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Optical Molecular Imaging Detects Changes in Extracellular pH with the Development of Head and Neck Cancer

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Abstract

Non-invasive localized measurement of extracellular pH in cancer tissues can have a significant impact on the management of cancer. Despite its significance, there are limited approaches for rapid and non-invasive measurement of local pH in a clinical environment. In this study, we demonstrate the potential of noninvasive topical delivery of Alexa-647 labeled pHLIP (pH responsive peptide conjugated with Alexa Fluor® 647) to image changes in extracellular pH associated with head and neck squamous cell carcinoma using widefield and high resolution imaging. We report a series of preclinical analyses to evaluate the optical contrast achieved after topical delivery of Alexa-647 labeled pHLIP in intact fresh human tissue specimens using widefield and high-resolution fluorescence imaging. Using topical delivery, Alexa-647 labeled pHLIP can be rapidly delivered throughout the epithelium of intact tissues with a depth exceeding 700 microns. Following labeling with Alexa-647 labeled pHLIP, the mean fluorescent contrast of increased 4 to 8 fold higher in clinically abnormal tissues as compared to paired clinically normal biopsies. Furthermore, the imaging approach showed significant differences in fluorescence contrast between the cancer and the normal biopsies across diverse patients and different anatomical sites (unpaired comparison). The fluorescence contrast differences between clinically abnormal and normal tissues were in agreement with the pathologic evaluation. Topical application of fluorescently labeled pHLIP can detect and differentiate normal from cancerous tissues using both widefield and high resolution imaging. This technology will provide an effective tool to assess tumor margins during surgery and improve detection and prognosis of head and neck cancer.

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Keywords

pH imaging; head and neck cancer; optical widefield imaging; confocal microscopy; topical delivery

Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is a significant public healthcare burden worldwide¹. HNSCC has a poor survival rate with only about half of patients surviving over 5 years². Currently, tumor and nodal status (TNM classification), histological tumor grading and HPV status are the most common indicators used to determine treatment course and prognosis for a particular patient^{3,4}. There is an unmet need to integrate molecular specific measurements made using non-invasive imaging approaches with current classification methods to improve the assessment of HNSCC⁴. In this study, we evaluated a non-invasive optical molecular imaging to detect changes in pH in clinically isolated biopsy samples using topical delivery of fluorescently labeled pH responsive peptides.

In tumors, development of hypoxia and a compensatory shift to anaerobic glycolysis for energy production^{5,6} can lead to a significant increase in production and release of acidic metabolites (such as protons and lactic acid) by tumor cells in the extracellular matrix. This results in reduction of extracellular pH within the tumor⁶⁻¹². Acidic microenvironments are closely associated with increased mutagenesis, metastasis and resistance to radiation and drug therapies^{6,7,10,11,13-18}. Thus, acidosis could be a unique marker to improve both the detection and prognosis of cancer including distinguishing aggressive from benign tumors and selection of appropriate therapy for a particular lesion.

Significant efforts have been made to measure changes in extracellular pH in cancer tissues using both invasive (pH electrodes) and non-invasive approaches^{8-12,14,19,20}. Non-invasive measurements have predominantly focused on PET (positron emission tomography) and MRI (magnetic resonance imaging) imaging approaches. pH measurements using PET imaging have used radiolabeled dimethadione, but due to limited accuracy this approach has not been translated to clinical practice²¹. One of the key reasons for limited accuracy of dimethadione in measuring extracellular tissue pH is the lack of exclusive localization of the probe to the extracellular environment in a tissue. pH measurements using MR imaging and spectroscopy have demonstrated significant success in measuring intra-tumoral pH based on changes in relaxation properties (T_1 and T_2) of contrast media such as dysprosium-DOTP⁵⁻ and gadolinium-DOTA-4AmP⁵⁻ and chemical exchange saturation transfer at high magnetic field strengths²¹. Ratiometric imaging based on a combination of pH sensitive (Gd-DOTA-4AmP⁵⁻) and insensitive (Gd-DOTP⁵⁻) contrast media was used to measure extracellular tissue pH in-vivo in mouse kidney¹⁰ and rat glioma models²². Despite significant developments in MR imaging and spectroscopy for pH measurements, there is a critical need to improve spatial resolution and sensitivity of detecting acidosis in tissue pH within tumor tissue^{10,21,23-26}.

Complementary to PET and MR imaging, an optical molecular imaging approach can provide a non-invasive measurement of changes in pH at a whole tissue level using

widefield imaging as well as provide a detailed map of pH changes at cellular resolution using confocal microscope. Recently, an innovative approach was discovered to selectively target plasma membrane of cells based on extracellular pH using pH (Low) Insertion Peptide (pHLIP)^{7, 27–31}. pHLIP is a 36-amino acid peptide derived from bacteriorhodopsin-C that inserts itself into the plasma membrane at acidic extracellular pH²⁸. This peptide has been validated for imaging acidosis in animal xenograft tumor model systems^{32–34}. The degree of pHLIP staining in these animal models has been correlated with tumor pH as measured by microelectrode³³.

This study is focused on evaluating the potential for clinical translation of topically delivered fluorescently labeled pHLIP to detect differences in tissue pH in paired sets of ex-vivo clinically normal and abnormal appearing biopsies, and its correlation with pathological diagnosis. To evaluate the clinical translational potential, the specific objectives of this study were: (a) to determine if topical delivery of fluorescently labeled pHLIP can provide specific optical contrast to distinguish clinically abnormal tissues from normal tissues from various anatomical locations in diverse patients; (b) to evaluate if the resulting optical contrast could be detected using both widefield (whole biopsy) and high resolution imaging modes; and (c) to evaluate variation in optical contrast resulting from binding of pHLIP changes with development of neoplasia (tumor to dysplasia) and its correlation with pathological diagnosis. Addressing these specific questions will demonstrate the potential of topically delivery of pHLIP for detection of head and neck cancer, delineating tumor borders, and assisting with forming prognostic or therapeutic decisions.

Material and methods

Materials

The human cervical carcinoma cell line HeLa was a gift from Professor Glenn M. Young (University of California, Davis). HeLa cells were maintained in a culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, Pittsburgh, PA) supplemented with 10% FBS (Fisher Scientific, Pittsburgh, PA) and 100 mg/L penicillin (Sigma, St Louis, MO). HeLa cells (4×10^4 cells/ml) were seeded into culture flasks, grown in a humidified atmosphere of 5% CO₂–95% air at 37°C, and sub-cultured with 0.05% trypsin (Invitrogen, Carlsbad, CA).

Biopsies of clinically normal and abnormal upper aerodigestive tract mucosa were obtained from patients undergoing surgery to remove suspected or confirmed head and neck tumors at the University of California, Davis, Department of Otolaryngology. Patients gave written informed consent to participate, and the study was approved by the University of California, Davis Institutional Review Board.

The pHLIP peptide (ACEQNPIYWARYADWLFTTPLL~~LD~~LALLVDADEGTG) and the mutated control peptide (ACEQNPIYWARYAKWLFTTPLL~~KL~~LALLVDADEGTG) with purity >95% (GenScript Inc., Piscataway, NJ) was dissolved in molecular grade water to achieve the stock concentration of 100 µM. The mutated control peptide has the same amino acid sequence as the pHLIP peptide except mutations of two key amino acids (Aspartic acid residues at position 14 and 25 from N-terminus was charged to lysines as marked in the

mutated sequence) that inhibits pH induced binding of the mutated peptide to cell membrane. The pHLIP peptide had a cysteine amino acid at penultimate position of N terminus (underlined in the peptide sequence). For fluorescent labeling of the pHLIP peptide, Alexa Fluor® 647 C2 maleimide (Invitrogen) was reacted with the thiol (SH-group) of cysteine at the N-terminus of pHLIP to form a stable thioether bond. The mixture of pHLIP (100 μ M) and Alexa Fluor® 647 (7 mM) with the ratio of peptide:dye of 5:1 was stirred at room temperature in the dark for 4 hours and kept at 4°C overnight. The conjugated peptide was purified from free dye by extensive dialysis (Slide-A-Lyzer® Dialysis Cassette G2 3,500 MWCO, Thermo, Rockford, IL) for over 48 hours with repeated exchange of buffer solution followed by column purification (Zeba™ Spin Desalting Column 7K MWCO, Thermo, Rockford, IL). The final concentration of fluorescently labeled pHLIP was determined based on UV-vis absorption spectroscopy ($\epsilon_{280}=12,300 \text{ M}^{-1} \text{ cm}^{-1}$ for pHLIP and $\epsilon_{652}=237,000 \text{ M}^{-1} \text{ cm}^{-1}$ for Alexa Fluor® 647). The mutated peptide was labeled and characterized using the same procedure as described above.

Cell culture model

To evaluate the specificity and localization of pHLIP uptake, initial studies were carried out using HeLa cells cultured in monolayer. Cells from a confluent T-25 flask were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) and re-suspended in culture medium to a concentration of 10^5 cells/ml. After incubation for 48 hours, cells were washed twice with phosphate buffered saline (PBS) (pH=7.4) and then maintained in PBS at pH 6.2, 6.6, 7.0 and 7.4 in the presence of Alexa-647 labeled pHLIP. After 1 hour of incubation, the cells were washed three times with PBS at their respective incubation pH. The cells were finally maintained in Phenol red free DMEM (pH=7.4) and imaged live using an Olympus IX71 confocal laser scanning microscope (Olympus Inc., Center Valley, PA).

Paired patient biopsies

Pairs of clinically normal and abnormal biopsies were obtained from the operating room while the patient was undergoing scheduled diagnostic mapping biopsies or surgical removal of an upper aerodigestive tract tumor. Patients over the age of 18 with known or suspected primary head and neck tumors were recruited. 11 patients provided 11 distinct normal and 16 distinct clinically abnormal biopsies. Each patient provided one clinically normal appearing biopsy, and one or two clinically abnormal appearing biopsies (2 mm – 6 mm in diameter). The paired biopsies isolated from patients were transferred to non-buffered saline at 4°C. Non-buffered saline was selected to limit influence of storage media on intrinsic pH of isolated biopsy sample. The paired biopsy samples in non-buffered saline were stored in an ice box (with cold packs and insulation) and transferred from the clinic to the laboratory within 20–30 minutes after isolation of biopsies from patients. Low temperature storage condition is expected to significantly reduce metabolic activity of tissue and limit significant changes in pH. This expectation is supported by previous studies that have used similar hypothermic metabolic protection approaches for ex-vivo analysis of fresh tissue samples^{35, 36}.

Topical delivery of fluorescently labeled pHLIP

To deliver the fluorescently labeled pHLIP, paired biopsies of normal and abnormal mucosa were labeled topically with the pH peptide in 24-well plates. Topical delivery method was based on our previous studies in which we have demonstrated successful delivery and specific labeling of oral biopsies^{37, 38} using both small molecules and fluorescently labeled peptides. Briefly, the biopsy samples were oriented to ensure that the epithelial surface was exposed to topical contrast media. Topical labeling was achieved by topical labeling of isolated biopsy samples using 5 μ M concentration of fluorescently labeled pHLIP in non-buffered saline with 10% DMSO. 10% concentration of DMSO was included as a permeation enhancer to facilitate intraepithelial delivery^{39, 40}. After 60 minutes at 37°C, the biopsies were rinsed in non-buffered saline for 10 minutes to remove any unbound contrast agent.

We acquired white light and widefield fluorescent images before and after staining for each pair of biopsies using a commercially available widefield imaging system (Maestro 2, Cri (Woburn, MA) at the Center for Molecular and Genomic Imaging, University of California, Davis). Pre-contrast images provide a measure of tissue autofluorescence background signal while the post-contrast images measure contributions from both autofluorescence and pHLIP labeling. Biopsies were placed side-by-side on a black plastic sheet. Widefield images were obtained using bandpass excitation (BP 640) and a bandpass emission filter (BP 670–800). The integration time for the Maestro system was maintained constant throughout the study at 100 ms. Both the pre and post contrast images were acquired using the same integration time.

Following widefield imaging, each biopsy was sectioned transversely into 200 μ m slices using an oscillating tissue slicer (EMS 5000, Electron Microscopy Sciences Inc., Hatfield, PA). These transverse slices were then imaged using a Zeiss LSM 510 confocal fluorescence microscope (Carl-Zeiss Inc., Thornwood, NY) using 633 nm laser excitation and 655–719 nm emission filter. White light and fluorescent images of paired normal and abnormal biopsies were imaged using identical detector gain, pinhole and input laser power.

Quantification of imaging data

Results of the fluorescent imaging studies were quantified by calculating mean fluorescence intensity (MFI) within a selected region using ImageJ (NIH, Public Domain). We have used this standardized approach in past molecular imaging studies for quantification of fluorescent imaging^{37, 38} in cells, isolated biopsies and resected tumors.

To quantify pHLIP binding under different extracellular pH conditions, HeLa cells were selected individually based on white light confocal images. The MFI was calculated for each cell. The average MFI was calculated by averaging the MFI from all individual cells incubated within each specific condition (results represent average of fluorescent contrast from 50–60 individual cells).

For quantification of widefield images collected using the Maestro 2 Imaging system (CRI, Woburn, MA), the whole biopsy was selected based on the white light images. To calculate the MFI using Image J, the fluorescence images were converted to grey scale, 8-bit images.

The MFI was calculated for both the pre- and post-contrast images using ImageJ software (NIH, public domain). Subtracting the MFI of the pre-contrast image of the biopsy sample (prior to staining) from the post-contrast imaging represents the difference () in MFI due to binding of pHLIP within the tissue sample ($\Delta \text{MFI} = \text{Post Contrast MFI} - \text{Pre Contrast MFI}$). Based on this difference in MFI, a differential contrast ratio for each paired biopsy set (clinically normal and abnormal biopsy from individual patient) was calculated. The differential contrast ratio is the ratio of increase in MFI of the clinically abnormal appearing sample to the increase in the MFI of clinically normal appearing sample (Differential Contrast Ratio = $\Delta \text{MFI} (\text{Post} - \text{Pre}) \text{ of Clinically Abnormal} / \Delta \text{MFI} (\text{Post} - \text{Pre}) \text{ of Clinically Normal}$). Results of fluorescence imaging were then compared with the pathologic diagnosis for each specimen. We have used this quantification approach in our previous studies^{37, 38} to characterize and compare increase in fluorescence contrast between clinically abnormal and normal biopsies.

High-resolution fluorescence images of the tissue slices were analyzed to quantify the relative average fluorescence intensity of clinically abnormal samples and clinically normal samples from the same patient. For quantification of high resolution imaging, the epithelial section of each labeled tissue slice was selected based on structural image (DIC image) of the tissue section. MFI was calculated within the selection using ImageJ software (NIH, public domain). The contrast ratio was calculated using the average MFI from two individual images per biopsy. The quantified imaging results from widefield and high resolution imaging were analyzed based on analysis of variance (ANOVA) Bonferroni post-test calculations using Stata 11® (StataCorp LP, College Station, Texas).

Pathological diagnosis

Following imaging, tissue samples were fixed in 36% Formalin, paraffin embedded and submitted for hematoxylin and eosin (H&E) staining and examined by a board certified pathologist at the University of California, Davis (RGE). Samples were assessed for degree of dysplasia (none, mild, moderate, severe), cancer differentiation (well, moderate, poor, invasive), inflammation (none, acute, chronic, mild moderate, severe), % necrosis, keratinization and overall diagnosis (normal, hyperplasia, dysplasia, cancer). Representative photographs were obtained.

Results

Determine sensitivity of imaging changes in pH using fluorescently labeled pHLIP in cell culture

To demonstrate sensitivity of fluorescently labeled pHLIP to detect changes in extracellular pH and specificity of binding cell membrane of individual cells in acidic environment, live HeLa cells were incubated with fluorescently labeled pHLIP under selected extracellular pH conditions, ranging from 6.2 to 7.4. Figure 1A shows representative images of cells incubated with Alexa-647 labeled pHLIP for one hour under different extracellular pH conditions. Imaging measurements showed a decrease in fluorescence staining of HeLa cells with an increase in extracellular pH and also demonstrated that the fluorescent staining is localized predominantly in the plasma membrane of cells. The average MFI of individual

cells after labeling with pHLIP in different extracellular pH conditions was quantified. Figure 1B shows the MFI decreased as the pH increased from 6.2 to 7.4. There was a significant drop in MFI between pH 6.2 to 6.6 (14.63 ± 0.44 vs. 9.44 ± 0.38 , $p < 0.001$) and from 6.6 to 7.0 (5.45 ± 0.34 , $p < 0.001$). There was a non-significant decrease in MFI between pH 7.0 and 7.4 (2.20 ± 0.12 , $p=0.462$). This result validates that the pHLIP peptide specifically binds to plasma membrane in acidic extracellular pH. The results are in agreement with the previously published results^{29, 30, 32, 33}.

Patient Characteristics

Table 1 shows the diverse anatomical locations from which the paired biopsies were obtained from and the corresponding pathologic diagnosis. Though paired set of biopsies were obtained from a variety of anatomical locations, there was a predominance of tonsillar tissue including both palatine tonsils and the base of tongue (81% of abnormal biopsies, 66% of normal biopsies). This reflects the current population of patients seen in the otolaryngology clinic. Nine of the clinically abnormal appearing samples were diagnosed as invasive squamous cell carcinoma based on pathological analysis, which is reflective that the head and neck cancers are often detected and treated at late stages of the disease^{3, 24}. Patients ranged from 51 to 84 years old, and 75% were male. All of the patients were white and had no prior history of oropharyngeal carcinoma.

Widefield imaging of topically applied pHLIP in paired normal and abnormal appearing biopsies

To demonstrate the sensitivity of imaging changes in extracellular pH in intact biopsies using topical delivery of pHLIP, widefield imaging of the paired biopsies was evaluated. The MFI was calculated for both clinically abnormal and normal biopsies before and after topical application of fluorescently labeled pHLIP (Figure 2). The MFI before staining represents the contribution from background tissue autofluorescence in the selected wavelength range (670 nm–800 nm). As illustrated in Figure 2A, in most of the samples the pre-contrast autofluorescence of cancerous tissues was lower than its paired normal tissue, but this was not always the case. Figure 2B shows a case in which the cancer biopsy's pre-contrast autofluorescence was higher than its paired normal biopsy.

The difference () between the post-contrast MFI and the pre-contrast MFI was calculated, and represents the specific contribution from the Alexa-647 labeled pHLIP staining. The differential contrast ratio (MFI (Abnormal)/ MFI (Normal)) was calculated for each pair of biopsies. For example, for the pair shown in Figure 2A, the cancerous biopsy was found to increase in intensity 3.58 times more than its paired normal biopsy increased (MFI of 37.25 vs. 10.41). Figure 2B shows another pair of biopsies, in which the cancerous biopsy was found to increase in intensity 5.88 times more than its paired normal biopsy increased (MFI of 21.50 vs. 3.66). These results highlight the sensitivity of widefield imaging approach to measure changes in extracellular pH in intact clinically abnormal biopsies. Figure 2C shows the results of paired biopsy set that was labeled with a mutated control peptide labeled with Alexa Fluor® 647 to validate specificity of binding of pHLIP in acidic extracellular pH. The results show that clinically abnormal and normal biopsies incubated with this mutated peptide do not show any significant increase in fluorescence contrast. It is

important to note that this mutated peptide has the same amino acid sequence as the pHLIP peptide except mutations at two specific amino acid residues (Aspartic acid was changed to lysine) as described in the materials and methods section.

Figure 3A shows the results of MFI across all cancerous biopsies irrespective of the anatomic location or patient of origin (i.e. the tissue samples are unpaired). Results showed a significantly increase in MFI after staining in cancerous samples ($n=9$; pre-contrast: 10.07 ± 3.07 vs. post-contrast: 34.95 ± 3.10 , $p < 0.001$), but no significant increase after staining in normal samples ($n=7$; pre-contrast: 20.60 ± 6.0 vs. post contrast: 28.53 ± 6.88 , $p = 0.329$). The unpaired average differential contrast ratio was then calculated by averaging increase in the mean fluorescence intensity of all 9 cancerous tissue's and dividing by the average increase in the mean fluorescence intensity of all 7 normal tissues. Thus, across all patients and anatomical locations there is a 3.13 fold increase in contrast in all cancerous tissues compared to all normal tissues upon staining with fluorescently labeled pHLIP. These results show that the cancerous tissue selectively binds pHLIP compared to normal tissue, and that this difference can be discriminated even when averaged over a heterogeneous sampling of anatomical locations and patients.

Figure 3B shows the differential contrast ratios of the paired biopsies separated by pathologic diagnosis of the clinically abnormal appearing biopsy. Results indicate that on average, cancerous biopsies ($n=9$) show a 5.91 fold increase in MFI as compared to increase in MFI of the normal tissue isolated from the same patient. Carcinoma in situ (CIS) diagnosed tissue biopsies show an increased fluorescence contrast (ranging between 4–2.5 fold) as compared to the paired normal samples. For the moderately dysplastic biopsy, the results showed an intermediate contrast ratio of 1.71 compared to its paired normal biopsy. In this study, we only had one patient with moderate dysplasia which is reflective of the advanced stage of disease presentation seen in head and neck cancer clinics. In addition, four clinically abnormal appearing biopsies were diagnosed as pathologically normal. Two of these clinically abnormal biopsies were diagnosed as normal with chronic inflammation. In these four set of paired biopsies, the increase in MFI of pathologically normal biopsies ($n=4$; initially identified as clinically abnormal biopsies) was similar to the increase in MFI in normal paired biopsies after labeling. This result illustrates the high specificity of the pH imaging approach to distinguish true clinical normal biopsy from abnormal biopsies including in cases with chronic inflammation.

Penetration of topically applied pHLIP in paired normal and abnormal biopsies

To demonstrate penetration depth of topically applied pHLIP within the epithelial tissue and to map spatial distribution of pH changes within the tissue, biopsy pairs were sliced transversely (approximately 200 μm thick) and imaged using confocal microscopy. Representative images from one of the pairs of normal and cancerous biopsies are shown in Figure 4. For both biopsies, the top epithelial surface is delineated in the images by the white border on the right. Figure 4A shows the normal biopsy, with low signal intensity throughout the thickness of the sample (MFI of 0.58, Figure 4C). The autofluorescence intensity in high resolution imaging of tissue sections is significantly lower as compared to the widefield imaging results of intact biopsies. This difference in the level of

autofluorescence results from ability of confocal imaging to optically section the tissue such that signal from few microns (2–3 microns) is integrated to form an image. In case of widefield imaging, the image integrates the autofluorescence from the entire tissue including the epithelium and the stroma, resulting in significantly high autofluorescence signal intensity as compared to the results of high resolution imaging. Figure 4B shows the paired cancerous biopsy with significant staining throughout the tissue (MFI 22.26, Figure 4C). High resolution imaging results also demonstrates heterogeneity of staining within clinically abnormal tissue. The pathological diagnosis of the normal biopsy in this case was normal base of tongue epithelium (Figure 4D). For the abnormal biopsy, the diagnosis was carcinoma in situ (CIS) of the base of tongue (Figure 4E).

Figure 5 compares the ratio of mean fluorescent intensity of paired sets of clinically abnormal and normal biopsies. The pathological diagnosis of these paired biopsies is shown on the x-axis of the graph. The results show that the ratio of the mean fluorescence intensity of cancer: normal (n=8, including carcinoma in situ) biopsies ranges from approximately 2–14. Comparison of pathologically normal (thought to be abnormal on clinical exam) to its normal paired biopsies show no significant increase in fluorescence contrast, hence a contrast ratio of approximately one. The variation observed in ratiometric contrast ratios of paired cancer and normal biopsies reflects both the biological differences among biopsies collected from different patients and different anatomical sites in the head and neck cavity, and the limitations of small tissue sections imaged with confocal microscopy (each section is 250×250 microns).

Discussion

In this study, we have evaluated non-invasive optical imaging of changes in extracellular pH in clinically isolated paired biopsies. Based on topical delivery of fluorescently labeled pHLIP in intact biopsies, the optical contrast in both widefield and high resolution imaging modes was measured. This combination of imaging modes (both widefield and high resolution) provides multiscale analysis of molecular contrast in tissue biopsies. Imaging based results were compared with pathological analysis to validate the molecular imaging approach can detect differences between normal and cancer tissues. In addition, ex-vivo approach was selected in this study to detect pH changes in head and neck cancers tissues isolated from diverse patients and across different anatomical sites within oral cavity. Analysis of fresh tissue biopsies provides an important step in translating molecular imaging approaches to human patients. This imaging approach based on topical application of contrast media can be translated to clinical environment as many optical contrast media have been previously delivered in-vivo using the same approach^{48–49}.

The results of widefield imaging show an average 3.13 fold increase in MFI after topical application of pHLIP between cancerous tissue and normal tissue (Figure 3A). This differential contrast ratio is particularly significant because it represents an average measurement across all patients, tumor locations and etiology reflecting the heterogeneity involved in a clinic setting. The average differential contrast ratio between cancer and normal tissues highlight that a large number of head and neck cancers have acidic extracellular environment. In paired comparison of biopsies isolated from individual

patients, fluorescence contrast in cancer biopsies was on average 5.9 fold higher as compared to normal paired biopsies (Figure 3B). These results are supported by results from previous studies^{41–43} that have measured extracellular pH using electrochemical methods in oral cancer patients and measured lactate concentration using quantitative immunohistochemistry in frozen tissue samples.

In many of the clinically isolated biopsies, the normal tissue had higher autofluorescence signal intensity (with an excitation wavelength of 640 nm) than the clinically abnormal tissue. This autofluorescence background can be significantly reduced by selecting excitation and emission wavelengths in the range of 700–800 nm^{44–47} as illustrated in the results presented in supplementary data section. Thus, the differences in pre-contrast of clinically abnormal and normal biopsy can be significantly reduced. In this study, we used Alexa Fluor® 647 (excitation at 647 nm) for labeling of pHLIP as it was the longest wavelength that could be used with a commercial confocal imaging system for high resolution imaging. It is important to note that although autofluorescence does provide good differentiation between the clinically abnormal and normal samples in many of clinically isolated biopsies; it has limited specificity as the differences in autofluorescence can result for a variety of physiological factors such as inflammation other than the presence of neoplasia. In addition, the pHLIP peptide provides a novel molecular approach to understand the microenvironment of clinical samples that can have significant impact on clinical evaluation of the disease.

In addition to detecting differences between cancer and clinically normal samples, the molecular imaging approach based on topical delivery of pHLIP had high specificity in identifying normal tissue that was considered clinically abnormal based on the clinical impression (Figure 3B). In these paired biopsies, the clinically abnormal appearing tissue (but pathologically normal) biopsy did not show any significant increase in fluorescence contrast compared to the paired clinically and pathologically normal tissues. This result is significant as it highlights the specificity of differentiating between true clinical abnormal and normal tissues including tissues with chronic inflammation.

In this study, two sets of clinically abnormal biopsies were diagnosed as carcinoma in situ (CIS) and one clinically abnormal biopsy was diagnosed as moderate dysplasia from a set of over 27 biopsies. Based on the results of widefield fluorescence imaging, the clinically abnormal biopsies (both CIS and moderate dysplasia) had increased fluorescence contrast as compared to paired normal biopsies. The fluorescence contrast was lower in these clinical abnormal biopsies as compared to cancer biopsies. These measurements indicate that early lesions can be detected, and possibly even differentiated, from advanced lesions. To further validate the results, more CIS and moderate dysplasia samples are needed. To address this, patients need to be recruited from lower acuity settings.

Topical delivery approaches are commonly used in clinical diagnostic applications^{48, 49} for localized delivery of contrast media such as toluidine blue. Topical delivery of molecular contrast agents has significant advantages including rapid and non-invasive access of probes to epithelial tissue (epithelial tissue is not vascularized in early stages of neoplasia, which limits intravenous delivery of probes to epithelial tissues); reduced non-specific

accumulation of contrast media in non-targeted tissues and decreased degradation of peptide based contrast media during delivery. With topical delivery, non-specific retention of contrast media within a local tissue is a potential limitation as it can reduce contrast between normal and abnormal samples. Using topical delivery in biopsy samples (Results in Figure 3A and supplementary data section), non-specific staining of tissue was not significant and differences between clinically abnormal and normal can be distinguished. Further studies are required to validate these results in-vivo.

The results of high resolution imaging demonstrate using topical delivery fluorescently labeled pHLIP (~3.5 kDa) can be delivered more than 600 microns deep in intact biopsies and selectively bind to cells in clinically abnormal tissue compared to paired normal tissue biopsies. These results are also in agreement with our previous study³⁸. The high resolution imaging results are in agreement with the widefield imaging results and demonstrate a significant increase in fluorescence contrast in clinically abnormal biopsies compared to clinically normal biopsies. The spatial variation in distribution of pHLIP binding within clinically abnormal biopsies indicates that there are variations in pH within a tumor tissue (Figure 4). These results are highly significant as this approach may provide a unique method to map relative variations in pH within tissues at a single cell resolution.

In summary, the results of this study have demonstrated that topically applied fluorescently labeled pHLIP has the potential to differentiate cancerous tissues from surrounding normal tissues in a variety of anatomical locations across a diverse group of patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HNSCC	Head and Neck Squamous Cell Carcinoma
CIS	Carcinoma in situ
MFI	Mean Fluorescent Intensity
pHLIP	pH (Low) Insertion Peptide

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Novelty and Impact Statement

The manuscript describes an optical molecular imaging approach to non-invasively detect changes in extracellular pH in clinically isolated paired biopsies from head and neck cancer patients. This molecular imaging approach will have significant impact on both detection and prognosis of head and neck cancer including detection of tumor margins.

Figure 1A **Figure 1B** **Figure 1.**

Validation of pHLIP to measure changes in extracellular pH in a cell culture model.

Figure 1A: Fluorescent labeling of HeLa cells with Alexa-647 labeled pHLIP at various extracellular pH conditions. Scale bars represent 10 µm.

Figure 1B: Quantification of fluorescence imaging measurements of HeLa cells at selected extracellular pH conditions (error bar represents standard error).

Figure 2.

Representative widefield imaging data from clinically isolated paired human biopsies and its quantification. Results show both the pre and post contrast imaging measurements.

Figure 2A: Fluorescence and corresponding pathological H&E images of paired biopsies incubated with Alexa-647 labeled pHLIP. In this case, the pre-contrast mean fluorescence intensity of clinically normal tissue was significantly higher than the pre-contrast mean fluorescence intensity of the clinically abnormal tissue (invasive squamous cell carcinoma).

Figure 2B: Fluorescence and corresponding pathological H&E images of paired biopsies incubated with Alexa-647 labeled pHLIP. In this case, the pre-contrast mean fluorescence intensity of clinically normal tissue was significantly lower than the pre-contrast signal from the clinically abnormal tissue (invasive squamous cell carcinoma).

Figure 2C: Fluorescence and corresponding white light images of paired biopsies incubated with Alexa-647 labeled mutated control peptide. Results show no significant differences in pre and post contrast in both clinically abnormal and normal biopsies.

Figure 3A **Figure 3B** **Figure 3.**

Analysis of widefield imaging measurements of clinically isolated abnormal and normal biopsies.

Figure 3A: Average mean fluorescence intensity of pre and post contrast widefield imaging measurements across all cancerous (invasive tumors and in situ carcinoma) and normal biopsies independent of the patient or the anatomical location from which the biopsies were isolated (unpaired averages).

Figure 3B: Differential contrast values calculated from widefield fluorescence images of fresh oral tissue incubated with Alexa-647 labeled pHLIP as a function of pathologic diagnosis. Each data point represents a pair of tissue specimens from a single patient. The

bars represent the average differential contrast and the standard deviation for paired tissue biopsies.

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Figure 4A Normal Biopsy **Figure 4B Cancer Biopsy** **Figure 4C** **Figure 4D Normal** **Figure 4E Carcinoma in Situ (Cis)** **Figure 4.**

Confocal fluorescence and white light images of a representative pair of clinically isolated biopsies incubated with Alexa-647 labeled pHLIP.

Figure 4A: Clinically normal biopsy.

Figure 4B: Clinically abnormal biopsy.

Figure 4C: Shows quantification of the mean fluorescent intensity in the cancerous biopsy compared to the normal biopsy.

Figure 4D: Shows the pathologic diagnosis and H&E stained image for both the clinically normal and abnormal biopsies.

Figure 5.

Ratiometric contrast measurements for the paired biopsy sets (clinically abnormal: normal) based on the ratio of the mean fluorescence intensity of clinically abnormal and normal sample. The contrast ratio represents the average ratio of two individual images per biopsy. The pathological diagnosis for these paired biopsies is shown on the x- axis.

Table 1

Details of fresh human biopsy samples from individual patients

Patient	Clinically “normal” location	Pathological diagnosis	Clinically “abnormal” location	Pathological diagnosis
1	Tonsil		Tonsil	
2	Base of tongue		Tonsil	
3	Post. pharynx		Epiglottitis	
4	Epiglottitis		Subglottitis	
5	Buccal		Hard palate	
6A and B	Tonsil		Tonsil	
7A and B	Tonsil		Tonsil	Invasive cancer
8A and B	Base of tongue		Base of tongue	Carcinoma <i>in situ</i>
9A and B	Base of tongue		Base of tongue	Normal
10	Soft palate		Tonsil	Moderate dysplasia
11A and B	Base of tongue	Normal	Base of tongue	Normal with chronic inflammation

The table shows the anatomical locations in the oropharynx from which biopsy samples were collected and the pathologic diagnosis of individual biopsies based on a pathological review by a board certified pathologist.