

Peptide discovery and characterization

We used phage display technology to identify peptides that bind specifically to the plasma membrane of human H460 adenocarcinoma cells. Non-neoplastic human Q-hTERT BE cells were used as control. This unbiased approach maximizes the fluorescence signal needed for real time imaging. After 4 rounds of biopanning, enrichment was found for 5 candidates (number of phages in parentheses): ASYNYDA (9), AQLSTLA (3), QLMSADS (2), LPLHSL (2), and TGPTIQH (2). ASYNYDA phage was found to have 5.3-fold greater binding for H460 than for Q-hTERT cells and >10-fold greater binding for H460 cells in comparison to that of wild-type (WT)-phage (no peptides expressed) on pfu counts (fig. S1). Thus, ASYNYDA was chosen for further validation.

The ASYNYDA peptide was labeled with FITC for in vitro, ex vivo, and in vivo imaging. The chemical structure of the FITC-labeled peptide is shown in Fig. 1A. The C-terminus of ASYNYDA is attached by a GGGSK linker to FITC to prevent steric hindrance, and is hereafter termed ASY*-FITC. The fluorescence spectra for ASY*-FITC and FITC at 100 μ M in PBS with 471 nm excitation revealed a peak emission at 519 nm, Fig. 1B. Thus no spectral shift was observed after FITC labeling. The purity of the GMP-synthesized product on HPLC was >97%, table S1. On mass spectrometry, the experimental mass-to-charge (m/z) ratio measured for ASY*-FITC agreed with that expected (Fig. 1C; table S1). On stability testing, performed every 3 months in the first year and every 6 months thereafter for 3 years, no change in the appearance by color or mass spectra of ASY*-FITC, stored as a lyophilized powder at -20°C , was observed. The purity on HPLC changed ~2% (97% to 95%) over 36 months.

The pharmacology/toxicology study for ASY*-FITC was performed in rats. They were administered one dose of ASY*-FITC by oral gavage ($n = 12$ animals per dose at 4 different doses), and showed no peptide-related acute adverse effects in clinical signs or chemistries or on necropsy up to 15 days after peptide administration (table S2).

Peptide binding to human cells and tissues

We measured an apparent dissociation constant (K_d) of 32 nM for ASY*-FITC, Fig. 1D. ASY*-FITC had an association rate constant (k) of 0.2 min^{-1} , Fig. 1E. These results were determined using a least squares fit of the data. On competitive binding, unlabeled ASY* inhibited binding of ASY*-FITC to H460 cells in a dose-dependent manner over concentrations ranging from 100 to 1000 μ M (Fig. 1F). No significant reduction in fluorescence intensity was observed with addition of unlabeled GGGAGGGAGGGK peptide (control) at concentrations up to 1000 μ M ($P > 0.05$, unpaired t-test) (Fig. 1F).

Flow cytometry was performed on adenocarcinoma and Q-hTERT cells incubated with ASY*-FITC, control peptides FITC-Ahx-ASYNYDA, GGGAGGGAGGGK-FITC, MNDPIPQ-GGGSK-FITC, AADYYSN-GGGSK-FITC (scrambled) or cells alone to validate peptide specificity (fig. S2). Compared with control peptides, ASY*-FITC showed stronger binding to the adenocarcinoma cells. The mean intensities for the controls were <3-fold lower than that for ASY*-FITC. No difference was observed between ASY*-FITC and the control peptides on binding to Q-hTERT cells. On confocal fluorescence microscopy, we could see binding of ASY*-FITC to the plasma membrane of H460, FLO1, OE33, and OE19 adenocarcinoma cells, but not to Q-hTERT cells (fig. S3A). Significantly less signal was measured for control peptides FITC-Ahx-ASY*, GGG*-FITC, MND*-FITC and AAD*-FITC (fig. S3B).

We isolated a possible protein target for the ASY*-phage from the H460 cell membrane using the Mascot protein search engine. On mass spectrometry, cyclophilin A (CypA) was found to have 50% and 82.3% coverage using photoactive amino acids (L-photo-leucine and L-photo-methionine) and BS3 crosslinking agents, respectively. CypA was also seen on mass spectrometry from the purified plasma membrane isolated from H460 cells. We investigated CypA as a target for the ASYNYDA peptide on competitive inhibition of a Cy5.5-labeled anti-CypA antibody (fig. S4, A and B). Furthermore, ASY* was found to align with CypA (2CPL.A in Protein Data Bank) ($P=0.0087$ by fit to Gumbel distribution) using Pepsite docking software (fig. S4C) (22).

Peptide binding to human esophageal specimens ex vivo

Specific binding of ASY*-FITC to Barrett's neoplasia was confirmed ex vivo using resected human esophageal specimens. On a representative white light stereomicroscopy image, squamous and HGD show differences in color but not in architecture (Fig. 2A). The corresponding fluorescence image collected after topical administration of ASY*-FITC revealed differences in intensity (Fig. 2B). Histology was performed along six sections of the esophageal specimen (Fig. 2C). By magnifying the red region in section 3, histological features of HGD, including increased nuclear-to-cytoplasmic ratio with stratification and lack of cytoplasmic mucin, could be seen on the epithelial surface (Fig. 2D). The results for squamous (yellow box) and Barrett's esophagus (blue box) were also confirmed on histology.

The mean fluorescence intensities from 1 mm² regions of surface epithelium for squamous ($n=73$), BE ($n=142$), HGD ($n=66$), and EAC ($n=22$) from 12 EMR specimens stained with ASY*-FITC are shown in Fig. 2E; a one-way ANOVA resulted in an F-value of 35.2 ($P<0.01$). Also included in Fig. 2E are the mean fluorescence intensities from 5 EMR specimens stained with the GGG*-FITC (control) peptide for squamous ($n=24$), BE ($n=26$), HGD ($n=12$), and EAC ($n=7$); the one-way ANOVA resulted in an F-value of 3.2 ($P<0.05$). The mean fluorescence intensities for both HGD and EAC were significantly greater than non-dysplastic BE and squamous. LGD was seen in some regions mixed with higher histological grades of disease, but the number was insufficient for statistical analysis.

Imaging and quantifying Barrett's neoplasia in vivo

We validated specific binding of ASY*-FITC to Barrett's neoplasia on confocal endomicroscopy in vivo in a total of 25 human subjects (22 males, 3 females) (Table 1). The surface pathology was assessed in the epithelium where the ASY*-FITC peptide was administered and imaged (depth 50 μ m), whereas the conventional pathology was evaluated from the full mucosal cross-section. All of the patients enrolled in this study were on proton-pump inhibitors, minimizing the effect of changes in pH on peptide binding. The clinical schema is shown in Fig. 3A. A white light endoscopy image from the distal

esophagus a BE patient with HGD/EAC showed a salmon-pink region of BE (black arrow) and normal-appearing squamous mucosa (white arrow) (Fig. 3B). A catheter (black arrow) was used to spray the region with ASY*-FITC (5 ml of 100 μ M solution) (Fig. 3C). Then, the endomicroscope was placed in contact with the mucosa to collect confocal fluorescence images (Fig. 3D).

The in vivo image of squamous epithelium showed no peptide binding (Fig. 3E). Some signal (yellow arrow) seen from BE may represent non-specific pooling of contrast in benign crypts (Fig. 3F). HGD (Fig. 3G) and EAC (Fig. 3H) showed specific peptide binding to neoplastic crypts (yellow arrows). H&E staining of the surface epithelium (Fig. 3, E and F) confirmed the presence of squamous and BE, respectively (Fig. 3I). Histology of Fig 3G showed HGD (Fig. 3J). On the higher-magnification view, the epithelium lining the tubules and covering the mucosal surface revealed large, irregularly shaped nuclei that were fully stratified with little cytoplasm (Fig. 3K). The result for EAC in Fig. 3H was also confirmed on histology (Fig. 3L).

The T/B ratio was calculated for each video in Fig. 3 by measuring the average fluorescence intensity in regions of interest around the outer rim of crypts and within the lumen (Fig. 4A). For squamous epithelium, images with a localized region of maximum contrast were chosen to overcome the autogain function of the imaging system, and the boxes were selected at random. In vivo images collected prior to peptide administration (autofluorescence) showed no detectable signal. The T/B ratio was quantified for autofluorescence ($n=13$) and for ASY*-FITC binding to squamous ($n=18$), BE ($n=17$), HGD ($n=8$), and EAC ($n=4$) from 25 patients (Fig. 4B). One region of LGD was classified as BE, and one patient (#12) had peptide administered but no biopsy or EMR was obtained. The receiver-operator characteristic (ROC) curve shows an optimum sensitivity of 75% (95% CI: 43%–95%) and specificity of 97% (95% CI: 85%–100%) at a T/B ratio of 4.2, with area-under-curve (AUC) of 0.91 (Fig. 4C). The performance of the peptide varied with T/B threshold (Fig. 4D). At a T/B ratio of 4.2, we found $n=9$ true positives, $n=1$ false positive, $n=34$ true negatives, and $n=3$ false negatives for identifying neoplasia, resulting in a PPV of 90% and NPV of 92% (Table 1).

Peptide safety in humans

The results of the baseline and post-procedural labs from all 25 patients are shown in table S3. Urinalyses for all subjects were within normal limits. We observed a transient increase in the mean white blood cell (WBC) count (7.0×10^3 to 11.1×10^3 , $P < 0.01$, unpaired t-test) at 24 hours for the 21 patients who had EMR, resulting in an increase in the mean post/pre-EMR WBC ratio from 1.0 to 1.7 ± 0.4 (SD) (Fig. 4E). This change was not associated with fever or pain. Leukocytosis was resolved in 14 out of 21 patients by day 5 [mean WBC count ratio 1.1 ± 0.2 (SD); $P < 0.01$, Mann Whitney test], and in the remaining 7 patients after day 5. We retrospectively analyzed the WBC count at 24 hours for 18 EMR procedures that did not involve peptide administration, and found a mean WBC ratio of 1.5 ± 0.4 (SD), which was not significantly different from subjects receiving both peptide and EMR. Thus, tissue trauma from the EMR procedure is the likely cause of leukocytosis. A total of 1 serious adverse event (SAE) and 22 adverse events (AEs) were reported to the FDA (table S4). One subject experienced significant bleeding after EMR that resulted in hospital admission, treatment, and subsequent discharge. This complication is a known risk of the EMR procedure.

Limitations of imaging with topical peptide administration

A limitation of confocal endomicroscopy is that disease $>50 \mu$ m below the mucosal surface cannot be identified. A white light endoscopy image of an ulcer in the distal esophagus is

shown (fig. S5A). The confocal image showed punctate fluorescence with T/B ratio of 1.25. On histology, the ulcer (arrow) could be seen on the mucosal surface, and the EAC below was not detected on imaging. An erythematous region identified on endoscopy showed fluorescence with T/B ratio of 1.01, consistent with that of squamous epithelium (fig. S5B). However, EAC (arrows) below neo-squamous epithelium was found on histology.

Discussion

Here, we present a first-in-humans clinical validation of a fluorescently labeled peptide that is specific for HGD and EAC in BE. Pre-malignant lesions in BE are often flat and thus “invisible” on conventional white light endoscopy, in particular the 0-IIb lesions (Paris classification). We show that topical application of ASY*-FITC is safe and effective for localizing disease in BE patients on imaging, thus demonstrating potential to perform “intelligent” biopsy. ASY*-FITC produced a 3.8-fold greater signal for HGD and EAC than for squamous and BE in vivo, highlighting areas for biopsy or therapy. This peptide demonstrated high binding affinity with rapid onset (~5 minutes), representing important properties for clinical use in a busy endoscopy unit. Topical administration allows for a high concentration (100 μ M) of the contrast agent to be delivered directly to the suspect mucosa. Targeting represents an improvement over current screening methods based on random biopsy that have been limited in effectiveness by sampling error. These findings address an important unmet clinical need for improved guidance of diagnostic biopsies in patients at increased risk for developing esophageal cancer.

We have previously identified peptide imaging agents for detection of pre-malignant lesions in the colon and esophagus (18, 20). The ASY* peptide was developed to identify new targets to address the genetic heterogeneity of EAC. We have preliminarily identified the cell surface target as CypA. Although CypA is primarily known to be cytosolic, it is associated with several cell surface proteins found in esophageal cancer, including CD147 (23, 24). This peptide was initially discovered in our laboratory with phage display on human lung adenocarcinoma H460 cells (25). The reactivity with esophageal cancer is not surprising because lung and esophagus are both endodermal-derived organs, and we have found that lung and esophageal adenocarcinomas share a subset of genetic alterations (26–28).

Validation of ASY*-FITC was performed on human esophageal specimens ex vivo with histological evaluation registered to fluorescence intensities with <1 mm resolution. Specific binding of the fluorescent peptide to HGD and EAC was demonstrated. This level of precision is necessary to validate the patchy and focal spatial distribution of this disease, and is difficult to achieve in vivo because of motion artifacts introduced by organ peristalsis, respiratory motion, and heart beating. Nevertheless, this correlation cannot be easily established for highly focal disease encountered in vivo. We further validated specific binding of ASY*-FITC to esophageal adenocarcinoma cell lines FLO1, OE19, and OE33. ASY*-FITC did not bind to Q-hTERT cells on any of these assays.

We controlled for non-specific binding on in vitro cell validation studies using 4 different peptides: 1) FITC-Ahx-ASY* was developed to block the ASY* binding moiety by moving the FITC fluorophore from the C- to the N-terminus; 2) GGGAGGGAGGGK-FITC was motivated by a previous control peptide that had functional groups removed and charge neutralized (29), demonstrating that net charge, hydrophobicity, and hydrophilicity of the peptide play an important role in binding; 3) MND*-FITC represents an unrelated peptide; and 4) AAD*-FITC is a scrambled version of the target peptide.

We used a simple regulatory strategy by performing the clinical study under an Investigational New Drug (IND) application (30). FITC was chosen to label the ASY* peptide because of its compatibility with the confocal miniprobe, which is FDA-approved. For surveillance in real-time, the ASY*-FITC peptide specifically highlights suspected regions of disease in a fixed horizontal plane below the mucosal surface, enabling quantitative evaluation. Use of this targeting agent resulted in high specificity of 97% at a T/B ratio of 4.2. The sensitivity of disease detection may be further improved with a panel of peptides to address the heterogeneity of target expression in a general patient population.

Recently, targeted imaging of Barrett's neoplasia has been demonstrated using topically applied fluorescently labeled lectins on human samples *ex vivo* (31). In this approach, HGD and EAC appeared with reduced fluorescence intensity compared to that for normal (squamous/BE) esophagus. Such a negative contrast strategy can be limited by false-positives *in vivo* because of overlying mucus, anatomic shadows, and mucosal folds. Furthermore, lectins have less diversity than peptides; thus, peptides can achieve binding with greater specificity and higher affinity. Fluorescently labeled molecules have also shown promise for intraoperative imaging of other cancers, such as ovarian. FITC-labeled folate was administered intravenously to target overexpressed folate receptor- α in patients with ovarian cancer to improve disease staging (32). Generalizability of this molecular probe to other cancers is limited by the high level of expression of the folate receptor needed for imaging. In addition, with systemic delivery, the probe concentration becomes diluted by the volume of distribution, requiring larger quantities, increased risk of toxicity, and higher costs. Topical administration of an activatable probe, γ -glutamyl hydroxymethyl rhodamine green, was demonstrated recently in a mouse model of ovarian cancer (33). Fluorescence activation occurred in ~1 minute—a rapid onset for this class of probe. Clinical use of this imaging agent will be challenged by high costs for GMP synthesis.

A rigorous validation process and focused regulatory strategy are needed to translate promising molecular probes for widespread clinical use. In this study, analytical validation and pharmacology/toxicology studies of ASY*-FITC was performed to maximize the likelihood for clinical success prior to seeking FDA approval. We first performed structural elucidation and synthesis of ASY*-FITC using fully validated assays on qualified analytical instrumentation (HPLC and mass spectrometry) at a GMP-certified facility. Additional confirmation of safety for ASY*-FITC was demonstrated comprehensively in a GLP animal study. Our work also highlights the safe use of peptide imaging agents in human subjects. All adverse events were likely to have resulted either spontaneously or from trauma associated with the endoscopic procedure, and were unlikely to have been related to the peptide.

We found topical application of ASY*-FITC to be an effective method for imaging cell surface targets in esophagus on endoscopy. However, this approach has limitations for detection of foci of disease present underneath the epithelial surface or mucosal ulcers at depths >50 μ m, and clinically important lesions could still be missed (Table 1). Images from regions of inflammation or indefinite for dysplasia on histology or that contained saturated fluorescence intensities were not used. Furthermore, rapid surveillance of the esophagus will require wide-field imaging instruments that are sensitive to the peptide-fluorophore conjugate over large mucosal surface areas. Confocal endomicroscopy can still be used as an adjunct to validate specific peptide binding to Barrett's neoplasia *in vivo*. Additionally, use of the FITC fluorophore is limited by hemoglobin absorption, tissue autofluorescence, and tissue scattering. Image contrast can be improved with use of near-infrared fluorophores and imaging instruments (34).

Targeted, peptide-based imaging agents are promising for future use in early detection of esophageal cancer, which is growing currently at a rate faster than that of any other cancer in industrialized countries. This strategy can be generalized to the detection of other cancers of epithelial origin that are challenged by flat morphology, heterogeneity, and patchy distribution, including colon, stomach, oropharynx, lung, cervix, and bladder, which in total comprise >80% of all deaths by cancer in the U.S.

METHODS

Cell culture

Human H460 lung adenocarcinoma cells were maintained in DMEM (Invitrogen) media supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100 U/ml each of penicillin/streptomycin (P/S). Human OE19, OE21 and OE33 esophageal carcinoma cells were grown in RPMI-1640 (Invitrogen) media supplemented with 10% FBS and P/S and 5 µg/ml plasmocin (Invitrogen) to prevent mycoplasma contamination. Human Q-hTERT BE cells were grown in keratinocyte serum-free media (Invitrogen) supplemented with 50 µg/ml bovine pituitary extract and 0.005 µg/ml human recombinant epidermal growth factor (Invitrogen) and P/S. Human FLO1 esophageal adenocarcinoma cells were grown in DMEM media supplemented with 10% FBS, P/S, and 5 µg/ml plasmocin (Invitrogen). All cells were grown as adherent monolayer cultures at 37°C in 5% CO₂.

Phage selection of specific peptide

Peptide selection was performed using a phage display library (Ph.D.-7, New England Biolabs) consisting of M13 bacteriophage that expresses ~10⁹ unique 7-amino acid sequences. First, non-specific binders were cleared from the library by biopanning 2×10¹¹ pfu of the original library against ~5×10⁵ OE21 cells cultured in 6-well plates for 30 min at room temperature (RT) with gentle agitation. Blocking was performed by adding 200 µl of PBS with 1% bovine serum albumin (BSA). The supernatant containing unbound phages was collected and added to the next well for another round of clearance. The resulting supernatant was then amplified once, precipitated with PEG-NaCl, and titered per manufacturer's instructions.

Positive selection was achieved by adding 2×10¹¹ pfu of the cleared library to H460 cells cultured in a 6-well plate. The unbound phages in the supernatant were discarded. The cells were washed 10X with PBS/0.1% Tween-20 (v/v). The bound phages were eluted with 1 ml buffer solution consisting of 0.2 M glycine (pH 2.2) and 0.1% BSA for 8 min. The resulting solution was immediately neutralized with 1 M Tris (pH 9.5). The eluted phage were then used for another round of positive selection against H460 cells, as described above, with no amplification in between rounds. This time, the bound phages were eluted with glycine for 2 min to remove the weaker binding phages. Then, fresh elution buffer was added for 6 min. The elute buffer was collected for a third round of biopanning with no amplification in between rounds. The binding efficiency from each round of biopanning was evaluated using 10 µl of elution buffer containing bound phage for titrating. The phage from the final round of selection was precipitated per manufacturer instructions, and 48 plaques were randomly picked for DNA sequencing. These sequences were analyzed with an ABI Automatic DNA Analyzer (Applied Biosystems) using the primer 5'-CCCTCATAG TTA GCG TAA CG-3' (−96 gIII sequencing primer, New England Biolabs) that corresponds to the pIII gene sequence of the M13 phage.

Peptide synthesis and subsequent binding assays are described in Supplementary Methods.

Synthesis, storage, and distribution of peptide

For the clinical and pharmacology/toxicology studies, ASY*-FITC was GMP synthesized (CPC Scientific). Stability was evaluated by appearance (color), HPLC, and mass spectrometry for 3 years. The pharmacology/toxicology study in rats was conducted in accordance with FDA CFR title 21, part 58 (Supplementary Methods). Aliquots of peptide were placed into amber vials and sealed using crimp tops with septa (Wheaton Scientific Products) to facilitate peptide reconstitution. Each vial was labeled (peptide name, lot number, manufacturer, storage conditions, and "Investigational Use Only"), and shipped to the Investigational Drug Service (IDS) research pharmacy at the University of Michigan for storage at -20°C . After patient consent was obtained, a prescription was submitted to IDS for a kit that contained: 1) a single-use vial of 0.80 mg of lyophilized peptide; 2) 5 mL of 0.9% NaCl; and 3) a syringe with needle for peptide delivery.

Validation on stereomicroscopy ex vivo

IRB approval was obtained from the University of Michigan, and informed consent was acquired from each subject. Patients with previously diagnosed BE with HGD who were referred for EMR were recruited. Resected specimens of esophagus were rinsed in PBS to remove debris. The mucosal surface was oriented to face the stereomicroscope (Olympus SZX-16) objective. An autofluorescence image was captured with 477–500 nm excitation and 500–630 nm emission at 12 ms exposure. The specimens were then incubated with 1 mL of ASY*-FITC at 100 μM for 5 min at RT followed by rinsing with PBS. Image calibration and registration were performed as described previously (20). The specimens were fixed in formalin overnight, and tissue sections were cut along the lines evaluated by fluorescence using the ink landmarks for registration. Histopathological interpretation of each section was performed by a gastrointestinal pathologist (H.A.) in 1-mm intervals, and classified as squamous, BE, LGD, HGD, or EAC. For sections with mixed histology, the most advanced grade was assigned.

Clinical study design

The clinical validation study aimed to enroll 25 human subjects. IRB approval was obtained from the University of Michigan. Patients previously scheduled for surveillance endoscopy per routine clinical care were recruited. Informed consent was obtained from each subject prior to the procedure. Study inclusion criteria included: 1) history of BE and biopsy-proven HGD or EAC; 2) no anticoagulation or co-morbidities; and 3) age between 18–100 years. Exclusion criteria included: 1) known allergy to fluorescein; 2) history of esophagectomy; 3) preparation for colonoscopy; and 4) on active chemotherapy/radiation protocol. This study was performed under IND#110,444 (sponsor C.O.) approved by the FDA, and was registered at ClinicalTrials.gov (NCT01391208).

Prior to the procedure, subjects who consented had baseline blood tests, urinalysis (dipstick), and if applicable, urine pregnancy test (β -HCG). Blood tests included CBC (WBC, RBC, hemoglobin, platelets), chemistries (sodium, potassium, chloride, bicarbonate, blood urea nitrogen, creatinine), and LFTs (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin). After peptide administration, the same labs were repeated in 24 ± 4 hours. The study coordinator then contacted the patients by telephone to record any side effects experienced, including nausea (>15 min), vomiting ($>2\times$ total), brown/dischored urine, jaundice, diarrhea (>4 stools/day) or bloody stools. Those with test results that differed by more than 10% of initial values had the abnormal tests repeated daily until the peak value was achieved and then weekly until they reverted to within 10% of baseline.

Validation of peptide binding using endomicroscopy in vivo

The upper endoscopy was performed per routine with standard white light. Regions suspicious for HGD or EAC, including nodularity, abnormal appearance, and prior location of HGD or EAC, were identified and rinsed thoroughly with saline to remove mucous. ASY*-FITC (5 ml of 100 μ M solution) was sprayed topically using a standard circumferential catheter (#PW-5V-1, Olympus). After 5 min for incubation, the area was rinsed with saline to wash away unbound peptide. Several regions of mucosa were then imaged with the confocal endomicroscope. A 2.6 mm diameter 1000X GastroFlex type MiniO miniprobe (Mauna Kea Technologies) was passed through the instrument channel of the endoscope, delivering <2 mW of excitation at 488 nm. This power level has been determined to be non-significant risk (NSR) by the FDA CFR title 21, part 812 (35). The instrument parameters include: FOV 240 $\times$ 240 μ m², lateral resolution 1.4 μ m, axial resolution 7 μ m, and working distance 50 μ m. Videos were collected at 8 frames per second. The average time required to perform the experimental portion (peptide spray and imaging) of the procedure was <15 min.

After imaging was completed, the resected specimen was placed in formalin and processed for routine histopathology. A gastrointestinal pathologist (H.A.) evaluated histology from the epithelial surface (50 μ m) (Table 1). Video streams with >8 consecutive images (1 sec) that had distinct structural features and negligible motion artifacts (blurring) were included in the analysis. Individual fluorescence images were exported from the videos in PNG format. Fluorescence images were registered to histology using landmarks defined by the Barrett's segment on white light endoscopy, summarized per Prague criteria (36) (Table 1).

Target identification and validation assays are described in Supplementary Methods.

Statistical analysis

The D'Agostino & Pearson omnibus test was used to evaluate data for normality. *P*-values were calculated using either unpaired t-test, Mann Whitney, or Dunn's multiple comparison using Prism 5.0 software (GraphPad Inc). For ex vivo tissue validation, the fluorescence intensities for each histological classification were analyzed for normality. Differences in the mean intensities for all classifications were first compared using the Kruskal Wallis test, then differences in the mean values between classifications were evaluated using Dunn's multiple comparison test. *P* 0.01 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Fluorescently labeled peptide specific for Barrett's neoplasia

(A) Chemical structure of ASYNYDA peptide (black) with a GGGSK linker (blue) and a FITC label (green). (B) Fluorescence emission spectra for ASY*-FITC and FITC with $\lambda_{ex} = 471$ nm shows emission peaks at 519 nm. (C) Mass spectrum of ASY*-FITC. (D) Binding affinity (apparent dissociation constant, k_d) for ASY*-FITC to H460 cells. Data are averages from 3 independent experiments. (E) Binding kinetics (apparent association time constant, k) for ASY*-FITC to H460 cells. (F) Relative fluorescence intensity (mean $\pm$ SD) from binding of ASY*-FITC to human H460 adenocarcinoma cells decreases in a concentration-dependent manner on addition of the unlabeled ASY* peptide. The control was unlabeled GGG* at two different concentrations. P -values determined by unpaired t-test.

Fig 2. Ex vivo validation of peptide specific for Barrett's neoplasia

(A) White light stereomicroscope image of resected esophageal specimen shows differences in color but not in architecture for squamous (yellow box), HGD (red box), and Barrett's esophagus (blue box). Histology was cut along dashed lines 1–6. Scale bar, 2 mm. Image is representative of 12 specimens. (B) Fluorescence image collected after staining (A) with ASY*-FITC. Scale bar, 2 mm. (C) Histology (H&E stain) from sections 1–6. Scale bar, 2 mm. (D) Expanded region of red box in (B) section 3 shows features of HGD, including large, stratified nuclei and lack of cytoplasmic mucin (black arrow). (E) Fluorescence intensities from ASY*-FITC and GGG*-FITC (control) binding to human esophageal mucosa ex vivo in 1 mm² intervals for squamous (Squ), BE, HGD, and EAC. Data are means ± SEM. **P<0.01, determined by Dunn's multiple comparison test.

Fig 3. Clinical validation of specific peptide binding in vivo

(A) Enrollment scheme for 25 patients with Barrett's esophagus (BE). (B) Representative white light endoscopy image of the distal esophagus shows a typical salmon-pink region of BE (black arrow) and normal-appearing squamous epithelium (white arrow). (C) Peptide was applied endoscopically to distal esophagus via spray catheter (arrow). (D) In vivo imaging with confocal endomicroscope (arrow). (E to H) Representative peptide-based fluorescence image of normal squamous epithelium (E), BE with a benign crypt (arrow) (F), HGD dysplastic crypts (arrows) (G), and EAC-transformed crypts (arrows) (H). (I to L) Histology (H&E). Squamous epithelium alongside BE is shown (I). Surface epithelium in (J) confirms HGD in (G). Magnified view of the epithelium lining the mucosa in (J) reveals large, irregularly shaped nuclei that are fully stratified and have little cytoplasm (K). EAC from (H) shows malignant cells with glandular differentiation (L).

Fig 4. Quantitative analyses of imaging performance

(A) Target-to-background (T/B) ratio for a representative confocal image of HGD was calculated by measuring the mean fluorescence intensity from 4 solid boxes (dimensions $25 \times 25 \mu\text{m}^2$) around the outer rim of crypts and 4 dashed boxes within the crypt lumen from 3 consecutive images. (B) T/B ratio (mean $\pm$ SD) on in vivo confocal images collected from autofluorescence, squamous, BE, HGD, and EAC. ** $P<0.01$, with P -values determined by unpaired t-test. (C) Receiver-operator curve (ROC) shows an optimum sensitivity of 75% (95% CI: 43%–95%) and specificity of 97% (95% CI: 85%–100%) for detection of HGD/EAC at T/B ratio of 4.2. (D) The sensitivity and specificity for peptide targeting HGD/EAC varies with T/B ratio as a function of detection threshold. Dashed line shows results at T/B ratio of 4.2. (E) Leukocytosis (average number of white blood cells measured as a ratio of post/pre-EMR) was quantified with and without peptide administration. ** $P<0.01$, with P -values determined by Mann Whitney test.

Table 1

Patient clinicopathological data

Prague criteria define the anatomic extent of Barrett's segment. Paris classification describes the level of mucosal elevation. Surface pathology was assessed in the epithelium where the ASY* peptide was administered and imaged (depth 50 µm), whereas conventional pathology was evaluated from the full mucosal cross-section of the specimen.

Patient	Age	Prague criteria	Paris classification	Therapy		Surface pathology					Conventional pathology					
				EMR	Biopsy	SQ	BE	LGD	HGD	EAC	SQ	BE	LGD	HGD	EAC	
1	59	C4M5	0-IIb,IIC	X	X		X						X			
2	66	C8M8II0	0-IIa,IIf	X	X	X	X			X			X	X		
3	49	C0M5	0-IIa,IIf	X	X	X	X					X	X			
4	82	C8	0-IIa,IIf	X		X	X						X		X	
5	86	C5M5	0-Is		X	X	X					X	X		X	X
6	81	C2M4I5	0-IIc	X		X	X				X		X	X	X	X
7	83	C1M1	0-IIa,IIf	X	X		X					X	X	X		
8	57	C2M4	0-IIa,IIf	X	X	X	X						X		X	
9	70	C7M8	0-IIa,IIf	X	X	X	X				X		X	X	X	
10	62	C1M0	0-IIa,IIf	X		X	X				X		X			
11	77	C1M3	0-IIb		X	X	X						X	X		
12	59	C2M4	0-Is													
13	74	C5M5	0-IIa,IIf	X	X	X	X				X			X		
14	59	C9M9	0-IIa,IIf	X	X	X	X						X	X		
15	53	C1M1	0-IIa,IIf	X	X	X	X							X	X	X
16	54	C0M1	0-IIb	X		X								X		X
17	72	C0M1	0-IIa	X	X								X	X	X	
18	84	C5M5	0-Is	X							X					
19	78	C10M10	0-IIb	X		X							X	X		
20	52	C2.5M2.5	0-IIa		X								X	X		
21	41	C0M2	0-IIa	X	X	X	X				X					X
22	79	C1M1	0-IIb	X	X	X	X									
23	77	C2M2	0-IIa,IIf	X	X						X	X				X

Patient	Age	Prague criteria	Paris classification	Therapy		Surface pathology						Conventional pathology					
				EMR	Biopsy	SQ	BE	LGD	HGD	EAC	SQ	BE	LGD	HGD	EAC		
24	87	C1M8I13	0-Is,IIa,IIb	X		X					X	X				X	
25	51	COM0.5I2	0-IIb	X	X	X	X	X								X	X

BE, Barrett's esophagus; EAC, esophageal adenocarcinoma; EMR, endoscopic mucosal resection; HGD, high-grade dysplasia; SQ, squamous.