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Confocal Microscopy and Molecular-Specific Optical Contrast Agents for the Detection of Oral Neoplasia

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Abstract

Using current clinical diagnostic techniques, it is difficult to visualize tumor morphology and architecture at the cellular level, which is necessary for diagnostic localization of pathologic lesions. Optical imaging techniques have the potential to address this clinical need by providing real-time, sub-cellular resolution images. This paper describes the use of dual mode confocal microscopy and optical molecular-specific contrast agents to image tissue architecture, cellular morphology, and sub-cellular molecular features of normal and neoplastic oral tissues.

Fresh tissue slices were prepared from 33 biopsies of clinically normal and abnormal oral mucosa obtained from 14 patients. Reflectance confocal images were acquired after the application of 6% acetic acid, and fluorescence confocal images were acquired after the application of a fluorescence contrast agent targeting the epidermal growth factor receptor (EGFR). The dual imaging modes provided images similar to light microscopy of hematoxylin and eosin and immunohistochemistry staining, but from thick fresh tissue slices. Reflectance images provided information on the architecture of the tissue and the cellular morphology. The nuclear-to-cytoplasmic (N/C) ratio from the reflectance images was at least 7.5 times greater for the carcinoma than the corresponding normal samples, except for one case of highly keratinized carcinoma. Separation of carcinoma from normal and mild dysplasia was achieved using this ratio ($p < 0.01$). Fluorescence images of EGFR expression yielded a mean fluorescence labeling intensity (FLI) that was at least 2.7 times higher for severe dysplasia and carcinoma samples than for the corresponding normal sample, and could be used to distinguish carcinoma from normal and mild dysplasia ($p < 0.01$). Analyzed together, the N/C ratio and the mean FLI may improve the ability to distinguish carcinoma from normal squamous epithelium.

Keywords

Confocal microscopy; Contrast agents; Epidermal growth factor receptor (EGFR); Fluorescence labeling intensity; Nuclear-to-cytoplasmic ratio; Oral cancer

Introduction

Over the past decade, advances in anatomic and functional imaging have revolutionized the field of oncology (1). Positron emission tomography (PET) and magnetic resonance imaging are now standard clinical tools for both tumor detection and monitoring response to therapy. Fluorodeoxyglucose PET can visualize the metabolic activity of tumors, providing crucial information for identifying therapeutic targets. There is now ample evidence that the metabolic response of a tumor to therapy precedes the anatomic response, so that PET may provide a tool to rapidly assess tumor response as well as the emergence of resistance (2). However, despite these advances in imaging, current clinical techniques cannot visualize tumor architecture and morphology at the cellular level.

In many clinical situations, such as diagnosing and characterizing suspected tumors, visualization of cellular morphology and architecture is necessary. This requires surgical excision of a tissue biopsy or needle aspiration for processing and histopathologic evaluation. Biopsies are invasive, time consuming, and costly, and results are not available immediately. Recent advances in optical molecular imaging technologies have the potential to address this clinical need by delivering real-time, *in vivo* images with sub-cellular resolution. This paper describes the use of real-time, dual mode confocal microscopy to image tissue architecture and cellular morphology in normal and neoplastic oral tissue. We also show that when used in tandem with optical contrast agents targeting receptors over-expressed in oral cancer, optical molecular images can be used to distinguish normal and mildly dysplastic oral tissues from oral carcinoma.

There are a growing number of biomarkers that have the potential to be used for cancer detection, and a variety of optically active contrast agents have been developed to target these biomarkers. Optical contrast agents, including quantum dots, metallic nanoparticles, and nanoshells, have been used to study the molecular pathophysiology of neoplasia *in vivo*, and to create more sensitive methods for imaging the presence of early disease. Quantum dots have been successfully used to label molecular targets in cell lines (3–6), *ex vivo* mouse tissues (6), and *in vivo* mouse models of disease (3,7). Antibody targeted quantum dots have been used to label the Her2 receptor on the surface of SK-BR-3 breast cancer cells in culture and in fixed mouse mammary tissue sections (6). Peptides conjugated to quantum dots have been used *in vivo* to target the lungs, blood vessels, and lymphatic vessels in mice (7). Fluorescent dyes conjugated to monoclonal antibodies have been used to label the epidermal growth factor receptor (EGFR) on the surface of SiHa cervical cancer cells and in *ex vivo* human oral cavity biopsies (8,9). Gold nanoparticles and nanoshells have also been used to target surface receptors in cell lines and *ex vivo* tissues. Nanoshells targeted with anti-Her2 have been used to label SK-BR-3 breast cancer cells in culture (10), and gold nanoparticles conjugated to EGFR antibodies have been used to target EGFR on the surface of SiHa cervical cancer cells and in human cervical cancer biopsies (11).

In order to translate the potential of these contrast agents closer to clinical application, it is necessary to develop imaging systems that provide images of both the distribution of the contrast agent as well as the tissue architecture and cellular morphology. This tandem approach enables images of contrast agent distribution to be placed in a histologically relevant context. Real-time optical imaging techniques, such as confocal microscopy (CM), have the ability to image tissue architecture with sub-cellular resolution in intact tissues at depths of up to several hundred micrometers beneath the tissue surface. For example, reflectance CM has been used to study the structure and morphology of the uterine cervix (12), skin (13–16), and oral cavity (17,18). Morphological features, such as nuclear size, nuclear density, and the nuclear-to-cytoplasmic ratio, which can be evaluated with reflectance CM, have been used to discriminate

between normal, precancerous, and cancerous tissues of the skin (19) and cervix (12). Confocal microscopy can also be used to image the distribution of molecular-specific optical contrast agents within tissue. Recently, the visualization of EGFR expression in *ex vivo* tissues of the oral cavity (9) and cervix (11) has been shown using CM and optically active contrast agents.

The goal of this study was to analyze the ability of dual mode reflectance and fluorescence confocal microscopy to image both the molecular and morphologic features of neoplasia in the oral cavity. Reflectance confocal microscopy was used after the application of 6% acetic acid; images were analyzed to derive the nuclear-to-cytoplasmic ratio. Fluorescence confocal microscopy was used after the application of an anti-EGFR targeted fluorophore; images were analyzed to assess the mean fluorescence labeling intensity. Results show that the morphologic and molecular information, which can be derived from optical molecular imaging, can be used to identify the presence of oral neoplasia.

Materials And Methods

Contrast Agents

A molecular-specific fluorescent contrast agent was used to target the epidermal growth factor receptor. This contrast agent consisted of an anti-EGFR antibody conjugated to a fluorescent dye. The antibody used was a mouse monoclonal anti-human EGFR antibody (clone 225, 1.8 mg/mL, Sigma-Aldrich, St. Louis, MO). It was directly conjugated to Alexa Fluor® 488 dye using a monoclonal antibody labeling kit (Molecular Probes, Eugene, OR). Conjugation of approximately 4 moles of dye per mole of protein was achieved for each 100 µg of protein labeled. The degree of dye conjugated was determined using absorption spectroscopy, following the manufacturer's instructions within the labeling kit. For tissue labeling, the contrast agent was used at a dilution of 0.02 mg/mL in a solution of 10% bovine serum albumin (BSA) in 1x phosphate buffered saline (PBS), with 10% dimethyl sulfoxide (DMSO).

A mouse IgG antibody of the same isotype as the EGFR antibody (1.0 mg/mL, Invitrogen, Carlsbad CA) was used as a control. The IgG antibody was also conjugated to Alexa Fluor® 488 dye using the antibody labeling kit and used at a concentration of 0.02 mg/mL, diluted in a solution of 10% BSA and 1x PBS and 10% DMSO.

Reflectance contrast was enhanced by application of 6% acetic acid solution. Weak acetic acid has been shown to increase the backscattered signal from cell nuclei (20,21).

Fresh Tissue Slices and Labeling

Clinically normal and abnormal mucosal biopsy pairs were obtained from consenting patients at The University of Texas M. D. Anderson Cancer Center. The Institutional Review Boards at The University of Texas M. D. Anderson Cancer Center, The University of Texas at Austin, and Rice University approved the clinical protocols. Immediately following removal, tissue biopsies were immersed in chilled phenol red-free culture media (Dulbecco's modified essential media, Invitrogen, Carlsbad, CA). Tissues were sectioned into 250 µm-thick transverse slices using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL). Tissues were sliced in 1x PBS and were placed in phenol red-free media.

Two slices per biopsy were blocked with a solution of 10% BSA in 1x PBS on ice for 30–45 minutes. After removing the blocking solution, 0.5 mL of the EGFR contrast agent was added to one slice and 0.5 mL of the IgG control solution was added to the second slice. The slices were incubated for 45 minutes on ice on a shaker plate, washed twice with 1x PBS for 5 minutes with constant shaking, and were imaged in 1x PBS. A third, unlabeled slice was imaged immersed in 6% acetic acid solution.

Imaging

Confocal images were obtained at 15 frames per second using the dual-mode reflectance and fluorescence confocal microscope (DCM), described in detail in (22). Briefly, the DCM is equipped with an argon laser at 488 nm with an emission bandpass filter allowing 500–550 nm fluorescence to be detected. It is also equipped with a 784 nm laser diode and a near infrared 50/50 beam splitter used for reflectance imaging. Images were acquired with a 40× magnification, water-immersion objective with a numerical aperture of 0.80. For reflectance imaging, the laser power incident on the sample was approximately 7.8 mW, a 20 µm-diameter pinhole was in the detection path, and a supply voltage of 370 V yielded a detector gain of approximately 5×10^2 . For fluorescence imaging, the laser power incident on the sample was approximately 200 µW, a 100 µm-diameter pinhole was in the detection path, and a supply voltage of 700 V yielded a gain of approximately 1.4×10^5 . The field of view of the system was 300×250 µm, and image tiling was performed so that images were captured covering the entire biopsy slice, typically several millimeters across.

Histology

After imaging, all tissue slices were fixed in 10% buffered formalin and paraffin embedded. Hematoxylin and eosin (H&E) stained sections were made from each tissue slice that was imaged. EGFR immunohistochemistry (IHC) stained sections, counter stained with hematoxylin to define the nuclei, were also prepared from the unlabeled slice that was imaged as well as an additional slice from the biopsy. The stained sections were examined by an experienced head and neck specialized pathologist for standard histopathology assessment. A single pathologist (AKE) analyzed the majority of the histology sections; however, a subset of the slides was analyzed by a second pathologist (MDW). In cases where both pathologists reviewed the same slide, there was good agreement between the diagnoses given by the two pathologists. In two cases, the level of severity of the diagnosed carcinoma differed by one category between the two pathologists; one pathologist diagnosed the section as moderately-differentiated carcinoma while the other gave a diagnosis of poorly-differentiated carcinoma. When this occurred, the more severe diagnosis was used. The following morphological classifications were used: normal squamous epithelium, hyperplasia, hyperkeratosis, dysplasia (mild, moderate, or severe dysplasia) with and without hyperkeratosis, and invasive carcinoma (well-, moderately-, or poorly-differentiated). For the purpose of the analysis, diagnoses of non-neoplastic hyperplasia and hyperkeratosis were considered normal.

Image Processing

Images were resized in the horizontal direction to account for the non-linear scan of the resonant mirror, as described in (22). The distortion in the images was corrected through pixel re-sampling, post-image acquisition, using Matlab (The MathWorks, Inc., Natick, MA). Individual images were built into a mosaic image using a macro written for Image J (NIH, Bethesda, MD), and the mosaic was saved as a large bitmap file. Photoshop CS2 (Adobe, San Jose, CA) was then used to select the region of the epithelium in each of the images. The epithelium was traced by hand in each image, and the selections were checked and verified by a board-certified pathologist (AKE). For the fluorescence images, a corresponding reflectance image was used to define the region of the epithelium. For the carcinoma samples, areas of neoplastic epithelial cells were selected. These selected regions were then used to create a mask so that only regions of epithelial cells were further analyzed. Two samples contained regions of submucosal carcinoma with overlying normal or mildly dysplastic epithelium. For analysis of these samples, the images were separated into the two histologically distinct regions and analyzed independently.

The fluorescence images obtained from the contrast agent-labeled tissue slice were used to determine the mean EGFR fluorescence labeling intensity (FLI) within the entire epithelium,

or in regions of neoplastic epithelial cells in the case of invasive carcinomas. The mean was calculated using Matlab code (The MathWorks, Inc, Natick, MA). The FLI was then used to determine if there was a correlation between the fluorescence labeling intensity and the histologic diagnosis.

The reflectance images, taken after the application of 6% acetic acid, were used to determine the nuclear-to-cytoplasmic (N/C) ratio in the superficial epithelium. The surface 100 μm of the tissue slice was cropped from the reflectance image mosaic and saved as a separate bitmap image. These images were then analyzed using Matlab code as described in detail in (23,24). Briefly, anisotropic diffusion was used to filter the image, and then Gaussian Markov random fields were used to segment the image. A threshold was then applied to separate nuclei from the background. The thresholded image was then filtered further to remove particles that would likely be too large or too small to be cell nuclei. The resulting image was then corrected further by hand (by ALC) to include nuclei that the image segmentation code missed and to remove particles that should not have been defined as nuclei. The person hand correcting the image was blinded to the histology classification of the sample. Other than cropping the desired region of interest, the confocal images were not altered further prior to processing. It was found that altering the brightness and/or contrast of the images did not improve the ability of the code to detect nuclei within the images.

Once the final, hand-corrected mask of the nuclei was obtained, the number of pixels occupied by the nuclei were counted and compared to the number of pixels in the entire region of interest (ROI). Everything within the ROI not defined as nuclei was considered to be cytoplasm. Comparing the number of pixels occupied by nuclei to the number of pixels within the ROI, therefore, was considered to give the N/C ratio. It is possible that the region of cytoplasm was overestimated using this method because there may have been other components within the ROI other than nuclei and cytoplasm, such as cell membranes. However, due to the difficulty of distinguishing the cytoplasmic area, this estimation was used.

Results

Overview of Patients

A total of 33 biopsies were obtained from 14 patients. Table I lists the anatomic location and the corresponding clinical impression and histologic diagnosis for each biopsy. Six of the clinically abnormal sites were found to be histologically normal and two of the clinically normal sites were determined to be histologically abnormal. One sample was considered histologically inevaluable, and was therefore excluded from further evaluation. When considering all samples diagnosed with any degree of dysplasia and carcinoma as abnormal, the sensitivity and specificity of visual recognition by the clinician were 86% and 67%, respectively. When considering only samples diagnosed with severe dysplasia and carcinoma as abnormal and all others as normal, the sensitivity and specificity of visual recognition by the clinician were 90% and 59%, respectively. Table II lists the number of biopsy samples per histologic diagnosis. For this study, there were 18 normal/non-neoplastic samples, five dysplastic samples, and nine carcinoma samples with two of those being submucosal carcinomas with overlying normal or mildly dysplastic epithelium.

Fluorescence Confocal Images

Figure 1 shows fluorescence confocal images of the clinically normal and abnormal biopsies from patient 1 stained with an anti-EGFR fluorophore, as well as corresponding H&E and EGFR IHC images for both. The clinically normal biopsy was obtained from the right side of the tongue and was diagnosed as normal with mild hyperkeratosis; the clinically abnormal biopsy was obtained from the left region of the floor of mouth and was diagnosed as invasive,

moderately-differentiated carcinoma. Mosaic images of the majority of each slice are shown in Figures 1a and 1e. Fluorescent staining is seen in the majority of the epithelial cells of the abnormal biopsy (Fig. 1e). However, in the normal biopsy (Fig. 1a), fluorescent labeling is limited only to the basal cells near the basement membrane, underscored by the white line, but is not seen in the superficial epithelium. Collagen autofluorescence can be seen below the basement membrane in Figure 1a, but endogenous cellular autofluorescence cannot be detected due to the low intensity of the autofluorescence signal and the near video-rate image acquisition speed which reduces the dwell time on each pixel. Fluorescence images were also taken of an unlabeled slice to ensure that no cellular autofluorescence could be detected from the epithelium of the biopsy samples (data not shown). Subsections of images 1a and 1e are shown in Figures 1b and 1f to highlight the labeling at the cell membranes, where the EGF receptors are located. The corresponding H&E stained sections are shown in Figures 1c and 1g, and EGFR IHC stained sections are shown in Figures 1d and 1h for the normal and abnormal biopsies, respectively. EGF receptors stain brown in IHC images. The IHC images show staining only at the basement membrane of the normal sample and increased labeling throughout the abnormal sample, corresponding well to the labeling that was seen in the confocal images.

Figure 2 shows fluorescence confocal, H&E, and EGFR IHC images for the clinically abnormal biopsy from patient 9, which was diagnosed as invasive, poorly-differentiated carcinoma with overlying focal mild dysplasia. In the overlying epithelium of Figure 2a, EGFR labeling can be seen near the basement membrane, in the lower third of the epithelium, corresponding to the area of mild dysplasia. However, the EGFR labeling of the underlying carcinoma is substantially brighter. Figure 2b shows a fluorescence image of an unlabeled slice to show that the signal below the basement membrane is indeed EGFR labeling and not autofluorescence from collagen. The corresponding EGFR IHC stained image (Fig. 2d) and the magnified image (Fig. 2e) show increased staining in the region of invasive cancer within the stroma compared to the overlying epithelium, corresponding very well to the fluorescence labeling intensity seen in the confocal images.

Mean Fluorescence Intensity of EGFR Labeled Epithelium

Figure 3a shows a plot of the mean fluorescence labeling intensity of the EGFR labeled epithelium for each specimen. Patient 11 was omitted because there was only one tissue slice available per biopsy, which was used for reflectance imaging, so no fluorescence labeling images were acquired. The clinically abnormal sample from patient 12 was also omitted because it was defined as histologically inevaluable, and therefore was excluded from further evaluation. The FLI of the histologically abnormal sample was generally greater than the FLI of the paired histologically normal sample for most patients, although the magnitude of the intensity increase differed between patients. The normal and mild dysplasia samples can be separated from the severe dysplasia and carcinoma samples at an FLI of 17, with the exception of the samples from patient 4 and one of the samples from patient 13. Figure 3c shows the sensitivity and specificity of the fluorescence labeling intensity as a Receiver Operating Characteristic (ROC) curve when separating severe dysplasia and carcinoma samples from normal and mildly dysplastic samples. The true positive rate (sensitivity) was plotted as a function of the false positive rate (1-specificity) for different FLI values ranging from zero to 67. At an FLI value of 17, the sensitivity and specificity were 1.00 and 0.86, respectively, when considering only samples diagnosed with severe dysplasia and carcinoma as abnormal. When separating mild dysplasia, severe dysplasia, and carcinoma from normal samples, an FLI of 17 yielded a sensitivity of 0.77 and a specificity of 0.84 (data not shown). Figure 3b shows the FLI for each patient also as a function of tissue type. Interestingly, the three normal samples that yielded higher fluorescence intensities were all from the gingival (indicated by arrows in Fig. 3b).

Figure 4 shows the fluorescence confocal image and corresponding H&E and EGFR IHC images for one of the gingiva biopsies from patient 4. The sample was diagnosed as normal with hyperplasia and hyperkeratosis, but yielded a high mean fluorescence labeling intensity. The fluorescence confocal image (Fig. 4a) shows EGFR labeling throughout the epithelium. The EGFR IHC stained image (Fig. 4c) shows staining throughout the epithelium with darker staining concentrated at the basement membrane. EGFR IHC staining was also seen throughout the epithelium of the other normal sample from patient 4, which was also from the gingiva (data not shown).

To reduce the impact of patient-to-patient variability in EGFR expression and labeling, the ratio of the FLI of the clinically abnormal sample to the clinically and histologically normal sample FLI was calculated for each patient. In cases where the clinically abnormal biopsy was diagnosed as normal, the ratio relative to the clinically and histologically normal biopsy was still calculated.

The ratio is plotted in Figure 5a for each patient; Figure 5b shows the average ratio as a function of the histological grade of the clinically abnormal sample. Patient 10 was excluded from this analysis due to the lack of a clinically and histologically normal sample for comparison. Patient 12 was also excluded because the clinically abnormal sample was histologically inevaluable. In general, the FLI ratio calculated for the carcinoma samples was greater than that of the normal or mild dysplasia samples. The ratio of each of the carcinoma samples ranged between 2.66 and 11.18, with an average ratio of 5.09 ± 2.78 . The ratio of the mild dysplasia and normal samples were all less two, except for one clinically abnormal, histologically normal sample from patient 13, taken from the gingiva, which yielded a ratio of 3.14. The average ratio of the mild dysplasia and normal samples was 1.36 ± 0.78 . We rejected the null hypothesis that the FLI ratio of samples diagnosed as carcinoma was equal to the ratio of samples diagnosed as normal and mild dysplasia. Using the Wilcoxon rank sum test, a significant difference was seen between these two groups (p-value < 0.01).

Reflectance Confocal Images

Figure 6 shows reflectance confocal images of tissue slices after the application of 6% acetic acid, as well as the corresponding H&E images, for both the clinically normal and abnormal biopsies from patient 1. The clinically normal biopsy was diagnosed as normal with mild hyperkeratosis and the clinically abnormal biopsy was diagnosed as invasive, moderately-differentiated carcinoma. The white circular features in the epithelial portion of Figure 6a, the region above the white line denoting the basement membrane, and all of Figure 6c are epithelial nuclei. The organization and differentiation expected of normal stratified squamous epithelium can be seen in Figure 6a. The structure of the tissue shows an ordered arrangement of cells and nuclei in all levels of the epithelium. The basal cells are small and orderly polarized along the basement membrane, underscored by the white line in the image. As the cells differentiate and move upward to the surface of the epithelium, the nuclear density and the nuclear-to-cytoplasmic ratio decrease. In contrast, Figure 6c shows the loss of this structure and organization that occurs with carcinoma. In this image, there is no defined structure and the cells appear to be disorganized and randomly distributed throughout the tissue section. In some regions of the image, nuclei are spaced very closely together and in other regions they appear to be separated by matrix. Figures 6b and 6d show the corresponding H&E images. The tissue structure and cell density assessed by confocal microscopy compares well with histology.

A reflectance confocal image taken after the application of 6% acetic acid and an H&E image for the clinically abnormal biopsy from patient 9, which was diagnosed as invasive, poorly-differentiated carcinoma with overlying focal mild dysplasia, are shown in Figure 7. The basement membrane is underscored by a white line in the confocal image (Fig. 7a). Above the basement membrane, the stratified structure of the epithelium can be seen. There is a slight

increase in the nuclear density within the bottom portion of the epithelium, corresponding to the area of mild dysplasia in the overlying epithelium. Beneath the basement membrane, an area packed with large, disorganized nuclei with an obvious increase in nuclear density and N/C ratio representative of invasive carcinoma is noted. Again, the confocal image corresponds well to the histology image.

Mean Reflectance Intensity of Epithelium

The mean reflectance intensity (RI) of the epithelium was determined for each sample. The RI did not provide separation of the histologically normal and abnormal samples. However, it is interesting to note that for 75% of the samples, the regions that were collected as clinically abnormal yielded a higher RI than the corresponding clinically normal sample taken from the same patient (data not shown).

Nuclear-to-Cytoplasmic Ratio

Figure 8a shows a plot of the nuclear-to-cytoplasmic ratio calculated from the reflectance confocal images for each patient. Patient 6 does not have a normal sample because the slice imaged with reflectance lacked epithelium. Patient 12 does not have an abnormal sample because it was histologically inevaluable and was omitted from further analysis. An N/C ratio of 0.11 can be used to separate the histologically normal and mildly dysplastic samples from the carcinoma samples. The single severe dysplasia sample, from patient 10, yielded an N/C ratio very close to zero because the surface of the tissue lacked proper orientation and was highly hyperkeratotic with very few identifiable nuclei.

The ROC curve for the nuclear-to-cytoplasmic ratio is shown in Figure 8b. The true positive rate (sensitivity) was plotted as a function of the false positive rate (1-specificity) for different N/C ratio values ranging from zero to 0.405. Classifying only samples diagnosed with severe dysplasia and carcinoma as abnormal yielded sensitivity and specificity values of 0.90 and 1.00, respectively, at an N/C ratio of 0.11. When separating mild dysplasia, severe dysplasia, and carcinoma from normal samples, an N/C ratio of 0.11 yielded a sensitivity of 0.60 and a specificity of 1.00 (data not shown).

Using the data in Figure 8a, the ratio of the clinically abnormal biopsy N/C ratio to the clinically and histologically normal biopsy N/C ratio was calculated for each patient. In cases where the clinically abnormal biopsy was diagnosed as normal, the ratio relative to the clinically and histologically normal biopsy was still calculated. The ratio was not calculated for patients 6, 10, or 11 because there was not a clinically and histologically normal sample from the same patient for comparison. Patient 12 was also excluded due to the lack of a histologically evaluable abnormal sample. The ratio is plotted for each patient in Figure 8c, and Figure 8d shows the average ratio as a function of the histological grade of the clinically abnormal sample. The ratio calculated for the carcinoma samples is greater than that of the normal or mild dysplasia samples. The abnormal-to-normal N/C ratios for the carcinoma samples are all greater than 7.47, except for one case of moderately-differentiated carcinoma (patient 1). This sample contained marked keratinization of the cancer cells, as can be seen in Figure 6d. This increased the region of the image defined as cytoplasm, and thus decreased the N/C ratio calculated for this image. The ratios of the carcinoma samples to their corresponding normal samples ranged from 7.47 to 25.79, with a mean of 14.42 ± 8.88 . The ratio of the mild dysplasia and normal samples were all less than two, except for one clinically abnormal, histologically normal sample from patient 14, which yielded a ratio of 4.92. The average ratio of the mild dysplasia and normal samples was 1.34 ± 1.40 . Using the Wilcoxon rank sum test, a significant difference was calculated between the ratio for the carcinoma samples and the ratio of the normal and mild dysplasia samples (p -value < 0.01).

Comparison of Mean FLI and N/C Ratio

Figure 9a shows a plot of the fluorescence labeling intensity versus the nuclear-to-cytoplasmic ratio derived for the same biopsy. Using these two parameters, there is fairly good separation of the carcinoma samples from the normal and mild dysplasia samples. The carcinoma samples are characterized by higher FLI and N/C ratio, as expected. Note that the normal gingiva samples show higher mean FLI, but lower N/C ratio. Figure 9b shows the points from the ROC curves calculated from the combination of the FLI and the N/C ratio when *either* the FLI was higher than the FLI cutoff value *or* the N/C ratio was higher than the N/C ratio cutoff value. Figure 9c shows the points from the ROC curves based on the combination of the FLI and the N/C ratio when *both* the FLI was higher than the FLI cutoff value *and* the N/C ratio was higher than the N/C ratio cutoff value. The sensitivity and specificity calculated using an FLI of 17 and an N/C ratio of 0.11, separating severe dysplasia and carcinoma from normal and mild dysplasia, were 1.00 and 0.85, respectively, for the case where *either* the FLI *or* the N/C ratio was greater than the cutoff, and 0.90 and 1.00, respectively, for the case where *both* the FLI *and* N/C ratio were greater than the cutoff. Similarly, a sensitivity and specificity of 0.77 and 0.82, respectively, were calculated for separating mild dysplasia, severe dysplasia, and carcinoma from normal samples for the case where *either* the FLI *or* the N/C ratio was greater than the cutoff and 0.69 and 1.00, respectively, for the case where *both* the FLI *and* N/C ratio were greater than the cutoff (data not shown).

Discussion

Various exogenous contrast agents have been used to enhance the native contrast observed in tissue. Studies have employed these contrast agents to gain a greater understanding of physiological and molecular processes occurring within tissue, as well as to create more sensitive methods for detecting and classifying neoplastic lesions. The two contrast agents used in this study were a 6% acetic acid solution, which is used routinely in the clinic to cause aceto-whitening of precancerous areas (25,26), and a molecular-specific fluorescent contrast agent targeting the epidermal growth factor receptor. The goals of this study were to obtain high-resolution molecular and morphologic images of normal and neoplastic tissues and to use these images to separate severely dysplastic and carcinoma samples from normal and mildly dysplastic samples, and to determine whether better separation could be achieved on the basis of morphologic features, molecular features, or a combination of both. Although we have focused on oral cancer within this analysis, similar clinical applications may be found in other malignancies, such as breast, cervical, and lung cancers.

A mean fluorescence labeling intensity value of 17 separated the severe dysplasia and carcinoma samples from the normal and mild dysplasia samples with a sensitivity of 1.00 and specificity of 0.86. We found that the ratio of the FLI of the clinically abnormal to normal biopsy was greater than 2.66 for the carcinoma samples and less than two for all but one of the normal and mild dysplasia samples. In this study, we did not observe a definitive increase in fluorescence signal with increasing severity of diagnosis. Hsu *et al.*, however, did find that the mean fluorescence intensity ratio of paired abnormal to normal tissue increased as the grade of the dysplasia and carcinoma increased (9).

Separation of carcinoma samples from normal and mild dysplasia samples was also achieved using a nuclear-to-cytoplasmic area ratio of 0.11 with a sensitivity and specificity of 0.90 and 1.00, respectively. A similar study of the nuclear-to-cytoplasmic areas has not been conducted previously in the oral cavity; however, a number of similar studies have been conducted for cervical tissue. Analyzing images taken parallel to the surface of cervical biopsies, Collier *et al.* found that an N/C ratio of 0.08 separated normal and mild dysplasia samples from moderate and severe dysplasia samples with high sensitivity and specificity. The measured N/C ratios for the moderate and severe dysplasia samples ranged between 0.10 and 0.22, with an average

of 0.14 (12). The difference between the current study and the study conducted by Collier *et al.* is that the images analyzed in this study were of transverse slices, taken perpendicular to the tissue surface. Walker *et al.* analyzed the N/C ratio of cervical epithelial nuclei in transverse histology sections. The epithelium was divided into four layers and the N/C ratio was analyzed for each layer. They found an average N/C ratio of 0.14 and 0.01 for the surface layers of severe dysplasia and normal epithelium, respectively. They also referenced three previous studies that yielded average N/C ratios between 0.27 and 0.39 for the surface layers of severely dysplastic lesions, while normal epithelium yielded N/C ratios between 0.04 and 0.22 (27). These N/C ratios correspond well to the N/C ratios calculated in this study of normal oral cavity tissue (ranged from 0.01 to 0.11) and oral carcinoma (ranged from 0.11 to 0.40).

The dual imaging modes used in this study provided images similar to histology H&E and immunohistochemistry staining, but from thicker slices of fresh tissue. The reflectance images provided information about both the architecture of the tissue as well as the cellular morphology while the fluorescence images yielded information about the expression level of EGFR. These images were acquired with near video-rate image acquisition, showing that detection of fluorescence from a molecular-specific contrast agent is possible at high scanning speeds, which yields promise for *in vivo* imaging applications that may be able to optically provide real-time H&E and IHC quality images for detection and diagnosis of disease.

For *in vivo* clinical application, safety issues regarding targeted contrast agents must be addressed, contrast agent formulations that will penetrate throughout the depth of interest will need to be developed, and limitations in confocal imaging must be overcome. The basement membranes within these images were outlined for visualization; however, it is more difficult to visualize the basement membrane when acquiring confocal images *in vivo*. In this study, transverse slices were imaged to allow comparison to traditional H&E stained sections; however, images acquired *in vivo* will be acquired *en face*, or parallel to the surface of the tissue. Therefore, defining the nuclear density at different depths of the tissue is critical to define areas of abnormality and to visualize tumor invasion. Studies of this feature are being conducted in cervical tissue and skin (12,28–31). Submucosal carcinomas with overlying epithelium present an additional challenge. These types of lesions might not be detected *in vivo* due to the limited penetration depth of confocal microscopy. However, surveying an area of tissue or pairing confocal microscopy with a macroscopic imaging system may improve the selection of areas to be analyzed microscopically with confocal imaging.

Quantitative analysis of the confocal images in this study yielded high sensitivity and specificity values for separating normal and mildly dysplastic samples from severely dysplastic and carcinoma samples, which is an important clinical distinction. A fluorescence labeling intensity of 17 yielded a sensitivity of 1.00 and a specificity of 0.86. A nuclear-to-cytoplasmic ratio value of 0.11 yielded a sensitivity of 0.90 and a specificity of 1.00. Combining the two modalities so that either value being higher than the cutoff classified the sample as abnormal (combined “or” analysis) yielded a sensitivity of 1.00 and a specificity of 0.85. Combining the two modalities so that a sample was classified as abnormal only when both values were higher than the cutoff (combined “and” analysis) yielded a sensitivity of 0.90 and a specificity of 1.00. The FLI and the combined “or” analysis performed similarly and yielded a higher true positive rate than the N/C ratio or the combined “and” analysis. Additionally, the FLI, N/C ratio, and the combination of the two yielded higher sensitivities and specificities than those achieved through visual clinical inspection (0.90 and 0.59, respectively). The separation between normal and mildly dysplastic samples and severe dysplasia and carcinoma is a clinically relevant distinction. In the clinic, the more severe diagnoses will lead to surgical excision and the lower levels of dysplasia will be clinically monitored. Therefore, having a more sensitive test is desired clinically so that no severe dysplasia or carcinoma is missed. In this pilot study, both the FLI and the combined analysis of the FLI and N/C ratio appear to improve the ability to

distinguish carcinoma samples from normal epithelium. A larger study with more moderate and severe dysplasia specimens is needed to definitively determine whether better separation can be achieved by FLI or by N/C ratio or by the combination of both.

Additionally, the information gained with fluorescence imaging of targeted contrast agents may add complementary information to that gained from the morphology of the tissue. For example, knowing the expression level of receptors such as Her2/neu for breast cancer could help to direct treatment of such lesions (32–35). This information cannot be gained from reflectance imaging of the morphology of the tissue. This study demonstrates that use of a targeted optically active contrast agent and combined imaging of both the contrast agent labeling and the acetic acid induced nuclear contrast has the potential to yield valuable clinical advantages for noninvasive early detection and molecular characterization of tissue. Additionally, an important application that could be quickly translated into clinical practice is the use of this technology for more rapid assessment of diagnostic biopsies. Thus molecular information, such as EGFR or Her2/neu expression to assist in identification of micrometastases in sentinel lymph nodes, for example, could be obtained quickly for use in surgical decision-making, rather than having to wait several days for this data using currently available IHC protocols.

Abbreviations

BSA, Bovine serum albumin; CM, Confocal microscopy; DCM, Dual-mode confocal microscope; DMSO, Dimethyl sulfoxide; EGFR, Epidermal growth factor receptor; FLI, Fluorescence labeling intensity; H&E, Hematoxylin and eosin; IHC, Immunohistochemistry; N/C, Nuclear-to-cytoplasmic; PBS, Phosphate buffered saline..

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Figure 1.
 (a, b, e, f) Confocal image mosaics, (c, g) H&E, and (d, h) EGFR IHC images of a normal (a, b, c, d) and abnormal (e, f, g, h) tissue slice from patient 1. The confocal fluorescence images were taken after labeling the tissue with a fluorescent contrast agent targeting EGFR. White lines in the confocal images underscore the basement membrane in the normal sample. Scale bars are 200 μ m.

Figure 2.

(a, b) Confocal image mosaics, (c) H&E, and (d, e) EGFR IHC images of an abnormal tissue slice from patient 9 diagnosed as poorly-differentiated carcinoma with overlying mild dysplasia. Confocal fluorescence image (a) was taken after labeling the tissue with a fluorescent contrast agent targeting EGFR. Confocal fluorescence image (b) was taken of an unlabeled tissue slice using the same settings to show that no autofluorescence was detected, and that the signal detected in image (a) below the basement membrane (indicated by the white line) was EGFR labeling of the underlying tumor. Scale bars are 200 μm for images (a, b, e) and 1 mm for images (c, d).

Figure 3.

(a) Plot of the mean fluorescence intensity of the EGFR labeled epithelium for each biopsy, as a function of histologic diagnosis and (b) tissue type. Arrows point to gingiva samples with high fluorescence labeling intensity. (c) ROC curve showing the true positive and false positive rates for different fluorescence labeling intensity values separating severe dysplasia and carcinoma from normal and mild dysplasia.

Figure 4. (a) Confocal fluorescence, (b) H&E, and (c) EGFR IHC images of a tissue slice from patient 4, from the gingiva, diagnosed as normal with hyperplasia and hyperkeratosis. The confocal fluorescence image was taken after labeling the tissue with a fluorescent contrast agent targeting EGFR. The white line underscores the basement membrane. Scale bars are 200 μm .

Figure 5.

(a) Plot of the ratio of the fluorescence labeling intensity of the histologically abnormal biopsy to the clinically and histologically normal biopsy fluorescence labeling intensity for each patient. (b) Chart of the mean ratio as a function of the histology diagnosis of the abnormal biopsy.

Figure 6.

(**a, c**) Confocal reflectance and (**b, d**) H&E images of tissue slices from patient 1. Confocal images were taken after applying 6% acetic acid to the tissue slices. The white line in image (**a**) defines the basement membrane. White circular structures in the confocal images are cell nuclei. Scale bars are 200 μm .

Figure 7. (a) Confocal reflectance and (b) H&E images of an abnormal tissue slice from patient 9 diagnosed as poorly-differentiated carcinoma with overlying mild dysplasia. The confocal image was taken after applying 6% acetic acid to the tissue slice. The white line in image (a) defines the basement membrane, and separates the overlying epithelium from the underlying carcinoma. Scale bars are 1 mm.

Figure 8.

(a) Plot of the nuclear-to-cytoplasmic ratio for each biopsy as a function of histologic diagnosis. (b) ROC curve showing the true positive and false positive rates for different nuclear-to-cytoplasmic ratio values separating severe dysplasia and carcinoma from normal and mild dysplasia. (c) Plot of the ratio of the nuclear-to-cytoplasmic ratio of the histologically abnormal biopsy to the clinically and histologically normal biopsy for each patient. (d) Chart of the mean ratio as a function of the histology diagnosis of the abnormal biopsy.

Figure 9.

(a) Plot of the nuclear-to-cytoplasmic ratio as a function of the mean fluorescence labeling intensity for each biopsy. (b) Points from ROC curves calculated from the combination of the fluorescence labeling intensity and the nuclear-to-cytoplasmic ratio such that when *either* value was higher than the cutoff value the sample was classified as abnormal. (c) Points from ROC curves calculated from the combination of the fluorescence labeling intensity and the nuclear-to-cytoplasmic ratio such that only when *both* values were higher than the cutoff values the sample was classified as abnormal. Sensitivity and specificity values were determined based on the separation of severe dysplasia and carcinoma from normal and mild dysplasia.

Table 1

Overview of the biopsy sites obtained from each patient and the corresponding histology diagnosis.

Patient	Clinically Normal	Location Clinically Abnormal	Clinically Abnormal	Clinically Normal	Clinically Abnormal	Histology Diagnosis	Clinically Abnormal
1	R Tongue	L Tongue	L FOM	normal, mild HK	normal	invasive, moderately-differentiated cancer	
2	L Buccal	R Post Buccal		normal, HP, HK	mild dysplasia, HP, HK		
3	L Tongue	R Tongue		normal, HK	invasive, well-differentiated cancer		
4	L Gingiva	R Gingiva		normal, HP, HK	normal, HP, HK		
5	L Tongue	R Tongue		normal, HP, HK	normal, HP, HK		
6	L Buccal	Retromolar Trigon		normal, HP, HK	well-differentiated cancer		
7	L Vent Tongue	R Vent Tongue	R Vent Tongue	normal, HP, HK	invasive, moderately-differentiated cancer, overlying normal, HP, HK	invasive, moderately-differentiated cancer	
8	L Tongue	R Ant Tongue	R Post Tongue	normal, HP, HK	normal, HK	focal mild dysplasia, HP, HK	
9	R Buccal	L Buccal	L Buccal	normal, HK	invasive, poorly-differentiated cancer, overlying focal mild dysplasia, HP, severe HK	invasive, poorly-differentiated cancer	
10	Central Lip	R Lip		severe dysplasia, HK	well-differentiated cancer		
11	R Buccal	L Buccal		mild dysplasia, HK	mild dysplasia, HP, HK		
12	R Palate	Soft Palate		normal, HK	inevaluable*		
13	L Gingiva	R Ant Gingiva	R Post Gingiva	normal, HP, HK	normal, HP, HK	well-differentiated cancer	
14	R Gingiva	L Gingiva		normal, HK	normal, HK		

* Biopsy was defined as histologically inevaluable when there was no epithelium present in the sample and a diagnosis could not be made.

Abbreviations used: L, Left; R, Right; Ant, Anterior; Post, Posterior; Vent, Ventral; FOM, Floor of mouth; HK, Hyperkeratosis; HP, Hyperplasia.

Table II

Overview of the number of biopsy samples per histology diagnosis.

Histology Diagnosis	Number of Samples
Normal	1
Normal with hyperkeratosis and/or hyperplasia	17
Dysplasia	
Mild	4
Moderate	0
Severe	1
Carcinoma	
Well-differentiated	4
Moderately-differentiated	2
Poorly-differentiated	1
Moderately-differentiated carcinoma with overlying hyperplasia / hyperkeratosis	1
Poorly-differentiated carcinoma with overlying mild dysplasia	1