Label-Free Assessment of Premalignant Gastric Lesions Using Multimodal Nonlinear Optical Microscopy

Lianhuang Li, Deyong Kang, Changyin Feng, Shuangmu Zhuo, Haohua Tu, Yongjian Zhou, and Jianxin Chen

***Abstract*—In this paper, a nonlinear optical microscopy employing two-photon excited fluorescence and second-harmonic generation was used for the detection of premalignant gastric lesions. It was found that gland morphology and collagen structure in mucosa will change with the progression of gastric diseases from normal to intestinal metaplasia, to low-grade intraepithelial neoplasia, and to high-grade intraepithelial neoplasia, and this microscopy was able to directly distinguish these warning symptoms. Furthermore, two features were quantified from nonlinear optical images to demonstrate the changes of gland size and collagen content during the development process of preneoplastic lesions. These results clearly show that nonlinear optical microscopy can effectively differentiate normal and precancerous gastric tissues without contrast agents, which would be helpful for early diagnosis and treatment of gastric diseases. This study may provide the groundwork for further application of nonlinear optical microscopy in clinical practice.**

***Index Terms*—Nonlinear optical microscopy, gastric intestinal metaplasia (GIM), low-grade intraepithelial neoplasia (LGIN), high-grade intraepithelial neoplasia (HGIN).**

I. Introduction

GASTRIC cancer is one of the most common malignant diseases of gastrointestinal tract [1], [2]. Currently, strategies to improve prognosis is strictly dependent on earlier detection of preneoplastic and neoplastic transformations since only gastric intestinal metaplasia, intraepithelial neoplasia or early gastric cancer can potentially be cured by endoscopic therapy [3]. Although it is commonly believed that gastric intestinal metaplasia and intraepithelial neoplasia are precancerous lesions, current medical imaging techniques lack precision and uniformity in discriminating these diseases, and therefore might hamper appropriate treatment decisions such as either long-term follow-up or endoscopic treatment.

At present, premalignant lesions can be readily identifiable by pathologists using routine hematoxylin and eosin-stained (H&E) sections of biopsy samples, but histological biopsies have several disadvantages including sampling error as well as time-consuming pathological procedure, and will result in substantial increase of additional pathology costs and possibly a higher rate of complications such as bleeding or perforation [4], [5]. Therefore, development of a new imaging technology, that is capable of identifying precursor lesions, will be of tremendous help.

Nonlinear optical microscopy using two-photon excited fluorescence (TPEF) combined with second-harmonic generation (SHG) is a potentially attractive technique for the diagnosis of precancerous changes. This imaging technique offers many advantages for biological tissues: TPEF and SHG are intrinsic signals and thus obviate the need for exogenous stains; near-infrared laser excitation minimizes scattering and absorption of the source and is very suitable for studying thick tissue samples [6]–[8]; additionally, SHG would not suffer from phototoxicity effects or photobleaching as it does not involve excitation of molecules [9], [10].

It is well known that the development of precancers is accompanied by changes in cellular and subcellular morphology as well as in extracellular collagen matrix [4], [11]. Nonlinear optical microscopy enables the visualization of cellular and subcellular structures and collagen structure with exceptional resolution because of the presence of endogenous autofluorescent molecules such as NADH and FAD and connective tissue known to induce SHG signal within gastric tissues. Thus, in this study, we try to examine whether this microscopy is useful for distinguishing both intestinal metaplasia and intraepithelial neoplasia from normal gastric mucosa.

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II. Materials and Methods

A. Sample Preparation

This study was conducted with the approval of the institutional review board at the Fujian Medical University Union Hospital, and signed informed consent was obtained from each patient. In this work, 20 fresh biopsy samples including 5 normal gastric tissues, 5 gastric mucosal intestinal metaplasia tissues, 5 low-grade intraepithelial neoplasia tissues and 5 high-grade intraepithelial neoplasia tissues were collected. Once samples were removed by surgeons, they were sent to the pathology laboratory immediately, and each specimen was serially sectioned at 10 μm by cryostat microtome. Then three serial tissue slices were chosen for research, where the middle slice was stained with H&E to confirm experimental results, and other two sections were used for nonlinear optical imaging.

All of the H&E-stained slices were reviewed by two certified pathologists, and images were then taken using a standard bright field light microscope (Eclipse Ci-L, Nikon Instruments Inc., Japan) with a CCD (DS-Fi2, Nikon). The results from nonlinear optical microscopy were then compared with the H&E images for confirmation. In addition, to avoid dehydration or shrinkage during the imaging process, a small amount of phosphate-buffered saline was applied to the specimen.

B. Nonlinear Optical Imaging System

The nonlinear optical imaging system used in this work has been previously described in detail [12], [13]. In brief, an inverted microscope (LSM 510 META, Zeiss, Germany) equipped with a mode-locked femtosecond Ti:sapphire laser (Mira 900-F, Coherent, Inc., USA) was used to obtain nonlinear optical images. An oil immersion objective (Plan-Apochromat 63×, NA = 1.4, Zeiss, Germany) was used for focusing the excitation beam into samples and also for collecting the backscattered intrinsic TPEF and SHG signals. The lateral and axial resolutions of this imaging system are 0.3 μm and 0.8 μm respectively at an excitation wavelength of 810 nm. The META detector with eight independent channels in this system consists of a reflective grating and an optimized 32-channel PMT array detector to collect emission signals within the random range from 377 to 716 nm. In this work, two independent channels were chosen to collect TPEF and SHG signals, where one channel covered the wavelength range from 430 to 716 nm for collection of TPEF signal and another channel covered the wavelength range from 389 to 419 nm for collection of SHG signal. To increase the contrast of TPEF/SHG images, the TPEF image was color coded in red and the SHG image was color coded in green.

C. Statistical Analysis

To quantitatively assess changes in gland morphology and collagen content in lamina propria during the development of premalignant gastric lesions, gland circumference and SHG mean intensity per unit area were measured, where the gland circumference was defined as the perimeter of basement membrane, and the SHG mean intensity per unit area was calculated for each image by dividing the sum of all intensities by the total area and SHG images were analyzed using ImageJ software. These values were expressed as means and standard deviations. All statistical analyses were done using the IBM SPSS Statistics 21. Statistical significance was determined using the student's t-test, and P values of ≤ 0.05 were considered statistically significant.

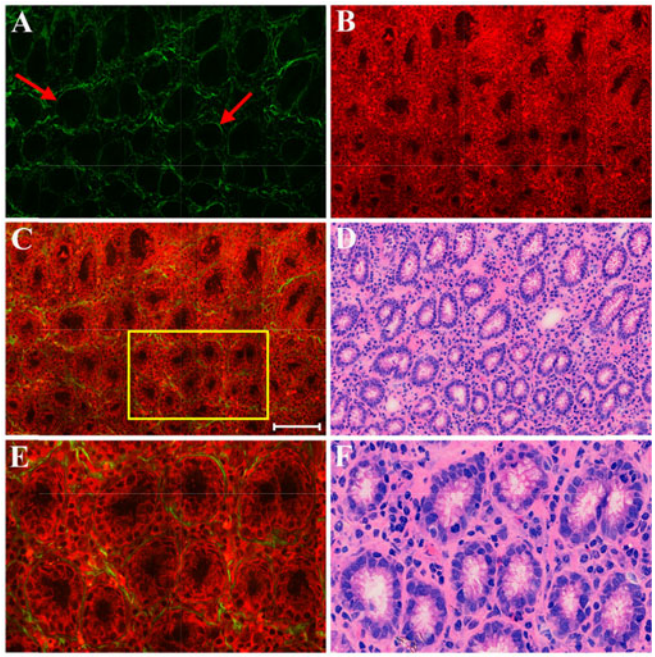

Fig. 1. Representative nonlinear optical images of normal gastric mucosa and corresponding H&E-stained images. (A) SHG image. (B) TPEF image. (C) Merging of SHG and TPEF images. (D) H&E-stained image. (E–F) Zoom-in image of the boxed region in (C) and corresponding H&E-stained image. Red arrow: basement membrane. Scale bar: 100 μm.

III. Results

A. Nonlinear Optical Imaging

The focus of the first part of this work is to qualitatively determine whether nonlinear optical microscopy can identify the morphologic differences between normal and precancerous gastric tissues. Fig. 1 shows representative nonlinear optical images of normal gastric mucosa and corresponding H&E-stained images. It reveals that normal mucosa is mainly composed of gastric glands and collagen fibers. More specifically, TPEF image demonstrates that individual gland composed of epithelial cells is readily differentiated, and cell nuclei with uniform size arrange regularly along basement membrane (red arrow in Fig. 1(A)), and these same details of cellular architecture are in agreement with the corresponding H&E-stained image of the paired histologic section (Fig. 1(D)). Collagen is an important extracellular matrix and plays a key role in maintaining cell behavior [14], [15]. SHG image shows that collagen fibers form a fine mesh of morphology to sustain the gastric glands, which cannot be observed directly from the H&E-stained image (Fig. 1(D)).

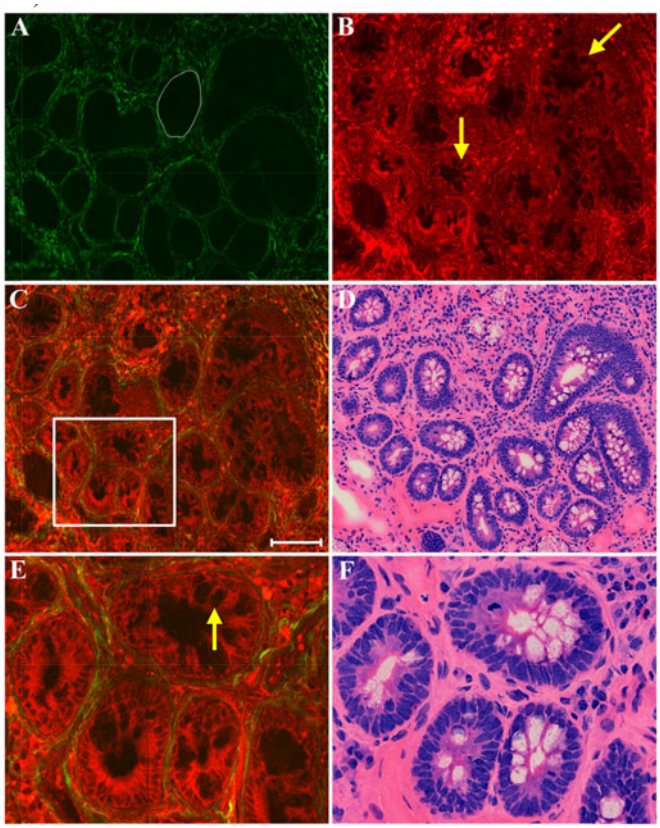

Fig. 2. Representative nonlinear optical images of gastric intestinal metaplasia and corresponding H&E-stained image. (A) SHG image. (B) TPEF image. (C) Merging of SHG and TPEF images. (D) H&E-stained image. (E)–(F) Zoom-in image of the boxed region in (C) and corresponding H&E-stained image. Yellow arrow: goblet cell; white circle: gland circumference. Scale bar: 100 μm.

Figure 2 shows representative nonlinear optical images of gastric intestinal metaplasia and corresponding H&E-stained image. Each gastric gland and individual epithelial cell nuclei can also be identified by TPEF image. The extracellular stroma is more prominent in SHG image (Fig. 2(A)) than in H&E image (Fig. 2(D)). For example, connective tissue separating glands could be detected too via SHG of collagen, and the basement membrane was readily identified as a thin band surrounding individual glands. However, unlike in normal mucosa, many goblet cells were detected by the obvious mucin droplets (yellow arrow in Fig. 2(B) and (E)) in glandular lumen. It is clear that goblet cells present distinctive characteristic, and are easily identified because the mucin droplets cannot generate nonlinear optical signal and are darker than surrounding structures. This feature proves that intestinal metaplasia occurs in the gastric mucosa, and is a major indicator for neoplastic transformation. The same details of cellular architecture correlate readily with the corresponding H&E image (Fig. 2(D)).

Figure 3 shows representative nonlinear optical images of low-grade intraepithelial neoplasia and corresponding H&E-stained image. Unlike what was observed in normal gastric mucosa, although the tubular-shaped architecture is maintained, the glands are enlarged and irregular in size and shape. During precancerous development in the mucosa, neoplastic cells will

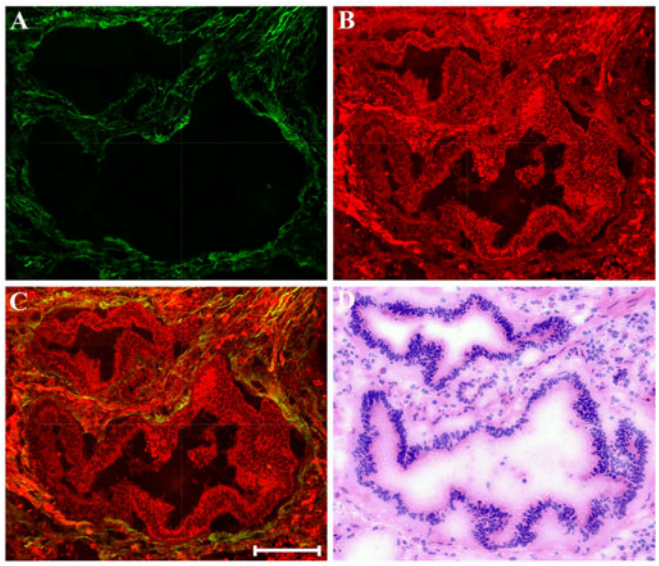

Fig. 3. Representative nonlinear optical images of low-grade intraepithelial neoplasia and corresponding H&E-stained image. (A) SHG image. (B) TPEF image. (C) Merging of SHG and TPEF images. (D) H&E-stained image. Scale bar: 100 μm.

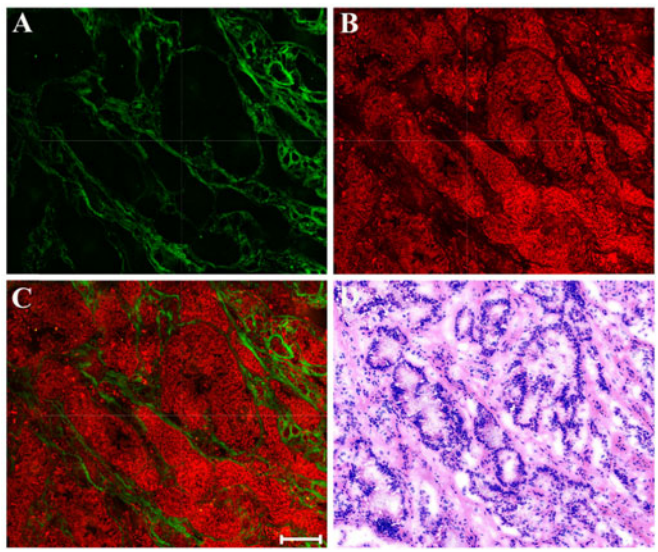

Fig. 4. Representative nonlinear optical images of high-grade intraepithelial neoplasia and corresponding H&E-stained image. (A) SHG image. (B) TPEF image. (C) Merging of SHG and TPEF images. (D) H&E-stained image. Scale bar: 100 μm.

destroy and then remodel the extracellular matrix. The interglandular space between gastric glands is apparently reduced, and the collagen content obviously decreases. These morphologic changes are consistent with those identified with the corresponding H&E-stained image (Fig. 3(D)).

Figure 4 shows representative nonlinear optical images of high-grade intraepithelial neoplasia and corresponding H&E-stained image. The architecture of glands is severely distorted, and it is difficult to identify a single gland because the glands begin to fuse together. The collagen fibers become disordered

TABLE I
QUANTITATIVE VARIABLES FOR DIFFERENTIATING NORMAL AND PRECANCEROUS GASTRIC MUCOSAL TISSUES WITH NONLINEAR OPTICAL MICROSCOPY

Samples	Quantitative variables	
	Gland circumference (μm)	SHG mean intensity per unit area (μm^{-2})
Normal	220.64 ± 51.34	930.90 ± 150.07
Intestinal metaplasia	308.30 ± 57.25	871.34 ± 182.32
Low-grade intraepithelial neoplasia	595.64 ± 308.97	563.06 ± 168.96
High-grade intraepithelial neoplasia	N/A	368.30 ± 218.49

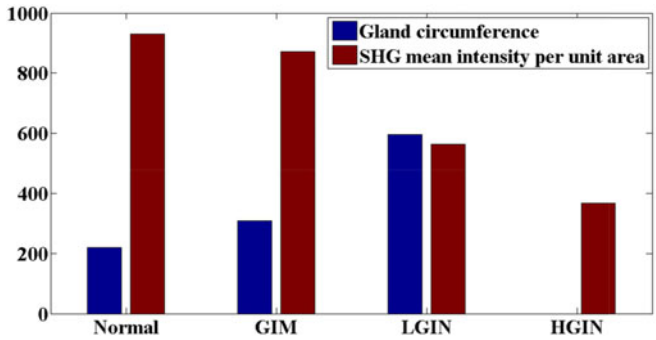

Fig. 5. The gland circumference and SHG mean intensity per unit area from normal gastric mucosa, intestinal metaplasia, low-grade and high-grade intraepithelial neoplasia, respectively.

and the glands only rarely are separated by collagen bands. A relationship seems to exist between the change of collagen matrix and the progression of early epithelial carcinogenesis. These microstructural changes correlate readily with the corresponding H&E image (Fig. 4(D)).

B. Quantitative Analysis

To determine whether these morphologic features were statistically different between normal and premalignant gastric tissues, two features were quantified from nonlinear optical images. The gland circumference was used to describe the change in gland size, and SHG mean intensity per unit area was used for assessing the change in collagen content with the development of preneoplastic lesions. Table I shows the mean and standard deviation (SD) of the gland circumference and SHG mean intensity per unit area for normal, intestinal metaplasia, low-grade intraepithelial neoplasia, and high-grade intraepithelial neoplasia samples.

The gland circumference was determined by measuring the perimeter of basement membrane (shown as the white circle in Fig. 2(A)) manually. For every sample from normal to intestinal metaplasia and to low-grade intraepithelial neoplasia, fifteen random glands were selected, and the gland circumference was determined as the mean perimeter of the seventy-five glands. It tends to increase as gastric mucosal tissues progress from a normal to intestinal metaplasia to intraepithelial neoplasia. Specifically, the gland circumference in normal mucosa is 220.64 $\pm$ 51.34 μm, in intestinal metaplasia is 308.30 $\pm$ 57.25 μm, in low-grade intraepithelial neoplasia is 595.64 $\pm$ 308.97 μm, however, in high-grade intraepithelial neoplasia is uncertain because the glands fuse together and thus boundary between them cannot be discerned accurately. Furthermore, we also provide a bar chart (Fig. 5) of the statistics to show the significance between different groups. Statistically significant differences were found between both normal and intestinal metaplasia and low-grade intraepithelial neoplasia, however, there was a lack of statistically significant difference in the circumference of gland between normal and intestinal metaplasia.

For each sample, three random images with the same size were chosen for calculating the SHG mean intensity. As shown in Table I, the mean and SD of SHG mean intensity per unit area from normal mucosa is 930.90 $\pm$ 150.07 μm^{-2}, from intestinal metaplasia is 871.34 $\pm$ 182.32 μm^{-2}, from low-grade intraepithelial neoplasia is 563.06 $\pm$ 168.96 μm^{-2}, and from high-grade intraepithelial neoplasia is 368.30 $\pm$ 218.49 μm^{-2}, indicating that the collagen content in mucosa tends to decrease when mucosal tissues progress from normal to intestinal metaplasia to low-grade intraepithelial neoplasia and to high-grade intraepithelial neoplasia. Statistically significant differences in the SHG mean intensity per unit area were also observed between both normal and intestinal metaplasia and both low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia, but there were no significant differences between normal and intestinal metaplasia as well as low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia. It is obvious that the detection of the signal from collagen could provide us an approach to identify the precancerous progression.

IV. DISCUSSION

Gastric intestinal metaplasia and intraepithelial neoplasia are generally considered to be precancerous lesions in the gastric carcinogenesis cascade. These lesions in the stomach increase the risk of gastric cancer, and the increased risk is proportional to their development extent. Early detection, accurate characterization, and resection of the precursor lesions are optimal for the prevention of this malignancy [16], [17]. In recent years, substantial interest has arisen in the diagnosis and management of premalignant lesions of the gastric mucosa because of the high cure rate achieved with treatment of these lesions [18]. Endoscopic biopsies are still the standard of practice for the histologic diagnosis of abnormal mucosal lesions. However, there are disadvantages to performing mucosal biopsy procedures, including sampling error, costs, risks to the patient, and the delay in obtaining results [19].

It has been previously shown that the development of precancers is accompanied by changes in cellular and subcellular morphology [20], [21]. At the same time, neoplastic cells will change the extracellular matrix during precancerous development in mucosa. Thus, visualization of these structures is important. It is possible to optically detect cellular and

subcellular detail and monitor collagen change in the mucosa using nonlinear optical microscopy because there are many endogenous fluorescent molecules within gastric tissues such as elastin, NADH and FAD, and collagen is known to induce second-harmonic generation [22]. Thus, we assess the feasibility of this microscopy for pathological evaluation of the progression of premalignant gastric lesions in this study.

Our results show that nonlinear optical microscopy is an acceptable and potentially useful technology for the identification of gastric intestinal metaplasia and grading of gastric intraepithelial neoplasia. It not only provides information on the microscopic morphologic changes that occur in intestinal metaplasia and intraepithelial neoplasia, but also provides quantitative information including the changes of gland size and collagen content in mucosa. Statistical data reflect that the gland circumference and SHG mean intensity per unit area will change at the different pathological stages of gastric mucosa. There are statistically significant differences in the circumference of gland and SHG mean intensity between normal gastric mucosa and intraepithelial neoplasia; however, statistically significant differences do not exist between the normal mucosa and intestinal metaplasia. Hence, these two variables may be helpful to distinguish normal from intraepithelial neoplasia tissues.

In summary, nonlinear optical microscopy can be used to assess morphologically whether a tissue is normal, intestinal metaplasia, low-grade intraepithelial neoplasia, or high-grade intraepithelial neoplasia. Moreover, this microscopy does not require staining and in the future could be applied in vivo in a clinical setting with multiphoton endoscopy [23], [24]. This work demonstrates that nonlinear optical microscopy employing two-photon excited fluorescence and second-harmonic generation has the potential to perform real-time diagnosis of dysplasia and suspicious lesions during surveillance examinations and lead to better management of these diseases.

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