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DIAGNOSTIC APPLICATION OF MULTIPHOTON MICROSCOPY IN EPITHELIAL TISSUES

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Epithelial cancer comprises more than 85% of human cancers. The detection and treatment at the early stage has been demonstrated to apparently improve patient survival. In this review, we summarize our recent research works on the diagnostic application of epithelial tissue based on multiphoton microscopy (MPM), including identification of the layered structures of esophagus, oral cavity, skin and bronchus tissues, establishment of the diagnostic features for distinguishing gastric normal tissue from cancerous tissue, linking collagen alteration and ectocervical epithelial tumor progression for evaluating epithelial tumor progression, and differentiating normal, inflammatory, and dysplastic ectocervical epithelial tissues. These results provide the groundwork for developing MPM into clinical multiphoton endoscopy.

Keywords: Epithelial tissue; multiphoton microscopy; two-photon excited fluorescence; second-harmonic generation.

1. Introduction

Epithelial cancer comprises more than 85% of human cancers.[1] The detection and treatment at the early stage has been demonstrated to apparently improve patient survival. For example, prognosis of gastric cancer is generally rather poor, with 5-year relative survival below 30% in most countries.[2] However, early gastric cancer has more than a 90% 5-year survival rate, even a 10-year survival rate of greater than 90% in high-incidence areas such as Japan.[3,4] Early cancer is usually defined as a malignant tumor limited to the mucosa or submucosa, irrespective of the presence of lymph node metastases. Moreover, the natural history of gastric cancer is clearly explained, the cancer cell appears at the middle or deeper layer of the gastric mucosa. The cancer cells increase in number rapidly and reach the mucosal surface. The total length of intramucosal carcinoma is estimated to be from 14 to 21 years. The duration after submucosal invasion to the patient's death is from 1.5 to 8 years.[5] These data indicate that the early-phase carcinoma will undergo a rather long period to develop into advanced cancer, providing enough time to diagnose gastric cancer in the early stage and the best opportunity to achieve a complete cure of gastric cancer. So, the clinical key question is the appearance of new imaging techniques that have the ability to perform real-time *in vivo* diagnosis for early epithelial cancer at the cellular level.

At present, the noninvasive medical imaging techniques consist of X-ray examination, endoscopy,

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computed tomographic (CT) scanning, endoscopic ultrasonography (EUS), magnetic resonance image (MRI) scanning, positron emission tomography (PET) imaging, etc. in the clinical environment. However, these imaging techniques are inadequate to detect early-stage cancer because they are only capable of providing morphological visualizations of tissue lesions at the millimeter-scale resolution. Confocal laser endomicroscopy can allow high-resolution *in vivo* histology assessment. The disadvantages of this technique, such as limited infiltration depth, intravenous injection of fluorescence contrast agent, and increased photobleaching and phototoxicity, limit widespread clinical use.

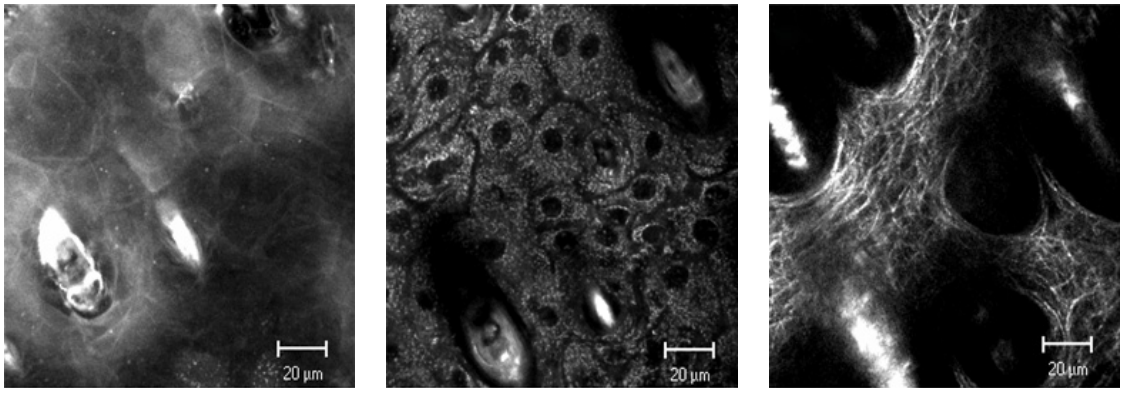
Multiphoton microscopy (MPM) based on two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) has tremendous potential to overcome the limitations of fluorescence confocal microscopy while retaining its merits. Coupled to near-infrared (NIR) femtosecond laser, it has apparent advantages over confocal imaging techniques in the imaging of thick tissue and live animals, such as greater imaging penetration depth and reduced out-of-focus photobleaching and phototoxicity.[6,7] The important advantage is that it is able to image unstained samples. When biological tissues are irradiated by intense NIR femtosecond laser pulses, the endogenous structural proteins with noncentrosymmetric structures can easily produce the optical SHG signal.[8,9] The SHG process changes two NIR incident photons into one visible photon at half the wavelength. At the same time, the native fluorophores in the biological specimen may also emit the autofluorescence through TPEF process.[10−12] TPEF process occurs when an electron is excited to a higher energy electronic state by the simultaneous absorption of two photons in the NIR wavelength. These make it possible to develop into multiphoton endomicroscopy. In this paper, we summarize our recent research works on the diagnostic application of epithelial tissue by use of MPM, which provide the groundwork for developing MPM into clinical multiphoton endoscopy.

2. Observing Layer Structure of Normal Epithelial Tissues by Use of MPM

Epithelial tissues with well-stratified structure include the esophagus, oral, bronchus, cervix, skin, stomach, and colon *et al.* Such tissues often consist of the topmost keratinizing epithelial layer, epithelial cell layer, and underlying stromal layer. MPM has the capability to identify the layered structures of esophagus, oral cavity, and bronchus including the keratinizing layer, epithelial cell layer, and stromal layer, which are strongly correlated to tissue pathology.[13−15] Coupled with several system analyzing tools, this method allows selective visualization of microstructural components based on their characteristics of intrinsic spectra in various layers, such as keratin, porphyrin derivatives, cells, elastin, and collagen, quantitatively providing some important depth-resolved information on the biomorphology and biochemistry of epithelial tissues. It can also offer a ratiometric redox fluorometry based on TPEF from cellular NAD(P)H and Fp to study mitochondrial energy metabolism. The exponential depth-dependent decay of the reflected SHG intensity that describes the optical property of the stroma can be potentially used for the characterization of normal and diseased tissue states. An optimum excitation wavelength for the estimation of epithelial cellular metabolism is 810 nm whereas, 850 nm excitation light is most suitable for acquiring high-contrast images of collagen and elastin and monitoring the change of collagen SHG over elastin TPEF in the stromal layer. Figures 1(a)−1(c) display the representative MPM images of the topmost keratinizing epithelial layer, epithelial cell layer, and underlying stromal layer from *in vivo* mouse skin, respectively.

3. Establishing Diagnostic Features for Distinguishing Cancerous Tissue from Normal Tissue

The establishment of diagnostic features is essential and significant for developing multiphoton endoscopy to facilitate early diagnosis of epithelial cancer at the molecular level. The microstructures of mucosa and submucosa from human normal stomach and that with gastric cancer were imaged by MPM.[16] The diagnostic features for identifying the mucosa and submucosa of human normal stomach and that with gastric cancer were established by investigating their MPM images. It is found that MPM has the ability to identify not only the mucosa and submucosa of normal stomach and gastric cancer but also the distribution and content of abnormal cells in these two layers. According to



(a) (b) (c)

Fig. 1. (a)−(c) display the representative MPM images of the topmost keratinizing epithelial layer, epithelial cell layer, and underlying stromal layer from *in vivo* mouse skin, respectively.

the observed results, several special markers were extracted to distinguish a stomach with gastric cancer from a normal stomach. First, the appearance of abnormal cells in the mucosa and submucosa is an important indicator of differentiating the normal tissue from cancerous tissue. In the normal gastric tissue, there are only normal epithelial cells in the gastric surface and the cells in the gastric glands, such as chief cells and parietal cells, showing nearly uniform size and shape and having regular arrangements. In case of gastric cancer, there are abnormal cells in the mucosa and submucosa, the nuclei of these cells all varied in size, had disorder arrangement, and sometimes tended to resemble the glandular tissue. The morphology and distribution of abnormal cells have obviously different MPM images when compared with the normal cells and they were easily identified from normal cells. The appearance of abnormal cells results in the structural variation of gastric mucosa. In the mucosa surface of a stomach with gastric cancer, the gastric pit and basement membrane disappeared, and in the lamina propria, the arrangement of gastric glands become more disordered, having random orientation. Second, the loss of extracellular matrix component in gastric cancer, collagen fibers and collagen bundles, is also a significant feature. In case of gastric cancer, the content of collagen fibers in mucosa and submucosa apparently decreased and lost fine mesh morphology, and collagen bundles in the submucosa became thin. The distribution of the residual collagen lost good arrangement in gastric cancer when compared with the normal gastric tissue. Last, the variation of blood vessels in the submucosa is also not negligible in normal tissue when compared with cancerous tissue. In case of gastric cancer, the lumen of blood vessels reveals distorted and irregular architecture.

4. Linking Collagen Alteration and Epithelial Tumor Progression for Evaluating Epithelial Tumor Progression

Collagen alteration is critical for epithelial tumor initiation and progression. Quantitatively linking collagen alteration and epithelial tumor progression is essential for developing an optical endoscopy to evaluate epithelial tumor progression. A total of 16 fresh ectocervical tissue samples obtained from 11 patients undergoing the loop electrosurgical excision procedure were examined, and correlation of collagen alteration and epithelial tumor progression was quantitatively linked by both qualitative label-free SHG imaging and quantitative image analysis.[17] It is found that the collagen presence decreases with ectocervical epithelial tumor progression. To be specific, the collagen presence in normal case is 0.89 ± 0.05, in precancer is 0.67 ± 0.11 and in cancer is 0.43 ± 0.12. The collagen−fibril bundles orientation increases with epithelial tumor progression. Specifically, the collagen−fibril bundles orientation in normal case is 0.21 ± 0.04, in precancer is 0.53 ± 0.08, and in cancer is 0.86 ± 0.07. The normal fibrils correlation fall off sharply with distance, indicating distinct linear fibrils, whereas the precancerous

and cancerous fibrils correlation levels remain elevated as distance increased, implying a less defined fibrillar structure. Furthermore, the correlation in the cancerous fibrils is even greater with distance in comparison with the correlation in the precancerous fibrils, suggesting that fibrils in the precancerous group still retain some normal fine structure. Quantitatively, the collagen fibrils structure in normal case is 0.19 ± 0.03, in precancer is 0.51 ± 0.07, and in cancer is 0.68 ± 0.10. So collagen presence, collagen−fibril bundles orientation, and collagen−fibril structure can be taken as quantitative features to link between collagen alteration and epithelial tumor progression. Moreover, collagen in the surrounding tumor regions is denser than that in tumor regions, and this feature may provide a novel indicator to locate the tumor and determine the margin of tumor regions.

5. Differentiating Normal, Inflammatory, and Dysplastic Epithelial Tissues

During the development of epithelial cancer, a number of biochemical and biomorphological changes have been observed in the epithelial tissues.[18,19] Probing alterations in intrinsic optical emission induced by either biochemical or biomorphological changes in diseased tissues has shown the promise of truly noninvasive, accurate, and rapid detection of precancerous and cancerous lesions. MPM can be used to isolate the intrinsic emission contribution of epithelial cellular origins and stromal collagen in normal, inflammatory, and dysplastic epithelial tissues. The depth-cumulated epithelial redox ratio and stromal collagen quantity were also established to quantify changes in diseased tissues. It is found that with the appearance of inflammation, a significant decrease in ectocervical epithelial redox ratio is observed; in contrast, epithelial dysplasia shows an increase in epithelial redox ratio; both inflammatory and dysplastic epithelial tissues display a marked decrease in stromal collagen quantity. Specifically, the epithelial redox ratio in normal case is 61.7 ± 4.3, in inflammation is 51.1 ± 3.8, and in dysplasia is 72.3 ± 6.1. Both inflammatory and dysplastic epithelial tissues display some voids in stroma, indicating a marked loss of collagen. The stromal collagen quantity in normal case is 26.1 ± 1.9, in inflammation is 20.4 ± 3.2, and in dysplasia is 19.8 ± 2.7. These results suggest that both inflammatory and dysplastic epithelial tissues display a large decrease in stromal collagen quantity but have very different epithelial redox ratio. Therefore, a possible way to differentiate normal, inflammatory, and dysplastic ectocervical epithelial tissues is to probe differences in epithelial redox ratio in addition to stromal collagen quantity.[20]

In conclusion, MPM has the ability to identify layer structure of esophagus, oral cavity, and skin, and bronchus tissues, establishing the diagnostic features for distinguishing gastric normal tissue from cancerous tissue, linking collagen alteration and epithelial tumor progression for evaluating ectocervical epithelial tumor progression and differentiating normal, inflammatory, and dysplastic ectocervical epithelial tissues. With the advancement of clinically miniaturized MPM and multiphoton probe, multiphoton endoscopy may assist in real-time *in vivo* early diagnoses of epithelial cancer at the cellular or subcellular level in the future.

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