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Distinct angiogenic changes during carcinogenesis defined by novel label-free dark field imaging in a hamster cheek pouch model

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Abstract

There remain gaps in knowledge concerning how vascular morphology evolves during carcinogenesis. In this study, we imaged neovascularization by label-free dark field microscopy of a DMBA-induced hamster cheek pouch model of oral squamous cell carcinoma (SCC).

Wavelength-dependent imaging revealed distinct vascular features at different imaging depths and vessel sizes. Vascular tortuosity increased significantly in high-risk lesions, while diameter decreased significantly in hyperplastic and SCC lesions. Large vessels preserved the same trends seen in the original images, whereas small vessels displayed different trends, with length and diameter increasing during carcinogenesis. Based on these data we developed and validated a classification algorithm incorporating vascular features from different vessel masks. Receiver operator curves generated from the classification results demonstrated high accuracies in discriminating normal and hyperplasia from high-grade lesions (area under the curve > 0.95).

Overall, these results provided automated imaging of vasculature in the earliest stages of carcinogenesis from which one can extract robust endpoints. The optical toolbox described here is simple, low-cost and portable and can be used in a variety of health care and research settings for cancer prevention and pharmacology research.

Keywords

Vascular imaging; head and neck cancer; cancer diagnosis; hamster cheek pouch; dark field

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Introduction

Angiogenesis is an important biological process through which new blood vessels sprout and grow from existing vasculature (1). Pathological angiogenesis occurs in various disease states ranging from macular degeneration to cancer (2–4). Pathological angiogenesis has been a major topic of interest in recent decades as a prognostic indicator and a potential target for cancer therapies (5–7). It is well established that tumor growth is limited in size if cancer cells cannot receive a sufficient supply of oxygen and nutrients to proliferate (5). The “angiogenic switch” is therefore essential to neoplastic progression and eventual invasion (8). By stimulating angiogenesis, rapidly proliferating cells create their own, albeit abnormal, blood supply to allow for continued growth.

Angiogenesis precedes the formation of a malignant tumor (9) as evidenced by immunohistochemistry (IHC). IHC of tissue biopsies from the head and neck regions have revealed structural and morphological changes in the local vasculature of dysplastic lesions (10–12). One study labeled several angiogenic factors and found a significant increase in vessel number and size in high grade human laryngeal dysplasia compared to normal tissues (10). Histopathology of neoplastic tissues in oral leukoplakia also revealed an increasing trend in micro-vessel density from low grade to high grade dysplasia, reflecting progressive neovascularization (11). A study comparing micro-vascular characteristics in different stages of oral carcinogenesis found increased number and volume of vessels in premalignant compared to normal tissue (12). Similar studies involving cervical pre-cancers and cancers also demonstrated an increase in micro-vessel density during carcinogenesis (13,14).

Although previous studies have shown that angiogenesis precedes malignant transformation (10–14), increased angiogenesis in pre-cancerous lesions has not been as extensively studied as it has in solid tumors, and the majority of previous work on pre-malignancies has focused on interpreting vascular changes from IHC. Further the angiogenic endpoints studied in these instances have been limited primarily to vascular density—no additional architectural changes such as diameter, length, or tortuosity have been characterized.

The overall goals of this study are to investigate the progressive evolution of angiogenesis over the course of dysplasia-carcinoma progression *in vivo* in a well-established model of epithelial carcinogenesis and to investigate the potential of leveraging quantitative vascular endpoints to differentiate between high grade pre-cancers, low grade pre-cancers, and normal/hyperplastic tissue. We employed a custom spectral dark field microscope (SDFM) to perform imaging at capillary level resolution *in vivo* and a Gabor-based segmentation algorithm to quantify vascular endpoints from the images. Although a variety of optical microscopy and imaging techniques such as multi-photon microscopy (15) and laser speckle microscopy (16) are well-suited to image vascular morphology, we elected to use SDFM because of its relative simplicity, low cost, and portability which enables a broad group of users in a variety of health care settings and research laboratories to use this technology.

The design, characterization, and validation of this toolbox along with proof-of-concept in a small set of pathologically-confirmed normal tissue and squamous cell carcinomas (SCC) in the hamster cheek pouch model has been described in (17). In this study, we build upon this

methodology to characterize and elucidate the angiogenic changes seen during the dysplasia-carcinoma sequence. Our previous paper considered all DMBA-treated tissues as a single group, and lacked the assessment by a board-certified pathologist to define distinct sub-stages of disease whereas in this particular study, pathology was obtained for tissues at different stages of the dysplasia-carcinoma sequence. Additionally, we incorporated spectral tunability into the SDFM to enable imaging of a wide distribution of vessel sizes from different tissue depths. We characterized vascular endpoints at specific stages throughout the pathologically-confirmed pre-cancer to cancer progression using the Gabor-based segmentation algorithm. We use this data to report on vascular endpoints as a function of diameter to identify how different sized blood vessels behave during angiogenesis. We demonstrated that differences between normal, pre-cancerous, and cancerous tissues were greatest when leveraging vascular endpoints stratified by vessel size.

Materials and Methods

Animal model

The animal study protocol was approved by the Duke University Institutional Animal Care and Use Committee (IACUC). 30 female Golden Syrian hamsters (5–6 weeks old and 80–100g in weight) obtained from the Frederick Animal Facility (NCI) were used in this study. All hamsters were individually housed at an on-site housing facility with standard 12-hour light/dark cycles and were provided *ad libitum* access to food (Purina Prolab RMH 1800) and water. The hamsters were divided into three groups that underwent DMBA treatment for 8 weeks (10 hamsters), 10 weeks (11 hamsters), and 12 weeks (9 hamsters), respectively. The right oral mucosa of each hamster was painted with 0.5% DMBA-mineral oil solution three times per week, while the left oral mucosa was painted with mineral oil to serve as an untreated control. Details of the painting procedures were previously described (17). At the end of the DMBA treatment, SDFM images were obtained from both DMBA-treated and control oral mucosae while the animals were anesthetized under inhaled 2–3% isoflurane.

Following final imaging, tissue from image sites was excised and fixed in 10% neutral buffered formalin, embedded in paraffin, and prepared as hematoxylin and eosin stained sections. Sections from each imaged site were evaluated by a board-certified veterinary pathologist (JE) and graded as either normal, hyperplasia, low grade dysplasia, high grade dysplasia, or SCC based on histologic features and degree of cytologic atypia. The pathologist was blinded to all imaging results. Hamsters were euthanized according to our IACUC protocol after biopsies were obtained.

Microscopy and Imaging protocol

The SDFM used for vascular imaging was modified from our previous study (17). Specifically, the polarizers were removed and a filter wheel was added after the optical fiber. The same white light LED light source, 4X objective lens, and CMOS RGB camera were utilized. Four bandpass filters with center wavelengths of 440, 540, 580, and 600 nm (XBPA440, XBPA540, XBPA580 and XBPA600, Asahi Spectra Co., CA, USA) were used in this study. The lateral resolution of the system was 3.1 μm and the field of view was 1.5 mm by 1.0 mm. Depending on the wavelength used, the SDFM system can interrogate a

tissue depth of approximately 500 μm at short end of the wavelength spectrum (i.e. 440 nm) versus approximately 1 mm at the long end of the wavelength spectrum (i.e. 600 nm) (18).

Hamsters were anesthetized immediately before imaging, and the cheek pouch was carefully inverted and stretched over the flat surface of a small cylindrical post. The oral mucosa was secured with Babcock clamps to reduce motion artifacts, and cleaned with sterile saline and a gauze pad. A drop of mineral oil along with a thin glass cover slip (No. 2) was added onto the exposed cheek pouch to reduce specular reflection during imaging. Five to six sites of interest were imaged at each wavelength (440, 540, 580, and 600 nm) with exposure times ranging from 500 to 1600 milliseconds depending on saturation. Images were collected through a customized LabVIEW (National Instruments, TX, USA) program.

Image processing and vascular feature extraction

The 440, 540, 580, and 600 nm SDFM images obtained at each site were automatically aligned to the 540 nm image using a built-in MATLAB function for image processing (imregister). The RGB channels from the aligned raw images were used to generate the ratiometric images in order to enhance vascular contrast (17). For each site, the blue channel of the 440 nm image and the green channel of the 540 and 580 nm images were divided by the red channel of the corresponding 600 nm image to generate three ratiometric images (400/600, 540/600, and 580/600 nm/nm). A Gabor-based algorithm was used to combine these images and segment the vasculature into a single binary mask (19). Details of the algorithm are in the supplementary data (Sup. Figure 1). The original binary vessel mask was skeletonized to extract tortuosity, diameter, length, and length density features. Area fraction was calculated directly from the vessel mask. For better visualization, inverse distance transforms were applied to the skeletonized tortuosity, diameter, and length maps using the diameter information. The averaged values were used for overlapping regions.

To enhance the vascular features, each original vessel mask was further split into large and small vessel masks. The threshold (14 μm) was selected based on criteria provided by Davis et al. (20). We defined the vessels whose diameters were in the capillary range ($<14 \mu\text{m}$), and the parent vessels to which they directly connect, as small vessels. The vessels with diameters greater than 14 μm as well as their connecting vessels were defined as large vessels. Details of the mask splitting are provided in Supplementary Figure 2. The same vascular features were computed from both large and small vessel masks, in addition to the original masks that contained all vessels.

Definition of Vascular Parameters

Five main vascular features (tortuosity, diameter, length, area fraction, and length density) were used as endpoints in our study and were extracted from each vessel in the skeletonized binary masks (17). Vascular length was calculated by summing the total number of pixels in a vessel segment. Tortuosity captures the twists and curves of blood vessels, and was derived by dividing vessel length by the Euclidean distance between the two vessel ends. Diameter was determined using the distance transform of the original binary vessel mask. Area fraction was calculated by dividing the number of vascular pixels by the total number of

pixels. Length density was defined as the sum of the lengths of the vessels divided by the total field of view area.

Statistical analysis

The mean of each vascular property (tortuosity, diameter, length, area fraction, and length density) was determined for a given cheek pouch by averaging values from all sites measured within the same cheek pouch. Wilcoxon rank sum tests were used to compare the mean vascular parameters from the normal group to vascular parameters from all other groups (hyperplasia, low grade dysplasia, high grade dysplasia, or SCC).

The pathologically normal and hyperplasia groups were combined to represent benign oral mucosa for classification purposes. A linear support vector machine (LSVM) classifier was trained to differentiate benign, low grade dysplasia, high grade dysplasia, and SCC using combinations of the mean vascular features (tortuosity, diameter, length, area fraction, and length density) obtained from original, large, or small vessel masks. The leave-one-out cross-validation technique was used to create receiver operating characteristic (ROC) curves for each classifier, and the area under the curve (AUC) was computed for each ROC. Statistical analyses and image processing were performed with MATLAB (MathWorks, MA, USA).

Results

Images taken over a range of wavelengths enhance visualization of different vessel sizes

The study design included control and DMBA painted oral mucosa. Figure 1(A) summarizes the pathology results for all mucosae. All 30 control oral mucosae were confirmed as normal based on histology. For the DMBA-treated cheek pouches, a total of 1, 7, 5, 9, and 8 oral mucosae were diagnosed as normal, hyperplasia, low grade dysplasia, high grade dysplasia, and SCC, respectively. High grade dysplasia includes moderate and severe dysplasia and carcinoma *in situ*. Figure 1(B) shows representative hematoxylin and eosin (H&E) images of the five different cancer subtypes as diagnosed by a board-certified pathologist. Figure 1(C) shows the ratio of images obtained at three different wavelength pairs, 440 nm/600 nm, 540 nm/600 nm, and 580 nm/600 nm from sites histologically confirmed to be normal, hyperplasia, low grade dysplasia, high grade dysplasia, and SCC. These sites correspond to the cheek pouch tissues in Figure 1(B). Shown in red are binary Gabor-based masks of vasculature corresponding to each of the grayscale ratiometric images. The clear vascular organization and hierarchy seen in the normal images is lost during the transition to SCC, where the vessels become progressively more disordered and crowded together. Vessels that show the greatest contrast at 440/600 have diminishing contrast at longer wavelength ratios, and the opposite is observed for those vessels that show the greatest contrast at 580/600, reflecting the importance of looking at a range of wavelengths to probe different depths, particularly as the oral mucosa thickens with neoplastic progression.

In order to leverage the contrast achieved from the shallow and deep vessels at short and long wavelength ratios, respectively, the ratiometric images for a given tissue site were combined into one integrated vessel mask. Figure 2(A) shows a representative example of

how the wavelength ratio images are combined to create a final mask for each tissue site. The details of the Gabor-based segmentation algorithm are described in the supplementary data (Sup. Figure 1). Representative combined masks from each of the five pathology groups are shown in Figure 2(B). The combined masks display a much wider distribution of vessel sizes than the masks created from ratios of images at any of the individual wavelength pairs.

Vascular endpoints differentiate normal, pre-cancerous and cancerous tissues

Figure 3(A) displays three extracted vascular parameters (tortuosity, diameter and length) quantified from the representative images of normal, hyperplasia, low grade dysplasia, high grade dysplasia, and SCC shown in Figure 1 and 2. Figure 3(B) shows the mean values of five endpoints (tortuosity, diameter, length, area fraction, and length density) for each animal and the overall mean for each group. The proportion of tortuous vessels clearly increases as tissue changes from normal to dysplasia to SCC. The majority of the tortuosity appears to be in smaller, sprouting vessels that are distinct in SCC. On the other hand, vascular diameter decreases during the progression from normal to SCC. The vascular tortuosity of dysplasia and SCC were significantly higher than that of normal ($p < 0.01$ for SCC and high grade dysplasia, $p < 0.05$ for low grade dysplasia), while the vascular diameters of hyperplasia and SCC were significantly lower than that of normal ($p < 0.05$ and < 0.01 , respectively). The area fraction of low grade dysplasia and SCC were significantly lower than that of normal ($p < 0.05$ and < 0.01 , respectively). There were no significant differences observed in the average vascular length between the normal and dysplastic/SCC groups. However, there was a decrease in the variance in the vascular length as tissue progressed from normal to SCC.

Vascular trends are distinct for large (arterioles, venules) vs. small vessels (capillary level vessels)

As observed previously, there was a significant decrease in vessel diameter during carcinogenesis. To further characterize and exploit the differences in vessel diameter, each original vessel mask was split into a large and a small vessel mask based on vascular diameter to quantify the behavior of different vessel types during carcinogenesis. Details of the methodology are provided in Sup. Figure 2. The diameter criterion was chosen to separate arterioles or venules from capillary level vessels. Specifically, a threshold reported in the literature ($14 \mu\text{m}$) was used in our study to differentiate large vessels from smaller capillary-type vessels (20). Varying the threshold by $\pm 25\%$ shows similar results (data not shown).

Figure 4(A) shows the natural log scale ($\ln$) distributions of vascular diameter for the original vessel masks, large ($>14 \mu\text{m}$) vessel masks and small ($<14 \mu\text{m}$) vessel masks, regardless of pathology. Note that 'pixel count' refers to the number of pixels within each diameter size distribution bin. The sum of the counts equals the total number of vascular pixels contained in all site images combined. The mean diameter for each group is shown in red. The means and standard deviations of vascular diameters for all masks, all large vessel masks, and all small vessel masks were 16.3 ± 19.14 , 25.7 ± 23.87 , and $7.0 \pm 3.56 \mu\text{m}$, respectively. Figure 4(B) shows representative vascular images overlaid with the corresponding large (shown in yellow) and small vessel masks (shown in red). Note that the large vessel masks contained some vessels with diameters less than $14 \mu\text{m}$, which are

connecting vessels. In general, it is straightforward to visualize the increased proportion of small vessels in dysplasia and SCC compared to that in benign tissues. Figure 4(C) shows vascular parameters obtained from the large and small vessel masks, as well as trends seen in the ratio of large and small vessels. The trends in vascular features of the large vessel masks are similar to those seen in the original masks. The tortuosity of the large vessels increased significantly from normal to dysplasia and SCC ($p < 0.01$). The diameter of the large vessels decreased significantly from normal to hyperplasia, high grade dysplasia, and SCC ($p < 0.01$ for each group). Average vascular length decreased significantly in high grade dysplasia relative to normal ($p < 0.01$). The area fraction of low grade dysplasia and SCC was significantly lower than that of normal ($p < 0.05$ for both). The small vessel tortuosity was significantly higher in the low grade dysplasia ($p < 0.05$), high grade dysplasia ($p < 0.01$) and SCC ($p < 0.01$) groups compared to normal, which was similar to that observed when using the original and large vessel masks. Surprisingly, the trends in vascular diameter, length, and area fraction from normal to SCC were the opposite of what was observed with the original and the large vessel masks. The small vessel diameter increased significantly from normal to hyperplasia, low grade dysplasia, high grade dysplasia, and SCC ($p < 0.01$ for all groups vs. normal). The small vessel length in SCC was significantly higher than that observed in normal tissues ($p < 0.05$), and the small vessel area fraction in SCC and high grade dysplasia were significantly higher than that observed in normal tissues ($p < 0.05$ for both). Taking the ratios of the vessel length obtained from the large and small vessel masks provided better contrast than any of the individual masks. Ratios of lengths were significantly decreased in hyperplasia, low grade dysplasia, high grade dysplasia, and SCC compared to that observed in normal tissues ($p < 0.05$ for low grade dysplasia, all others $p < 0.01$).

Vascular endpoints provide a robust strategy to differentiate SCC and dysplasia from benign tissues

Figure 5 shows the results from leave-one-out cross-validation using a linear support vector machine (LSVM) algorithm to discriminate normal and hyperplasia from SCC (left column), normal and hyperplasia from high grade dysplasia and SCC (middle column), as well as normal and hyperplasia from low grade dysplasia, high grade dysplasia, and SCC (right column) using the vascular features from large or small vessel masks or the ratios of the two (large/small). During each iteration in the leave-one-out cross-validation, we left out images collected from 1 cheek pouch to build our model and tested the classifier on these excluded images. This set of approximately 24 images included 6 image sites and 4 wavelengths (440, 540, 580, and 600 nm) per site. Vascular features obtained from all vessel masks performed comparably well, with the highest AUC obtained for the identification of SCCs as would be expected. It is encouraging that using endpoints from any size of vessels (rows) yielded comparable performance to distinguish multiple advanced pathologies from normal + hyperplasia (columns). In summary, tortuosity, vessel length, and diameter are robust parameters for differentiating dysplasia and carcinoma from non-neoplastic tissues regardless of vessel size. The last row of ROCs in Figure 5 demonstrates the benefit of utilizing vascular features from different vessel sizes in the LSVM algorithm. To generate the ROCs, tortuosity from only large vessels, diameter from only small vessels, and length from L/S ratios were chosen as classification parameters for discriminating between

pathological groups. These three parameters resulted in ROC curves with the highest AUCs. The AUC values from the ROC curves using each individual parameter as well as their combinations are presented in Supplementary Table 1.

Discussion

While much of the literature has focused on the aberrant vasculature of solid tumors, this study examines vascular changes during the earliest manifestations of carcinogenesis. Further, while previous studies have demonstrated changes in micro-vessel density early in the dysplastic process using tissue biopsies, our methodology combines SDFM and a wavelength-based ratiometric Gabor algorithm to enable high-resolution *in vivo* imaging and vessel segmentation methods to quantify a multiplicity of vascular endpoints that describe evolution in the vessel architecture during the process of carcinogenesis. The DMBA-treated hamster cheek pouch model of the dysplasia-carcinoma sequence was well-suited for this investigation as it presents a model in which there are progressive degrees of pre-malignancy characterized by cellular atypia that can be imaged prior to the development of invasive cancer. Because penetration depth and vascular contrast are closely related to the wavelength of the illuminating light (21,22), using a spectrally-tunable illumination source and integrating the features obtained at different wavelengths provided a wider distribution of vessel sizes from which to quantify vascular features compared to a single-wavelength approach. The ability to transform the imaging data into robust endpoints can be used for pharmacology research and cancer screening/diagnostic applications.

Spectral tunability allowed for the sampling of a broader range of vessel sizes than any single wavelength ratio pair. Small vessels were qualitatively more apparent at a short wavelength ratio (440 nm/600 nm) reflecting a shallow depth, while large vessels were qualitatively more apparent at a longer wavelength ratio (580 nm/600 nm) indicating that they are deeper within the tissue. The normal hamster oral epithelium is approximately 20 μm thick and can increase to 50 μm in SCC (23). It is possible that blood vessels could be too deep for SDFM to image in a very advanced tumor that exceeds 1–2 mm in thickness. However, our results in the hamster study showed that during the 12 weeks of DMBA treatment, blood vessels that were visible for all pathologically-confirmed disease stages and their area fractions served as useful parameters to differentiate between normal tissue, pre-cancers, and early cancers.

The proportion of large vessels decreased while the proportion of small vessels increased during the process of carcinogenesis (Figure 4). An increase in cell density, which increases optical scattering, is a common feature of neoplastic tissues (24). The gradual obscuration of deep vessels due to epithelial thickening may contribute to the significant decrease in diameter of large vessels seen during the progression from normal mucosa to SCC (Sup. Figure 3). On the other hand, the increase in growth of small vessels in pre-cancer and cancer, which is characterized by rapid and chaotic sprouting of new, usually immature vessels, serves to provide nutrients to the proliferating cancer cells (25). Overexpression of VEGF has been observed in DMBA-treated hamster oral mucosa (26,27) as a marker of angiogenesis (28,29), suggesting that the changes we observed here are due in part to increasing VEGF over the course of neoplastic progression. Upregulation of other pro-

angiogenic growth factors and endothelial receptor tyrosine kinases such as Tie2 likely contribute to these vascular abnormalities as well (30).

Vascular endpoints quantified from the original masks revealed increased tortuosity in dysplasia and SCC compared to normal tissue. Tortuosity increased significantly in high grade dysplasia and SCC compared to benign conditions for the original, large, and small vessel masks. Several *in vivo* studies have previously demonstrated that tumor vasculature is more tortuous than normal vasculature (31–33), and that tortuosity increases with tumor growth (33). Li et al. injected 20–50 tumor cells into mice window chambers to mimic natural tumor-induced angiogenesis and observed qualitative increases in vascular tortuosity during tumor progression (31). Conroy et al. utilized optical coherence tomography to quantitatively demonstrate that tumor-induced vasculature is significantly more tortuous than normal vasculature in a window chamber model (32). Another study used magnetic resonance angiography (34) to show that tortuosity was a more effective endpoint than vessel count for distinguishing early choroid plexus carcinomas. Although no studies have directly examined tortuosity during the normal to premalignant state and premalignant to malignant transformation, our findings are consistent with those reported for solid tumors.

Interestingly, opposite trends were observed in diameter and vessel length in the original and large vessel masks compared to the small vessel masks. Specifically, vessel diameter and vessel length both decreased in pre-cancer and cancer in the case of the large vessels but increased in pre-cancer and cancer when the small vessels were evaluated. Increasing trends were observed in diameter, standard deviation of the diameter (Sup. Figure 3), and length during the course of tumor progression in the small vessels. Dilation in response to nitric oxide happens early in the angiogenic process (1), which could account for the increase in small vessel size; others argue that “vessel dilation” is an unfortunate misnomer and that small tumor vessels instead increase in size due to extensive remodeling of the host vasculature (35). Though counterintuitive, the small vessels were able to increase in both length and tortuosity from normal to SCC. It is interesting to note the decrease in large vessel diameter, as most existing literature identifies vasodilation as a common characteristic of tumor-induced angiogenesis (36–38). However, the majority of previous studies have utilized implantable tumor models, so it is difficult to predict how vascular morphology in our spontaneous DMBA-induced model of carcinogenesis may differ. It is possible that the decrease in vessel diameter could be due to tissue pressure caused by proliferation of tumor cells and/or stromal growth (39,40). However, future studies using chemotherapy agents or enzymes such as hyaluronidase to relieve this tissue pressure would be necessary to verify causality.

A classifier utilizing a combination of the five quantitative *in vivo* vascular parameters (tortuosity, diameter, length, area fraction, and length density) was able to differentiate high and low grade lesions from normal tissues and hyperplasia with high accuracy. Integrating vascular features from large and small vessel masks as well as their ratios performed significantly better than utilizing vascular endpoints from a single mask, reflecting the importance of imaging different vessel sizes and parsing the results by diameter

The ability to detect and track changes starting early in disease progression is critical, since early detection more than doubles a patient's chance of survival (41). For example, 60% of head and neck squamous cell carcinomas (HNSCCs) are diagnosed when they have already progressed to advanced stage disease (42), with a particularly devastating impact in low and middle income countries (43). Therefore, having a non-invasive method such as ours to assess superficial mucosa at risk for malignant transformation would be an invaluable tool for the detection and treatment of these lesions in their early stages.

Magnetic resonance imaging (MRI) is an excellent technology for vascular imaging in invasive lesions, and it is already used as a diagnostic tool for HNC in advanced healthcare settings (44). However, MRI requires expensive, specialized imaging instrumentation, which may limit its use, and is likely not an appropriate tool for imaging superficial pre-cancers for which optical technologies, which are far less complex and expensive, can be readily used. Multiple optical techniques can be used for vessel-based cancer diagnosis (45) and could even potentially be coupled with our modular vascular algorithm toolbox. However, like MRI, the utility of these tools is often limited because of the specialized equipment and high-powered lasers needed for advanced optical imaging systems. The need for contrast agents in some optical vascular imaging techniques also limits their suitability (46). SDFM is low-cost, label-free, and requires relatively simple instrumentation, making it a suitable method for a variety of healthcare and research settings, as well as affordable and accessible to a broad group of users.

To enable use of our SDFM for clinical applications, we have developed a low-cost, portable "Pocket" microscope that is capable of imaging tissue vasculature at high resolution (47). This technology closely mimics our dark field microscope and is well suited for visualization of oral mucosa in clinical applications. Further, we have demonstrated that the Pocket microscope provides comparable contrast and resolution compared to the dark field microscope used in this study (Sup. Figure 4).

We believe that our technology is translatable for the detection of epithelial pre-cancers in a clinical setting. Human epithelial tissue typically ranges from 75 to 550 μm and the lamina propria can extend to 2 mm in depth (48,49). Despite the differences in epithelial thickness between humans and our hamster model, early dysplastic changes arise in the superficial epithelia and can be visualized with microscopy techniques. In fact, a study using similar dark field illumination demonstrated the feasibility of imaging superficial vasculature in the healthy human oral cavity (50).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Dark field spectral images reveal depth-sensitive vascular contrast.

(A) Pathology for the oral mucosae. Legend indicates the total length of DMBA (or control) painting. Numbers in parentheses indicate the number of animals in each category. (B) Representative hematoxylin and eosin (H&E) images of five different cancer stages. (C) Representative ratiometric images of five different cancer stages corresponding to the sites shown in B taken at three wavelength ratios (440 nm/600 nm, 540 nm/600 nm, 580 nm/600 nm) are shown. Dashed arrows indicate deeper blood vessels with better contrast in images obtained at 540 and 580 nm. Solid arrows indicate shallow blood vessels with better contrast in images obtained at 440 nm. Gabor-based masks for each representative image are shown in red below the corresponding wavelength ratio. Scale bars in C: 500 μ m. SCC: squamous cell carcinoma.

Figure 2. Merging different wavelength images to maximize vascular information.

(A) Schematic of three Gabor filter responses at 440/600 (blue), 540/600 (green) and 580/600 (red) combined into an integrated Gabor image. The sensing depths at 450 nm and 600 nm are approximately 500 μ m and 1 mm, respectively. This multi-wavelength Gabor image is then segmented using the Dijkstra algorithm to obtain the final binary mask. (B) Segmented Gabor-based masks of five different cancer stages incorporating vascular information from each of the three wavelength ratios. Scale bars: 500 μ m. SCC: squamous cell carcinoma.

Figure 3. Cancerous and pre-cancerous hamster oral mucosa display vascular features that are significantly different than normal.

(A) Quantitative vascular parameters (tortuosity, diameter, length, area fraction, and length density) extracted from representative images of normal tissue, hyperplasia, low grade dysplasia, high grade dysplasia and squamous cell carcinoma in hamster oral mucosa. (B) Significantly increased tortuosity and decreased diameter were observed in oral vasculature during the malignant transformation. **: $p < 0.01$, *: $p < 0.05$. LD: low grade dysplasia, HD: high grade dysplasia, SCC: squamous cell carcinoma.

Figure 4. Stratifying blood vessels by diameter enhances the changes in vascular features during cancer progression.

(A) Diameter distribution from the original vessel masks and resulting diameter distributions from the large and small vessel masks that were split with a threshold diameter of 14 μm . Some vessels with a diameter smaller than 14 μm were included in the large vessel masks because they connect two large vessels. Mean diameters shown in red. (B) Representative overlay images of large and small vessel masks on top of ratiometric images (540 nm/600 nm). (C) Distinct vascular trends were seen in large vessels and small vessels, as well as in their ratios. Significant increases in tortuosity were observed for both large and small vessels

during malignant transformation. However, significant decreases in diameter and length were observed in large vessels, while opposite trends were observed in small vessels. **: $p < 0.01$, *: $p < 0.05$. LD: low grade dysplasia, HD: high grade dysplasia, SCC: squamous cell carcinoma. Scale bar: 500 μm .

Figure 5. Enhanced *in vivo* vascular features can be used to detect both pre-cancerous and cancerous lesions

Representative receiver operating characteristic curves (ROCs) computed from classifying normal and hyperplasia versus low grade dysplasia and high-risk lesions (high grade dysplasia and/or squamous cell carcinoma) using five vascular features (tortuosity, diameter, length, area fraction, and length density) for large masks (green), small vessel masks (red), and L/S ratios (black). The ROCs in blue classify the same groups using tortuosity from large vessels only, diameter from small vessels only, and length from L/S ratios as parameters. Numbers in parentheses indicate the areas under the curves (AUCs). SCC: squamous cell carcinoma.