Establishing diagnostic features for identifying the mucosa and submucosa of normal and cancerous gastric tissues by multiphoton microscopy

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Background: Establishing diagnostic features is essential and significant for developing multiphoton endoscopy to make an early diagnosis of gastric cancer at the cellular level. Until now, these diagnostic features have not been clearly described and understood.

Design: Study of diagnostic features based on multiphoton microscopy (MPM).

Objective: Establishing diagnostic features to identify the mucosa and submucosa of human normal and cancerous gastric tissues by investigating their multiphoton microscopic images.

Setting: Fujian Normal University and Fujian Provincial Tumor Hospital.

Patients: Ten pairs of normal and cancerous specimens were obtained from 10 patients (ages 51-68 years) undergoing radical gastrectomy.

Interventions: MPM was performed on specimens.

Main Outcome Measurements: Establishment of diagnostic features.

Results: MPM has the ability to exhibit not only the mucosal and submucosal microstructures of normal and cancerous gastric tissues but also the distribution and content of abnormal cells in these 2 layers. More importantly, it can provide the diagnostic features to qualitatively and quantitatively differentiate between normal and cancerous gastric tissues.

Limitations: The selection bias and preparation of specimen.

Conclusions: These findings provide the groundwork for further establishing diagnostic criteria. (Gastrointest Endosc 2011;73:802-7.)

Endoscopically determined diagnosis in real time in vivo at the cellular level is the clinical key to increasing the survival rate of early gastric cancer. Confocal laser endomicroscopy can allow high-resolution in vivo histology...
assessments.\textsuperscript{1,2} Moreover, confocal reflectance microscopy shows the potential for diagnosing cancer without the use of contrast agents.\textsuperscript{3} However, the emergence of multiphoton microscopy (MPM) based on intrinsic 2-photon excited fluorescence (TPEF) and second harmonic generation (SHG) has attracted more attention. TPEF is a nonlinear process in which the fluorophore absorbs 2 photons with lower energy simultaneously and emits a single photon of fluorescence with higher energy. SHG is a coherent scattering process in which 2 photons with lower energy are combined to create a single photon of exactly twice the lower energy.\textsuperscript{4,5} These elements of MPM offer significant advantages over confocal imaging techniques for imaging in thick tissue and live animals, such as greater imaging penetration depth and reduced out-of-focus photobleaching and phototoxicity, which permit more long-term fluorescence observation.\textsuperscript{6,8} Recently, it has been applied to imaging the gastric mucosa\textsuperscript{9,10} and was developed into a multiphoton endoscope for neurobiology research.\textsuperscript{11,12} In addition, the miniaturized multiphoton tomography DermaInspect (JenLab GmbH, Jena, Germany) and multiphoton probe allows the clinical use of multiphoton endoscopy for diagnosing cancer.\textsuperscript{9,13,14} Thus, establishing diagnostic features is essential and significant for developing multiphoton endoscopy to allow early diagnosis of gastric cancer. Until now, these diagnostic features have not been clearly described and understood. In this report, we attempted to establish diagnostic features to distinguish between normal and cancerous gastric tissues.

MATERIALS AND METHODS

Sample preparations

Ten pairs of normal and cancerous specimens with mucosal and submucosal structures 1 to 1.5 cm wide and 0.2 cm thick were obtained from 10 patients (ages 51-68 years) undergoing radical gastrectomy at the Fujian Provincial Tumor Hospital. Written informed consent was obtained from each patient. The normal gastric tissue was 6 cm away from the cancer margin. Each gastric specimen was divided into 2 parts: 1 part of each specimen was cut into 5-μm transverse tissue slices for MPM imaging and 1 part was stained with hematoxylin and eosin for histological images, showing that 10 pairs of gastric specimens were 10 normal tissue and 10 were cancerous tissue, respectively. During our investigations, we usually confirmed the observed results from the MPM images by comparing with the histological images of paired sections.

The MPM microscopic imaging system

The multiphoton microscope used in this study was described previously.\textsuperscript{15} In short, the TPEF/SHG images were acquired by using an LSM 510 META system (Zeiss, Jena, Germany) coupled with a Ti:sapphire laser (Mira 900-F; Coherent Inc, Santa Clara, Calif). An oil immersion objective (Plan-Apochromat 63X, NA 1.4; Zeiss) was used for focusing the excitation beam on the samples and collecting the backscattered TPEF/SHG signals. Each channel of the META detector covers a spectral width of approximately 340 nm (range 377-716 nm) and the detected wavelength range depends on the collected signals. In this study, to simultaneously obtain high-contrast TPEF and SHG images, the 800-nm excitation light was chosen. Two channels were selected to image collagen and fluorescence components, respectively. One channel corresponding to the wavelength range of 387 to 409 nm showed the microstructure of collagen, whereas another channel covered a range from 430 to 708 nm to collect TPEF signals. The TPEF images of intrinsic components were revealed in the first column (green color coded) and the SHG images of collagen were shown in the second column (red color coded), whereas the overlay image showing their high-contrast images were displayed in the last column in Figures 1 and 2. All images had a 12-bit pixel depth. The images were obtained at 2.56 μs per pixel.

Quantification of morphological features

To quantitatively describe the differences in morphological features between normal and cancerous gastric tissue, the nuclear area was defined as the area of nuclear boundary. The collagen area was defined as the ratio of the SHG pixels over the whole pixels in each image to show the variation of collagen. Gastric gland orientation (GGO) was defined as the angle difference between gastric glands. Specifically, the inclination angle between each gastric gland relative to vertical axis was defined as each gastric gland direction angle (<90 degrees); the angle difference was the difference between 2 adjacent direction angles. In this study, each quantitative analysis was performed on all the samples by 2 experienced individuals in identification of MPM images. For each sample, 3 random positions were selected.

RESULTS

MPM images of normal gastric mucosa and submucosa

Figure 1 shows the representative TPEF/SHG images of normal gastric mucosa and submucosa. Three rows from
top to bottom correspond to the microstructures of the mucosa epithelium, lamina propria, and submucosa, respectively. In the mucosa epithelium (row 1), the boundary of epithelial cells and regular arrangement are quite distinctly observed. A small amount of collagen fibers from the lamina propria extends to the epithelium and forms numerous ridges. The area between the 2 ridges is the gastric pit. The basement membrane separating the lamina propria from the epithelium is also identified. The lamina propria is composed of collagen fibers and many gastric glands in row 2. Their distributions have a very good orientation. Many cells can be identified in each gastric gland based on black holes originating from nonfluorescent nuclei. In the gastric submucosa (row 3), there are 4 completely different microstructures: collagen fibers, collagen bundles, elastic fibers, and blood vessel. Collagen fibers show a fine mesh of morphology, elastic fibers present a morphology of long ropes, and blood vessels form a circular structure. A very interesting result of the collagen bundle was found in this layer. The collagen bundle has comparable SHG and TPEF signals. To exactly show this characteristic, its spectrum was obtained by using the image-guide spectral analysis method, as shown in Figure 3. Except for the SHG signal at 400 nm, the TPEF signal between 430 nm and 650 nm with a peak around 500 nm was also detected. These are consistent with the results of a purified collagen sample. This can be a sign to identify the observed collagen, whether in the mucosa or the submucosa.

**Multiphoton images of cancerous gastric mucosa and submucosa**

Figure 2 displays the representative TPEF/SHG images of cancerous gastric mucosa and submucosa. Rows
1 and 2 correspond to the microstructures of the mucosa epithelium and lamina propria, respectively. Rows 3 and 4 all come from submucosa. In comparison with normal epithelial cells, the abnormal epithelial cells apparently enlarge and vary in size and form the arrangement of a circular lumen. Around the body of the cavity, some cells with circular nuclei and irregular sizes can be clearly discerned. These cells are possibly inflammatory cells or cancer cells or lymphocytes. In this study, these cells were called abnormal cells. Compared with normal tissue, the collagen fibers, basement membrane, and gastric pit disappear in cancerous epithelium. The gastric glands in gastric cancer demonstrate an irregular shape and disordered arrangement in row 2. The collagen contents obviously decrease and many abnormal cells infiltrate and displace the locations of collagen fibers. In each gastric gland, the gland cavity with an irregular morphological structure shows a large black hole. In the submucosa (row 3), the collagen displays a significant loss compared with normal tissue.

Figure 2. Representative TPEF/SHG images of cancerous gastric mucosa and submucosa. Rows 1 and 2 correspond to the microstructures of the mucosa epithelium and lamina propria, respectively. Rows 3 and 4 are from the submucosa. In row 1, yellow circles indicate abnormal epithelial cells, and red circles indicate the nuclei of abnormal cells. In row 2, pink circles indicate the boundary locations of representative gastric glands; red circles indicate the nuclei of representative cells; blue circles indicate the position of the gland cavity. In row 3, blue arrows, pink arrows, red circles, and the blue circle indicate collagen bundles, elastic fibers, abnormal cell nuclei, and the lumen of the blood vessel, respectively. In row 4, red circles indicate the nuclei of abnormal cells and blue circles indicate the cavity of glandular tissue. Scale bar is 50 μm.
The residual collagen bundles become very thin, whereas elastic fibers become thick. Many abnormal cells aggregate in this layer, showing the same morphological characteristics as those of the mucosa. The blood vessel wall becomes distorted and uneven. In row 4, the abnormal cells tend to form the glandlike tubular structure, which is a typical characteristic of adenocarcinoma. These qualitative morphological variations correlate well with the paired histological sections.

**Quantitative analyses of normal and cancerous gastric tissues**

The quantitative results reveal that the abnormal epithelial cells (160.60 ± 77.48 μm²) enlarge by a factor of 2 on average compared with normal epithelium cells (80.31 ± 11.19 μm²). The nuclear areas of other abnormal cells reveal a similar value: 29.58 ± 15.50 μm² in the mucosal epithelium, 33.34 ± 12.69 μm² in the lamina propria, 36.49 ± 16.46 μm² (row 3), and 34.18 ± 14.06 μm² (row 4) in the submucosa, showing completely different sizes compared with cells in the gastric glands (26.25 ± 3.78 μm²). All the abnormal cells have large standard deviation, indicating that nuclei of different cells vary more in size. The calculated results of GGO show that the GGO in normal tissue is 3.4 ± 1.6 degrees, whereas in gastric cancer, it is 53.6 ± 15.8 degrees, indicating that the arrangement of the gastric glands in gastric cancer is more disordered. The collagen area in these 2 layers was also determined, as shown in Table 1. Compared with normal tissue, the collagen content displays a very apparent loss in gastric cancer. The same tendency of quantitative results is also seen in the histological images.

**DISCUSSION**

In this study, we used MPM to obtain the microstructures of the mucosa and submucosa of human normal and cancerous gastric tissues. Our results show that the content, distribution, and morphology of the main components in these 2 layers have apparently different properties. Several diagnostic features can be extracted to distinguish between normal and cancerous gastric tissues. First, the appearance of abnormal cells in gastric cancer is an important indicator. The formation of cancer cells is usually accompanied by the appearance of inflammatory cells and lymphocytes. Their major features show that cell nuclei vary in size, with an especially marked nuclear enlargement. The appearance of abnormal cells also changes the structures of the gastric mucosa, such as the absence of the gastric pit and basement membrane. In particular, the abnormal cells are partially polarized or nonpolarized, leading to the disordered arrangement of gastric glands.

Second, the significant loss of collagen in gastric cancer is also a significant feature. Neoplastic transformation is accompanied by some alterations in biosynthesis of extracellular matrix proteins, especially type I collagen. During the carcinogenesis, many abnormal cells infiltrate the mucosa and submucosa and replace the collagen, which promotes the expression of matrix-degrading proteases leading to the breakdown of collagen. The same result is also observed in the inflammatory and dysplastic oral tissues. The significant loss of collagen results in the disappearance of its regular arrangement.

Last, the distorted and uneven structure of blood vessel wall in cancerous submucosa is not negligible. In cancerous tissue, proliferating cancer cells may compress the immature blood vessel wall. Furthermore, some endothelial cells in the blood vessel wall do not express common endothelial markers and may undergo apoptosis, thus resulting in the exposure of cancer cells to the lumen. This causes the irregular architecture of the blood vessel.

In this study, the examined normal tissues were obtained from patients with gastric cancer. There may be little difference compared with normal tissues from healthy individuals. To study the microstructures of the mucosa and submucosa, the sample preparation of transverse tissue slices may cause unclear imaging of cell boundary in gastric glands.

However, in conclusion, we demonstrated that MPM has the ability to establish the diagnostic features to distinguish between normal and cancerous gastric tissues. These features are essential and significant for developing multiphoton endoscopy to make an early diagnosis of gastric cancer. With the advancement of clinically miniaturized MPM and the multiphoton probe, combining MPM with standard endoscopy will therefore allow us to make
a real-time in vivo early diagnosis of gastric cancer at the cellular level.

REFERENCES


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**TABLE 1. Comparison of the collagen area in the mucosa and submucosa of normal and cancerous gastric tissues**

<table>
<thead>
<tr>
<th>Tissue layer</th>
<th>Normal tissue</th>
<th>Lamina propria</th>
<th>Submucosa</th>
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<tbody>
<tr>
<td>Mucosa epithelium</td>
<td>0.042 ± 0.007</td>
<td>0.317 ± 0.021</td>
<td>0.629 ± 0.008</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>0.075 ± 0.014</td>
<td>0.061 ± 0.002</td>
<td>0.226 ± 0.019</td>
</tr>
<tr>
<td>Submucosa</td>
<td>0.226 ± 0.019</td>
<td>0.226 ± 0.019</td>
<td>0.226 ± 0.019</td>
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</tbody>
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