Basic Study

Multiphoton microscopy for tumor regression grading after neoadjuvant treatment for colorectal carcinoma

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Abstract

AIM: To evaluate the feasibility of using multiphoton microscopy (MPM) to assess a tumor regression grading (TRG) system.

METHODS: Fresh specimens from seven patients with colorectal carcinoma undergoing neoadjuvant radiochemotherapy at the Fujian Medical University Union Hospital were obtained immediately after proctectomy. Specimens were serially sectioned (10 µm thickness) and used for MPM or stained with hematoxylin and eosin for comparison. Sections were imaged by MPM using 810 nm excitation, and images were collected in two wavelength channels corresponding to second-harmonic generation (SHG) and two-photon excited fluorescence (TPEF) signals. The ratio of these signal intensities was used to distinguish fibrosis from normal mucosal and serosal tissues.

RESULTS: TRG of specimens assessed by MPM...
were in complete agreement with histologic grading performed by a consulting pathologist. SHG and TPEF images clearly revealed collagen fibers and fragmented elastic fibers in the muscularis propria specimens following neoadjuvant radiochemotherapy. Additionally, blood vessel hyperplasia was observed as thickening and fibrosis of the intima and media, which was accompanied by minimal inflammatory cell infiltration. Furthermore, the SHG/TPEF ratio in stromal fibrosis (4.15 ± 0.58) was significantly higher than those in the normal submucosal (2.31 ± 0.52) and serosal (1.47 ± 0.10) tissues (P < 0.001 for both). Analysis of emission spectra from cancerous tumor cells revealed two peaks corresponding to nicotinamide adenine dinucleotide hydrogen and flavin adenine dinucleotide signals; the ratio of these values was 1.19 ± 0.02, which is close to a normal metabolic state.

CONCLUSION: MPM can be used to perform real-time diagnosis of tumor response after neoadjuvant treatment, and can be applied to evaluate TRG.

Key words: Multiphoton microscopy; Neoadjuvant treatment; Second-harmonic generation; Tumor regression grading; Two-photon excited fluorescence

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Core tip: This study evaluated the feasibility of using multiphoton microscopy for the assessment of a tumor regression grading system. Multiphoton microscopy allows diagnostic features of colorectal carcinoma treated with neoadjuvant therapy to be visualized. Quantitative image analyses can be used to distinguish fibrotic tissue from normal submucosal and serosal tissues. This is the first study demonstrating the application of multiphoton microscopy for tumor regression grading.

INTRODUCTION

For patients with locally advanced gastrointestinal cancer, neoadjuvant therapy followed by surgery or perioperative treatment provides a survival benefit over surgery alone, particularly for patients with complete or subtotal tumor regression[1-5]. As such, assessment of therapeutic response and evaluation of residual disease are very important. The response to therapy can be evaluated histologically via a tumor regression grading (TRG) system, which has been shown to correlate with survival[6,7]. The TRG system aims to categorize the extent of regressive changes with consideration of the percentage of the tumor that is residual and the degree of therapy-induced fibrosis[7-10]. This system provides valuable prognostic information, and may serve as a morphologic indicator for neoadjuvant treatment and surgery[11,12].

Histopathologic evaluation of resected specimens can be subject to crush artifacts and sampling error, and involves time-consuming pathologic procedures[13,14]. In contrast, multiphoton microscopy (MPM), which relies on the nonlinear optical processes of second-harmonic generation (SHG) and two-photon excited fluorescence (TPEF), provides high resolution visualization of cell morphology and tissue architecture without the use of exogenous contrast agents[15,16]. The value of this method for use in the TRG system has not been examined. Therefore, the purpose of this study was to evaluate the accuracy and feasibility of MPM for optical diagnoses with the TRG system.

MATERIALS AND METHODS

Specimen preparation

Fresh specimens were obtained immediately after proctectomy from seven patients with colorectal carcinoma undergoing neoadjuvant radiochemotherapy at the Fujian Medical University Union Hospital. Normal tissue specimens were also obtained 6 cm away from the cancer margin. This investigation was approved by the Institutional Review Board of the Fujian Medical University Union Hospital. Written informed consent was obtained before study participation from patients. Specimens were sectioned (10 µm thickness) and five serial slices were selected. The middle section was stained with hematoxylin and eosin (HE) for histologic comparison and imaged under a standard bright-field light microscope (Eclipse Ci-L; Nikon Corp., Tokyo, Japan) with a CCD (DS-Fi2; Nikon). The remaining four sections were used for MPM imaging.

MPM

MPM was performed using an LSM 510 META imaging system (Carl Zeiss AG, Jena, Germany) equipped with a femtosecond Ti:sapphire laser (110 fs, 76 MHz, Mira 900-F, Coherent Inc., Santa Clara, CA, United States) mode-locked at a wavelength of 810 nm as described previously[17,18]. A Plan-Apochromat oil immersion objective (× 63, numerical aperture = 1.4) was used for image acquisition to collect the backscattered intrinsic SHG and TPEF signals. SHG signals were collected in one channel with a wavelength range of 387-419 nm, and TPEF signals were collected in another channel with a wavelength range of 430-698 nm.

Histopathologic evaluation

TRG from MPM was performed by two independent
investigators who were blinded to the results. Tumor regression was classified into five histologic grades according to vital tumor tissue at the ratio of fibrosis:\[8\]: TRG-1: fibrosis without detectable tumor tissue (complete regression); TRG-2: fibrosis with scattered tumor cells; TRG-3: fibrosis and tumor cells with preponderance of fibrosis; TRG-4: fibrosis and tumor cells with preponderance of tumor cells; TRG-5: tumor tissue without regression. TRG scores were confirmed by comparison with corresponding HE-stained sections that were reviewed by a consulting pathologist (Table 1).

Table 1  Tumor regression grading scores as determined by histology and microscopy

| Patient | Sex | Age (yr) | Cancer classification | HE | MPM |
|---------|-----|----------|-----------------------|----|-----|
| 1       | Male| 44       | Adenocarcinoma        | TRG-1 | TRG-1 |
| 2       | Male| 38       | Adenocarcinoma        | TRG-2 | TRG-2 |
| 3       | Female| 67     | Adenocarcinoma        | TRG-2 | TRG-2 |
| 4       | Female| 59     | Adenocarcinoma        | TRG-1 | TRG-1 |
| 5       | Male| 59       | Adenocarcinoma        | TRG-2 | TRG-2 |
| 6       | Male| 57       | Adenocarcinoma        | TRG-3 | TRG-3 |
| 7       | Male| 47       | Adenocarcinoma        | TRG-2 | TRG-2 |

HE: Hematoxylin and eosin staining; TRG: Tumor regression grading; MPM: Multiphoton microscopy.

Quantification of morphologic features
All quantitative analyses were performed by two individuals experienced in identification of TPEF/SHG images. Collagen and elastin changes and metabolic status after neoadjuvant radiochemotherapy were quantified as SHG/TPEF and redox ratios.

Statistical analysis
One-way analyses of variance were conducted to compare differences using SPSS, version 15.0 statistical software (SPSS Inc., Chicago, IL, United States). Data are presented as mean ± SD, and a P-value < 0.05 was considered significant.

RESULTS
Stromal fibrosis
Representative MPM images depicting the predominant fibrosis with minimal inflammatory cell infiltration replacing large parts of a previous tumor in the muscularis propria following neoadjuvant radiochemotherapy are shown in Figure 1. The smooth muscle was divided due to prior cancer invasion, and the tumor underwent complete regression and was replaced by fibrous tissue mainly composed of collagen fibers, which simultaneously generated SHG (blue...
DISCUSSION

A recent meta-analysis found that partial tumor regression is associated with improvement in disease-free survival\[^{21}\]. Furthermore, TRG can serve as an independent predictor of disease-free and metastases-free survivals\[^{6,11,22}\]. The prognostic value of this measure may even exceed that of currently used staging systems (e.g., tumor-node-metastasis staging), which are based on characteristics of untreated tumors\[^{12}\]. The evaluation criteria of the TRG system incorporate the ratio of tumor cells to fibrosis. However, evaluating TRG using current approaches such as computerized tomography, magnetic resonance imaging, and positron emission computed tomography is challenging, as these medical imaging technologies lack sufficient resolution\[^{20}\].

MPM relies on nonlinear optical processes to achieve high resolution imaging of biologic tissues, and can detect cellular and subcellular tissue microstructures. Compared with its single-photon counterpart, TPEF offers an inherent optical sectioning property and deep penetration, and the nonlinear scattering from non-centrosymmetric structures provides complementary information to visualize endogenous structures in intact tissues. Residual tumor cells are detected by the TPEF signal, and the SHG signal is used to detect fibrotic tissue. The SHG/TPEF ratio can be used to distinguish fibrosis from submucosal and serosal tissues, as well as to quantify the fibrotic change, which has been proposed as a diagnostic indicator for gastrointestinal diseases\[^{24,25}\].

Neoadjuvant radiochemotherapy can result in significant morphologic changes, including disrupted muscles, tumor regression, and extensive fibrosis\[^{26}\]. Moreover, tumor cells in post-treatment rectal carcinoma can become more solid or retain a glandular growth pattern similar to untreated colorectal adenocarcinoma\[^{27}\]. These alterations were readily detected in the specimens evaluated by MPM in this study. Also observed was the division and damage of muscular tissue by invasion of the adenocarcinoma, which can cause destruction or elimination of collagen and elastic fibers\[^{24}\].

The redox ratio is an indicator of cellular metabolic state\[^{28}\]. As this is known to be accelerated in cancerous cells, the redox ratio can be used to quantitatively monitor tumor regression\[^{29}\]. In this work, the redox ratio in specimens was close to the metabolic state of normal cells\[^{29}\]. This result supports the notion that abnormal cells undergo significant regression after neoadjuvant radiochemotherapy, which can be assessed via MPM.

In conclusion, MPM was used to evaluate TRG in colorectal cancer after neoadjuvant treatment. Spectral analyses provided quantitative evaluations of tumor regression and fibrosis, which corresponded to TRG via histopathologic investigation. Given the advantages of this method, including the capacity to produce real-time, label-free images that can be acquired in the near-infrared range, MPM may represent a valuable tool in clinical practice.

![Figure 2: Ratios of second-harmonic generation and two-photon excited fluorescence intensity. Error bars indicate the standard deviation. SHG: Second-harmonic generation; TPEF: Two-photon excited fluorescence.](https://www.wjgnet.com)
tool to evaluate TRG after neoadjuvant therapy to treat colorectal carcinoma. This is the first demonstration of the use of MPM to estimate TRG of colorectal carcinoma following neoadjuvant treatment. The results suggest that MPM has a promising future for real-time optical biopsy diagnosis of tumor regression.

Figure 3 Representative multiphoton microscopy images from a muscularis propria specimen after neoadjuvant radiochemotherapy, color-coded green represents the second-harmonic generation signal and color-coded red corresponds to the two-photon excited fluorescence signal. Remaining malignant glands are dispersed deep within the rectal muscularis propria. A: Multiphoton microscopy of second-harmonic generation from collagen; B: Two-photon excited fluorescence of carcinomatous cells, inflammatory cells, and elastin; C: Overlay image (scale bar: 100 μm); D: Hematoxylin and eosin staining of an adjacent section (magnification × 40). Tumor cells in post-treatment rectal carcinoma show marked changes, and can become more solid (blue arrows) or retain a glandular growth pattern (white arrow). Fragmented elastic fibers are denoted by pink arrows.

Figure 4 Normalized emission spectrum of residual tumor cells. Excitation wavelength (810 nm) revealed two emission peaks (red arrows).

and should be included in histopathologic reports of colorectal carcinoma after neoadjuvant treatment. However, the resolution of current imaging approaches, such as computerized tomography, magnetic resonance imaging, and positron emission tomography, is insufficient for accurate and easy assessment. The purpose of this study was to evaluate the feasibility of using multiphoton microscopy (MPM) to obtain optical diagnoses for the TRG system.

Research frontiers
The TRG system provides highly valuable prognostic information for evaluating colorectal cancer after neoadjuvant treatment.

Innovations and breakthroughs
This is the first study evaluating the use of MPM for optical diagnosis of tumor regression.

Applications
These results are essential and significant for developing MPM for TRG, and to perform real-time diagnosis of tumor response after neoadjuvant treatment.

Terminology
MPM incorporates nonlinear optical processes to generate second-harmonic generation and two photon excited fluorescence signals.

Peer-review
The article has good characteristics, value, and significance.

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