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TRIOLOGICAL SOCIETY CANDIDATE THESIS

Operative Margin Control With High-Resolution Optical Microendoscopy for Head and Neck Squamous Cell Carcinoma

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Objectives/Hypothesis: High-resolution microendoscopy (HRME) provides real-time visualization of the mucosal surface in the upper aerodigestive tract. This technology allows noninvasive discrimination of benign and neoplastic epithelium and has potential applications for intraoperative margin detection.

Study Design: Single institution, prospective, feasibility trial (phase I) of in vivo optical imaging.

Methods: The study was conducted on patients with squamous cell carcinoma of the upper aerodigestive tract. High-resolution microendoscopy images obtained during surgery were correlated with histopathologic diagnosis to determine the ability of HRME to differentiate between benign and malignant mucosa. Blinded reviewers evaluated HRME images and made determinations of the status of the mucosa. Accuracy, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and interrater agreement between multiple raters were calculated to determine the accuracy of HRME imaging.

Results: The mean accuracy of reviewers in differentiating neoplastic or benign mucosa was 95.1% (95% confidence interval [CI], 94%–96 %). Sensitivity and specificity were 96% (95% CI, 94%–99 %) and 95% (95 % CI, 90%–99 %), respectively. The NPV was 98% (95% CI, 97%–99%), and PPV was 91% (95% CI, 85%–98%). The Fleiss kappa statistic for interrater reliability was 0.81, with a standard error of 0.014 and a 95% CI (0.78–0.84).

Conclusion: High-resolution microendoscopy allows real-time discrimination between benign and neoplastic mucosa. High levels of sensitivity and specificity can be obtained with this technology when interrogating mucosal surfaces. Despite several technical limitations, HRME shows promise as a technique for intraoperative margin control and platform for molecular imaging technologies.

Key Words: HRME, diagnostic, head and neck cancer, frozen section, surgical margins, squamous cell carcinoma.

Level of Evidence: 3b.

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INTRODUCTION

Oncologic surgical resection remains a foundation in the management of head and neck squamous cell carcinoma. One of the most widely applied concepts is the concept of an appropriate surgical margin, which is the normal tissue interface that is resected when oncologic surgery is performed. The actual definition of an appropriate surgical margin remains elusive, however, and varies by tumor histology, anatomic location, histologic criteria, and tradition. Unfortunately, appropriate margins are difficult to achieve, most notably due to the inability to visualize individual cancer cells during surgery and the heterogeneity in the biologic behavior of the malignant cells.[1,2] What is widely accepted is that malignant disease left behind after oncologic surgery leads to recurrent disease and poor outcomes.[3,4] Several investigations examining the impact of surgical margins have demonstrated poor oncologic outcomes associated with positive margins.[5] Obtaining negative margins remains an important tenet for improved oncologic outcomes, and failure to achieve negative margins constitutes has a significant impact on therapy and the patient's quality of life.[6–10]

Currently, the majority of surgeons worldwide rely on intraoperative frozen-section analysis of the margins in an attempt to obtain an adequate resection.[11–13] Although this technique is currently the gold standard in many institutions around the world, technical problems including errors in margin sampling, pathological processing and shrinkage, and interpretation errors have been reported.[13–19] Additionally, the concept that

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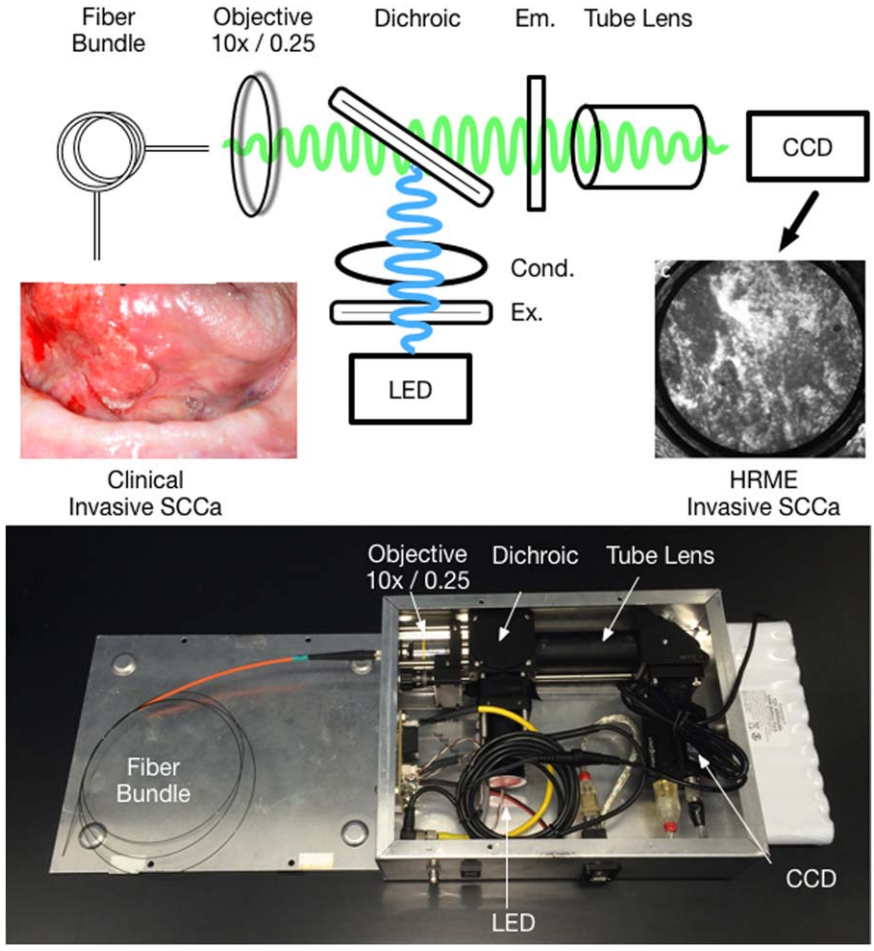

Fig. 1. The high-resolution microendoscope, shown in schematic (A) and fully assembled (B). Note that the associated computer tablet/viewing screen is not shown. CCD = Charge-coupled device; Cond = Condenser Lens; Ex = Excitation Filter; Em = Emission Filter; HRME = High Resolution Microendoscopy; LED = Light emitting diode; SCCa = squamous cell carcinoma.

malignant cells follow an organized radial growth pattern in all cases, allowing the surgeon to merely select the appropriate surgical margin and eradicate the disease, has been disproven.[20] In addition, genomic and molecular mechanisms in adjacent normal tissues from a histopathologic and morphologic standpoint may drive recurrence regardless of the margin status.[21] Malignant stem cells, or dormant tumor cells, may also contribute to local and regional recurrence, despite the surgeon's attempts at achieving an adequate resection margin.

Therefore, with the current knowledge regarding surgical margins and tumor biology, many surgeons accept the fact that in many situations the achievement of negative surgical margins does not equate to eradication of malignant disease. Furthermore, perhaps the most compelling finding is the recognition of the complexity of the molecular, genetic, epigenetic, and immune-related factors associated with squamous cell carcinoma. Thus, it would appear that for the near future, surgeons will continue to rely on surgical resection of malignant disease of the head and neck; therefore, novel technology allowing for improved oncologic control at the time of surgery is needed. High-resolution microendoscopy (HRME) is a fiber optic endomicroscopic technology that has been evaluated for mucosal interrogation in the field of head and neck oncology and in other specialties.[22–26] The objective of this investigation was to determine the accuracy of HRME for the discrimination of benign versus malignant mucosa in the upper aerodigestive tract. HRME is a novel imaging technology that utilizes a flexible fiber optic probe to obtain images of tissue in real time, allowing visualization of epithelial architecture and cellular morphology.[23,27–29] This technology may allow for a form of optical biopsy, which would allow real-time decision making with regard to surgical margins at the time of oncologic resection. The hypothesis of the investigation is that HRME is sufficiently accurate to discriminate benign mucosa from malignant disease in real time at the time of surgical resection.

MATERIALS AND METHODS

The study design was a single institution, prospective, in vivo clinical investigation in 33 adult patients with biopsy-proven squamous cell carcinoma of the head and neck who were to undergo surgical resection. Subjects with tumors amenable to surgical resection, imaging protocol, and control punch biopsies were consented for the study. All tumors were confirmed squamous cell carcinoma via biopsy prior to enrollment in the study. Patients were prospectively enrolled, and written informed consent was obtained from all study participants prior to surgery under an institutional review board-approved protocol.

Imaging System

Technical details of the HRME have been described in detail previously in the literature.[23,27] The HRME device

TABLE I.
Site, Subsite, and Obtained Images Associated With This Trial.

Site	No. Patients	Subsite	HRME Images	Anatomical Sites	Included in Test Set
Oral Cavity	23	Cancer	204	22	21
		Margin	127	21	11
		Ipsilateral normal	80	22	20
		Contralateral normal	71	19	18
Oropharynx	9	Cancer	81	8	8
		Margin	41	7	4
		Ipsilateral normal	36	7	7
		Contralateral normal	36	7	7
Hypopharynx/ Larynx	1	Cancer	5	1	1
		Margin	8	1	0
		Ipsilateral normal	3	1	0
		Contralateral normal	3	1	1
Total	33		695	117	98

HRME = high-resolution microendoscopy.

essentially operates as a fluorescence microscope coupled to a fiber optic imaging probe (Fig. 1). This probe consists of a 1-mm outer diameter fiber bundle (Fujikura, FIGH-30–850N, 1-5-1, Kiba, Kouto-ku, Tokyo 135-8512, Japan), which is comprised of 30,000 optical fibers. The cost of the device is less than $5,000, with a probe that can be sterilized and reused.[22] After topical application of a fluorescent contrast agent, the probe is placed on the mucosal surface, transmitting an image to a charged-coupled device connected to a tablet. A 0.01% solution of proflavine (Sigma-Aldrich, St Louis, MO) was used as the fluorescent contrast agent.[24,30] Proflavine has been tested and used extensively in Europe and Australia for in vivo studies of the gastrointestinal tract without any reported adverse events.[31,32]

Prior to resection, proflavine hemisulfate was applied to the visible margin of the tumor, as well as contralateral normal mucosa, by clinical examination to serve as controls. All sampled sites were from single tumors in individual subjects. Subsequently, HRME images were immediately acquired. Images were captured at 10 frames per second in real time (Table I). Correlative biopsies from each imaged site were obtained using a 4-mm punch biopsy at the site of image acquisition immediately after imaging, placed in formalin, and then submitted for histopathologic processing using standard criteria. Four biopsies were taken from each subject (Table I). The pathologist reviewing the biopsies was blinded to the corresponding HRME images. This allowed for comparison of the HRME-imaged epithelium to the histopathologic interpretation without investigator bias.

Image Quality Control

Selected images were then reviewed for quality control by a single observer who was blinded to the clinical impression and the histologic diagnosis, and were included if at least 50% of the frame contained nuclei and minimal motion artifact was present. Representative images were selected that correlated with each biopsy site. Images of benign and malignant mucosa were included in the analysis; images of necrotic debris, severe inflammation were excluded. Still images were excluded if the final histology showed hyperkeratosis or submucosal spread because these situations preclude high-quality HRME image interpretation (See Table II).

TABLE II.
Basis for Excluding Images From the Final Test Set.

Reason for Image Exclusion	No. Sites	% Sites Excluded (n = 19)	% Total Sites (n = 117)
Excessive artifact (> 50% of the frame without visible nuclei)	3	15.80	2.56
Oversaturation with proflavine	3	15.80	2.56
Hyperkeratosis	3	15.80	2.56
Submucosal spread	3	15.80	2.56
Motion artifact	2	10.50	1.70
Radiation changes	2	10.50	1.70
Dysplasia	2	10.50	1.70
Insufficient tissue for analysis	1	5.30	0.85
Total	19	100%	16.19%

Training and Test Sets

A training set of images was assembled from previous ex vivo data to illustrate HRME characteristics of normal and neoplastic tissue. In addition to the training set, a test set of 98 separate images was assembled from the in vivo data passing quality control to test the hypothesis of the investigation and determine if HRME imaging technology accurately distinguished benign from malignant mucosa.

Measurement of Diagnostic Accuracy and Interrater Reliability of HRME Images

Training set images were presented to a group of 11 blinded head and neck cancer specialists, together with a summary of features associated with each diagnostic category. Table III lists the HRME imaging characteristics associated with benign and cancerous mucosa. These classifiers were chosen a priori based on principles of conventional histopathology and were verified by review of representative HRME/biopsy pairs from each anatomical site imaged.[24]

After administration and review of the training set, raters were then administered the testing set of 98 images (33 benign, 65 cancer) blinded to anatomical site, tumor subsite, and final

TABLE III.
HRME Imaging Characteristics Associated With Benign Versus Malignant Mucosa.

	Histopathologic Classification	
Cellular Feature	Benign	Malignant
Nuclear size	Normal	Enlarged
Nuclear-to-cytoplasmic ratio	Normal	Increased
Overall cellular architecture	Regular and symmetric	Absent
Nuclear dispersion	Regular, widely spaced	Irregular, tightly packed

HRME = high-resolution microendoscopy.

histopathologic diagnosis. The panel of reviewers was asked to classify each image as either benign or malignant mucosa. The reviewers evaluated the images for nuclear size, nuclear-to-cytoplasmic ratio, and overall cellular architecture. Images were positioned randomly in the testing set, with a variety of study subjects, anatomical sites, and histologic diagnosis to prevent any pattern recognition on the part of the reviewers. Reviewers documented their interpretation of images in isolated settings to avoid the influence of additional reviewers' impressions of the images.

Statistical Analysis

Accuracy, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and interrater agreement between multiple raters, or Fleiss kappa statistic using the MAGREE function, were calculated with SAS version 9.2 (SAS Institute, Cary, NC). Two-tailed P values < 0.05 were considered to be statistically significant.

RESULTS

Thirty-three patients were enrolled in the trial. A total of 695 images were obtained from 117 unique sites; images from 98 of these sites passed the quality control review and were used for further analysis (Table I).

Representative examples of samples diagnosed as normal squamous epithelium are shown in Figure 2, and samples diagnosed as invasive squamous cell carcinoma are shown in Figure 3. The HRME images of normal squamous epithelium show small, bright nuclei with dark cytoplasm and relatively large and evenly spaced internuclear separation. Images from invasive carcinoma show high nuclear-to-cytoplasmic ratio and a distinct loss of cellular organization with haphazard, unevenly spaced, and abnormally large nuclei. In addition, a transition zone often could be observed at the interface of the tumor and adjacent benign mucosa (Fig. 4.).

Statistical analysis revealed that the mean accuracy in correctly diagnosing neoplastic or benign mucosa was 95.1% (95% confidence interval [CI], 94%–96%). The mean sensitivity and specificity were 96% (95% CI, 94%–99%) and 95% (95% CI, 90%–99 %), respectively. The negative predictive value was 98% (95% CI, 97%–99%), and PPV was 91% (95% CI, 85%–98%). The Fleiss kappa statistic for interrater reliability was 0.81, with a standard error of 0.014 and a 95% CI (0.78–0.84) (Fig. 5.)

DISCUSSION

As mentioned previously, oncologic surgery is likely to remain a cornerstone of the management of head and neck squamous cell carcinoma in the foreseeable future. Although the excitement of genomics and targeted immune and molecular therapy offers insight into the future of the management of cancer, currently surgical resection remains a foundation of management of this disease. Technological advancements related to surgery offer significant promise in the near future for patients with malignant disease of the head and neck. The field of image-guided oncologic surgery is an emerging area of research, and several optical imaging modalities have been proposed to improve surgical outcomes.[33–35]

The data presented allow for acceptance of the study hypothesis, namely that HRME provides sufficient imaging data to determine benign from malignant mucosa during oncologic ablative surgery. High-resolution microendoscopy, as well as other optical technologies such as confocal microscopy, have been evaluated by many investigations to determine the utility for tumor interrogation. Although the variety of optical technologies currently under investigation is somewhat overwhelming, none of the clinically available technologies are without limitations. The HRME is a simple, low-cost, portable diagnostic tool that has the ability to obtain real-time microscopic information,[22–24] which can be successfully delivered via a variety of delivery devices/instrumentation within the upper aerodigestive tract. High-resolution microendoscopy offers comparable resolution to other optical technologies such as contact endoscopy and confocal microscopy, allowing an evaluation of nuclear morphology. The findings of this investigation support the findings of previous work with HRME evaluating the discrimination of benign and malignant mucosa both in ex vivo laboratory studies and in vivo investigations in the field of head and neck oncology, and in other specialties such as gastroenterology.[22–26] Based on our findings, HRME appears to have excellent sensitivity and specificity to determine if mucosa is benign or malignant. When coupled with molecular and targeted contrast agents, HRME has the potential to allow for accurate, real-time surgical margin control.

Although our understanding of cancer biology is changing at a rapid pace, the impact of positive margins in oncologic surgery has been investigated, and the quest for improved surgical margin control is the subject of a variety of approaches. It is interesting that we still rely on techniques such as light field microscopy developed in the 19th century to determine the surgical margins, despite the major advances in technology and our understanding of disease since that period. It is for this reason that this is an area of great interest in the field of image-guided surgery.[35,36] The benefit of technology that would allow the surgeon to establish an immediate, real-time diagnosis that is consistent with the histologic diagnosis is obvious. Providing margin control during surgical procedures is only one facet of this technology, with screening programs, monitoring of lesions at risk, tumor surveillance, and interrogating for unknown primary lesions being other areas that would benefit from these technological advancements. With growing interest in minimally invasive surgical techniques in the head and neck, such as transoral robotic surgery,[37,38] advanced imaging modalities are likely to play an increasingly important role in oncologic surgery.

Despite the fact that HRME was shown in this study to have high specificity in determining mucosal status between benign and malignant disease, several technical issues prevent this technology from being widely accepted and replacing the current standard frozen section analysis. The first limitation is obvious when

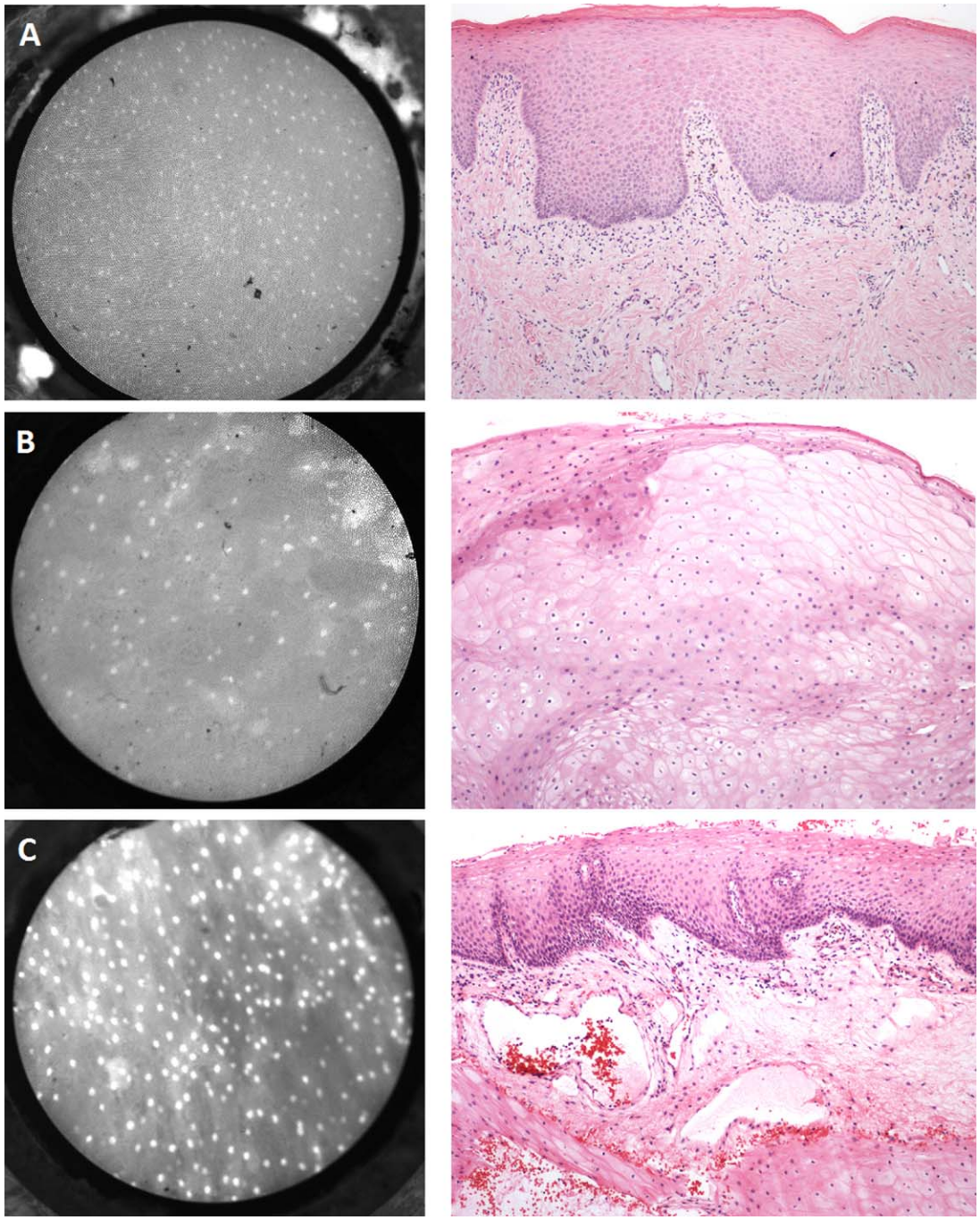

Fig. 2. Representative high-resolution microendoscopy images (100 μm) obtained intraoperatively from normal squamous epithelium of the oral tongue (A), buccal mucosa (B), and oropharynx (C), with corresponding hematoxylin and eosin histopathologic images. The bright, evenly spaced nuclei and normal cellular morphology can be observed in the images.

examining Table II. There exist several issues with image acquisition that render the images difficult to interpret. Severe inflammation, keratin debris, bleeding, and artifacts during imaging will impair the ability of optical devices to obtain accurate, interpretable images. Similarly, mucosa that has been radiated previously has been imaged ex vivo by our research group, and depending on the anatomic site may have significantly more artifact than surfaces that have not been radiated. This is likely due to changes in cellular architecture due to radiation fibrosis; currently we have not tested the technology in pilot trials involving salvage surgery after radiation therapy.

The second glaring limitation is that, at the current time, this technology is limited to surface imaging. The HRME has a limited depth of penetration (roughly 50–

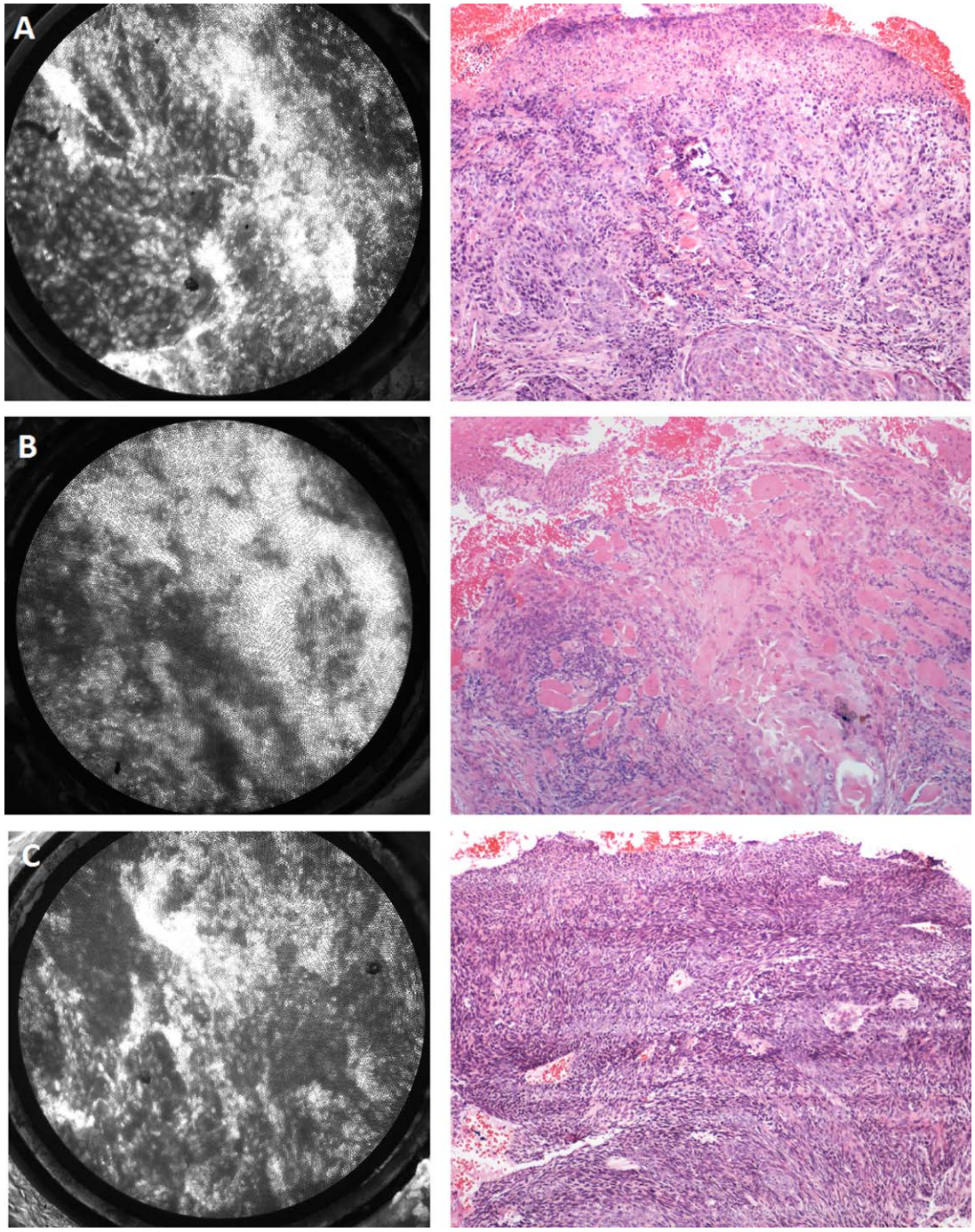

Fig. 3. Representative high-resolution microendoscopy (100 μm) obtained intraoperatively from invasive squamous cell carcinoma of the oral tongue (A), buccal mucosa (B), and oropharynx (C), with corresponding hematoxylin and eosin histopathologic images. Note the tightly packed, abnormally shaped nuclei with significant cellular heterogeneity, high nuclear nuclear-to-cytoplasmic ratio, and irregular imaging pattern characteristic of malignant epithelium.

100 μm), which makes it difficult to detect submucosal tumor spread when using proflavine contrast media.[39] This creates two problems when using the device for intraoperative margin control, the inability to determine submucosal spread of disease and the inability to image deep resection margins. Alternative fluorescent contrast media may allow for deeper imaging capability, and currently the investigational group is gathering preliminary data with a new HRME system designed to detect epidermal growth factor receptor (EGFR)+ fluorescent labeled cells to enhance the sensitivity of the system. High-resolution microendoscopy imaging of deep muscle margins remains unexplored and is the subject of ongoing investigation by our research group; however, preliminary muscle images have shown that nuclear labeling and nuclear morphology can be observed with

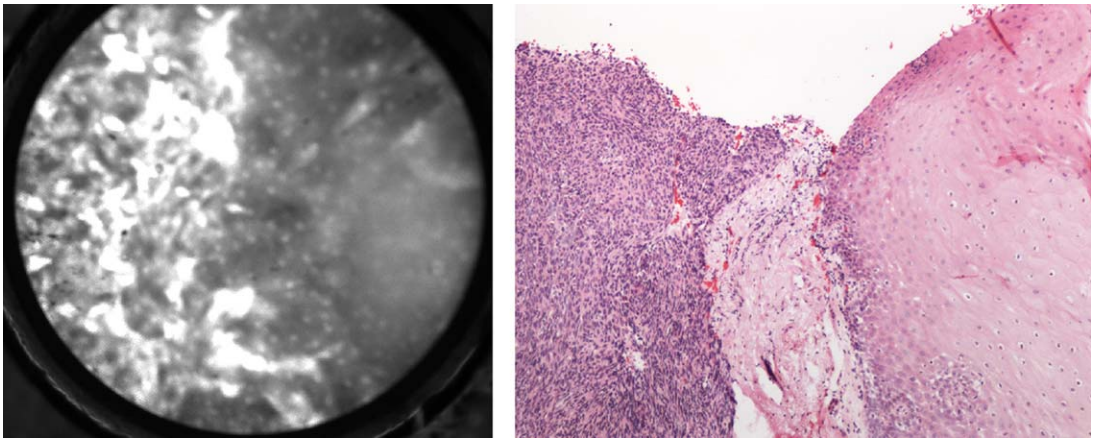

Fig. 4. Representative high-resolution microendoscopy image (100 μm) and corresponding hematoxylin and eosin image of the transition zone between malignant and normal mucosa.

the HRME system. Strategies that permit greater depth of penetration and the interrogation of deep margins are active areas of ongoing research; perhaps deep margin imaging may be possible by using targeted contrast media or spectroscopic algorithms to distinguish malignant cells from normal surrounding cells. Such strategies include inserting the HRME probe into a cannula or needle to image subsurface cells. This may be specifically relevant in HPV-related disease, which has a submucosal pattern of invasion in the lymphatic crypts. In our study population, we included nine HPV+ oropharyngeal squamous cell carcinomas for imaging. Whereas the HRME device performed quite well in this population, the device was applied to the surface of visible tumors of sufficient size for imaging. Certainly, small tumors hidden in the lymphatic crypts or submucosal invasion may not be detectable by the device. In this situation, imaging needles or probes and HPV-specific contrast media may allow for interrogation of HPV-related tumors, or for detection of unknown primary lesions. The investigational group is currently considering such strategies related to these limitations.

The third limitation of the technology is inherent in the fact that this is an anatomic imaging device. Therefore, accuracy and specificity rely on imaging characteristics and interpretation. Targeted contrast imaging of labeled malignant cells would be far superior when compared with interpretation of high-resolution images of epithelial cells. The contrast agent (proflavine hemisulfate) used in this investigation gives excellent visualization of the nuclei of epithelial cells but is not specific for malignant cells—and in addition has a strong affinity for keratin, which can result in artifact that can mask the underlying mucosa. Additionally, there is a significant clinical challenge in determining dysplastic mucosa versus normal mucosa visually; technology that could determine early dysplastic changes in real time would be beneficial. Preliminary data indicates that, although HRME has the ability to detect dysplastic mucosa, the dysplasia must be relatively severe to result in nuclear changes and morphologic changes reliably detected by the device. This is a significant limitation in the technology because detection of early dysplastic changes would be of value clinically. In order to address these issues, current research at our institution and others is focusing on creating more targeted approaches, such as NIR HRME probes, to image the mucosal surface.[40–43] Future research in alternative targeted contrast agents labeling markers, as noted above, such as EGFR or human papilloma virus (HPV) or other earlier molecular changes prior to the onset of severe dysplasia or frank malignancy, may allow for selective visualization of cancer cells with optical imaging technology.[44,45]

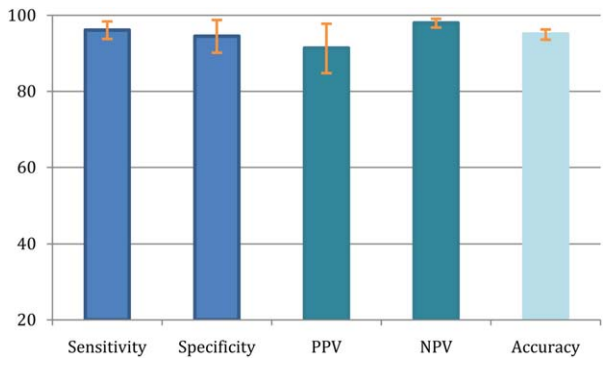

Fig. 5. Results of the interpretation of high-resolution microendoscopy images of benign mucosa vs. malignant mucosa.

Perhaps the most interesting limitation of this technology for intraoperative margin control lies in the general concept of the surgical margin. Current data indicates that molecular and genetic changes within cells occur before they exhibit the morphologic and biologic characteristics of malignant cells.[46–48] Regardless of the sensitivity of any optical imaging system, cells that may have genetic alterations and are predisposed to become malignant may appear completely normal, regardless of the technology employed. A currently relevant example would be that an epithelial cell infected by high-risk serotype HPV virus might be morphologically normal but destined to become malignant in the future.

Cancer stem cells, dormant tumor cells, and cells that appear morphologically normal may not be amenable to current imaging technology but may pose significant risks to patients. Perhaps in the future, optical techniques such as laser spectroscopy, gene analysis, or vibrational molecular imaging may allow us to interrogate groups of cells for these genetic changes[49,50]; however, this technology does not exist in a clinically applicable form at the present time.

CONCLUSION

In conclusion, this investigation has demonstrated that microendoscopy can be safely and successfully used to acquire high-quality, high-resolution images of cellular morphology and architecture in real time during oncologic surgery for head and neck mucosal squamous cell carcinoma. High levels of sensitivity and specificity can be obtained with this technology when interrogating mucosal surfaces for malignant disease. Despite the high levels of accuracy in this investigation, several technical and biological limitations currently prevent optical technology from supplanting traditional frozen section margin analysis. With advances in optical technology, target-specific contrast agents, and novel delivery systems with molecular or genetic resolution, this innovative technique may serve as a valuable adjunct to ablative oncologic surgery, potentially improving margin discrimination and oncologic outcomes.

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