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Original Article

Confocal laser endoscopy in the diagnosis for abdominal lymph node metastasis of gastric cancer

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Abstract: Confocal laser endoscopy (CLE) diagnostic criteria for lymph node metastasis of gastric cancer was established and evaluated to provide a basis for CLE clinical application in the diagnosis of abdominal lymph node metastasis. CLE scanning (surface scanning and sectional scanning) and pathology examination were conducted in gastric cancer tissues and lymph nodes of 5 cases. Characteristics of lymphatic metastasis in CLE imaging were observed and summarized in combination with pathology. The diagnostic criteria were corroborated in 124 lymph nodes of another 14 cases and CLE detection time needed for diagnosis was recorded. The CLE diagnostic criteria were tested and evaluated, and the effect of lymph node size on the diagnosis accuracy was determined. All the 19 participants were confirmed as gastric cancer. Sectional scanning can get comprehensive observation for internal structures of lymph nodes, in which abnormal large heterocyst appeared with special structural changes. CLE scanning could detect 88.75% of the positive metastasis and 68.18% of the negative metastasis examined by the pathology methods based on the established CLE diagnostic criteria. In comparison with pathological diagnosis, specificity, sensitivity and accuracy of CLE diagnosis were 88.75%, 68.18% and 81.45%, respectively. Accuracies of CLE diagnosis on the lymph nodes grouped by size were 85.29%, 77.78% and 88.89%, respectively, with no significant difference between groups ($P > 0.05$). Complete internal structures of lymph nodes can be observed clearly by CLE sectional scanning. The size of lymph nodes had no effects on diagnosis accuracy. CLE shows better sensitivity and specificity than traditional pathological diagnosis.

Keywords: Confocal laser microscopy (CLE), gastric cancer, abdominal lymph node metastasis, diagnostic criteria, application value

Introduction

Gastric cancer is a common malignant digestive carcinoma with high mortality in China [1]. Epidemiological survey has showed that the annual newly confirmed cases of gastric cancer in China accounted for about 42% of cancer cases globally. It has been documented that recurrence and metastasis are the main causes of high mortality in patients with gastric carcinoma [2-5]. The lymphatic metastasis and the number of metastasized lymph nodes are significant negative prognostic factors for the patients, which can provide important guidance for surgical procedure and postoperative chemotherapy to improve survival rate of patients with gastric cancer. Although the rapid histopathological examinations, such as frozen section, has been considered as the gold standard to detect the lymph node metastasis and guide the surgery, it is time-consuming and technically complicated for pathologists to handle the histological tissues in the fixing and dyeing procedures [6]. Therefore, it is of great clinical significance to develop new technologies for improving or even replacing the conventional histologic examinations. Confocal laser endoscopy (CLE) is a new kind of endoscopical technology featured in the ultraminiaturized laser scanning confocal microscope integrated in the endomicroscope [1, 7-14]. Under contrast of the fluorescent agent, CLE can observe the microstructure of cells, glands and blood vessels, require virtual histological images of the similar submucosal lesions magnified for 1000 times, and realize the intravital “optical biopsy”. The high-definition of cellular and subcelluar structures displayed by CLE is close to that of

the HE staining method. Recent researches revealed that CLE presents important diagnostic value for gastrointestinal cancers, but its practical value in clinical application still awaits further study [8].

The existing CLE diagnostic criteria for malignant tumors indicated that it is crucial to determine the symptoms when the abnormal tumor cells are present [2]. And the alien tumor cells in lymph nodes are the histological criteria for diagnosing the lymphatic metastasis. Although the nucleocytoplasmic ratio of CLE examination is not as good as that of the HE staining method, the nuclear structure can be clearly observed by CLE under the contrast medium with cellular pleomorphism featuring in the increased nuclear fission and nucleus volume [15]. These CLE detection characteristics can provide dependable diagnostic basis and application feasibility for lymphatic metastasis.

There are few researches that can systematically investigate the CLE diagnostic value for lymphatic metastasis in patients with gastric cancer, especially the lymph node metastasis in abdominal cavity. In this paper, the diagnostic criteria of lymphatic metastasis scanned by CLE were established according to the CLE imaging features and the diagnosis value were evaluated, with purpose of providing evidence in diagnosing the lymphatic metastasis and determining its clinical application.

Materials and methods

Patients

19 patients screened for gastric carcinoma and admitted to general survey in Chinese PLA General Hospital from December 2011 to December 2012 were enrolled in this study. The ages of the patients ranged from 18 to 75 years old without limitation in gender, who had been considered or diagnosed as the advanced distal gastric cancer before operation, regardless of the lymph node metastasis, and scheduled to undergo gastric carcinoma surgery. The following patients were excluded from the study, including those undergone severe liver disease and cardio-cerebrovascular disease, those who had renal injury, those pregnant or breastfeeding females, and those with other related operation contraindications. All the participants gave their signed consent form before engaging in this research.

Experimental design

The experiment was designed to get implemented in two phases. For Phase I, the gastric cancer tissues and 20 lymph nodes of 5 cases received CLE scanning and pathology examination, and both surface and sectional scanning methods were used in the CLE. Then the CLE diagnostic criteria of lymphatic metastasis were defined based on the summarized characteristics of CLE images in terms of the relevant pathological diagnosis standard.

For Phase II, 124 lymph nodes of 14 patients with gastric cancer were examined with CLE method and pathological method separately, and the lymph node metastases were diagnosed according to the CLE diagnostic criteria established in Phage I. The diagnostic effects were evaluated. And the time needed for CLE diagnosis through the whole CLE examination process was recorded.

CLE system

A confocal laser endomicroscope (Pentax ISC-1000, Pentax, Tokyo, Japan) was used in this study. With a confocal laser microscope integrated in the distal tip of the digestive endoscope, it can diagnose gastrointestinal diseases at the magnification of 1000 times accurately. CLE was performed to scan the lymph node from the top layer to 250 μm beneath the surface. And the CLE images were captured and stored for further analysis.

Examination procedure

In addition to the mucosal surface anesthesia and defoaming agent, patients took 50 mL warm saline containing 1 g sodium bicarbonate and 20000 units of chymotrypsin orally 20 min before the examination. And 10 min after that, patients were treated by the intramuscular injection of 20 mg butyl bromide scopolamine or 10 mg mountain scopolamine. The IV allergy test was done with 1 mL of the 2% fluorescein sodium and then the venous pathway was established. CLE was performed by the endoscopist experienced with the system. The white light endoscopy (WLE) was performed to inden-

Table 1. Demographic data and CLE image evaluation of patients enrolled in this study

Characteristics of patients	
Age (yr), median (range)	57.5 (44-69)
Gender	
Female	9
Male	10
Image quality of lymph nodes in Phage II (for 95.36% of the total images)	
Good	3389
General	2677
Size of the lymph nodes (mean ± SD) (cm)	0.72±0.11
Pathology grouping in patients with gastric cancer (19 cases)	
Poorly-moderately differentiated adenocarcinomas (case)	8
Highly differentiated adenocarcinomas (case)	11

tify the lesions firstly. The mucus and other attached objects on the surface of the lesions were rinsed with clear water. Then the patients were conducted the CLE scanning after intravenous injection with 5 mL of the 10% fluorescein sodium. The targeted biopsy and the conventional histopathological examinations were performed with the completion of CLE scanning. The lesions were removed surgically the next day according to the scope and extent. Under contrast of the 0.05% acridine yellow, both the surface and the sections of the lymph nodes cleared according to the location of lesions were observed by CLE after being dyed *in vitro*. Each node was collected the image data, tagged and then sent for pathology.

Image quality assessment of CLE detection

CLE images of each scanning part and lymph node were evaluated independently by two endoscopic physicians experimented with CLE imaging recognition. According to judgment standard established by Kiesslich [17], the CLE image quality can be divided into three levels: (1) the non-motion artifact for the image in high quality, where individual cells can be distinguished; (2) the motion artifact for the image in common quality, where the organizational structures can be detected; and (3) the significant artifact for the image in poor quality, which can not be evaluated and should be removed once judged.

CLE clarification of the lymphatic metastasis related with gastric cancer

In this study, two experienced endoscopic physicians compared CLE images of gastric cancer tissues and lymph nodes with the histopathological findings to summarize characteristics of the lymphatic metastasis associated with gastric cancer in CLE imaging.

Sensitivity, specificity, positive predictive value and negative predictive value of CLE diagnosis were calculated respectively in terms of the pathological diagnosis standard [18]. 124 lymph node metastasis cases were divided into 3 groups according to the relationship between the size of lymph node and the standard parameter ($\frac{\text{maximum diameter + minimum diameter}}{2}$), and they were Group A for lymph nodes less than 0.5 cm in size, Group B for those ranging from 0.5 cm to 1.0 cm and Group C for lymph nodes larger than 1.0 cm. The CLE diagnostic accuracy was evaluated for each group, on which effects of the lymph node size were also discussed.

Statistical analysis

Data were collected from CLE images and histopathological examinations. Statistical software SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 7.0 (Microsoft, Redmond, WA) were applied for statistical analysis. Taking the results from histopathological diagnosis as the "gold standard", the fourfold table was used to analyze evaluation indicators of the CLE diagnostic criteria, including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Comparisons of rates were based on chi-square test. And $P < 0.05$ was considered statistically significant.

Results

Collection of data

Demographic data of participants and CLE image quality assessment were listed in **Table 1**. 19 cases were confirmed as gastric carcinoma by endoscopy and biopsy pathological examination before surgery. According to WHO classification of gastric carcinoma [2], 19 cases of gastric carcinoma were studied and divided

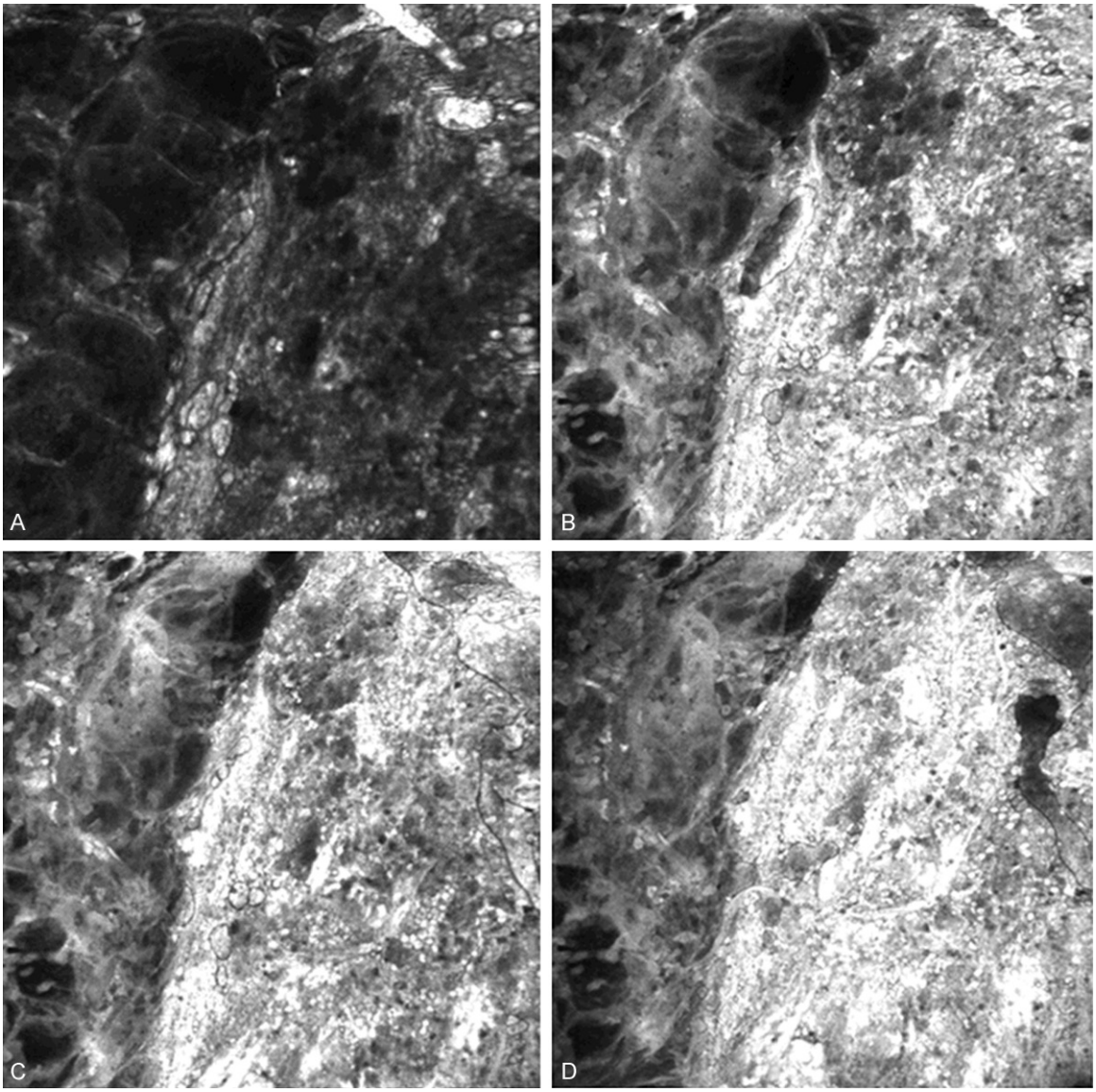

Figure 1. Confocal laser endomicroscopy surficial scanning of the lymph node. A-D: The internal superficial structure of lymph nodes can be observed through thinner envelops progressively.

into 8 cases of well differentiated tumor and 11 cases of poorly-moderately differentiated tumor, all of whom had no serious adverse reactions during inspections.

CLE diagnosis of lymph nodes

The average CLE examination time required for 5 cases in Phase I was (20±3.7) min prior to the operation, and the total time ranged from 12 min to 35 min. 323 CLE images were retained, and they were all confirmed as patients with gastric cancer by pathology.

20 removed lymph nodes were scanned by the CLE system after being stained with acridine yellow *in vitro*. 521 CLE images were retained for lymph nodes, of which 120 were those for surface scanning and the rest were those for sectional scanning, for 26 images per lymph node on average. When lymph nodes were performed CLE surface scanning, the internal structures were visible in only 8 lymph nodes (40% of the total) beneath the envelope (**Figure 1**). But the internal structures of all the 20 incised and dyed lymph nodes could be examined clearly by CLE layer scanning (**Figure 2**).

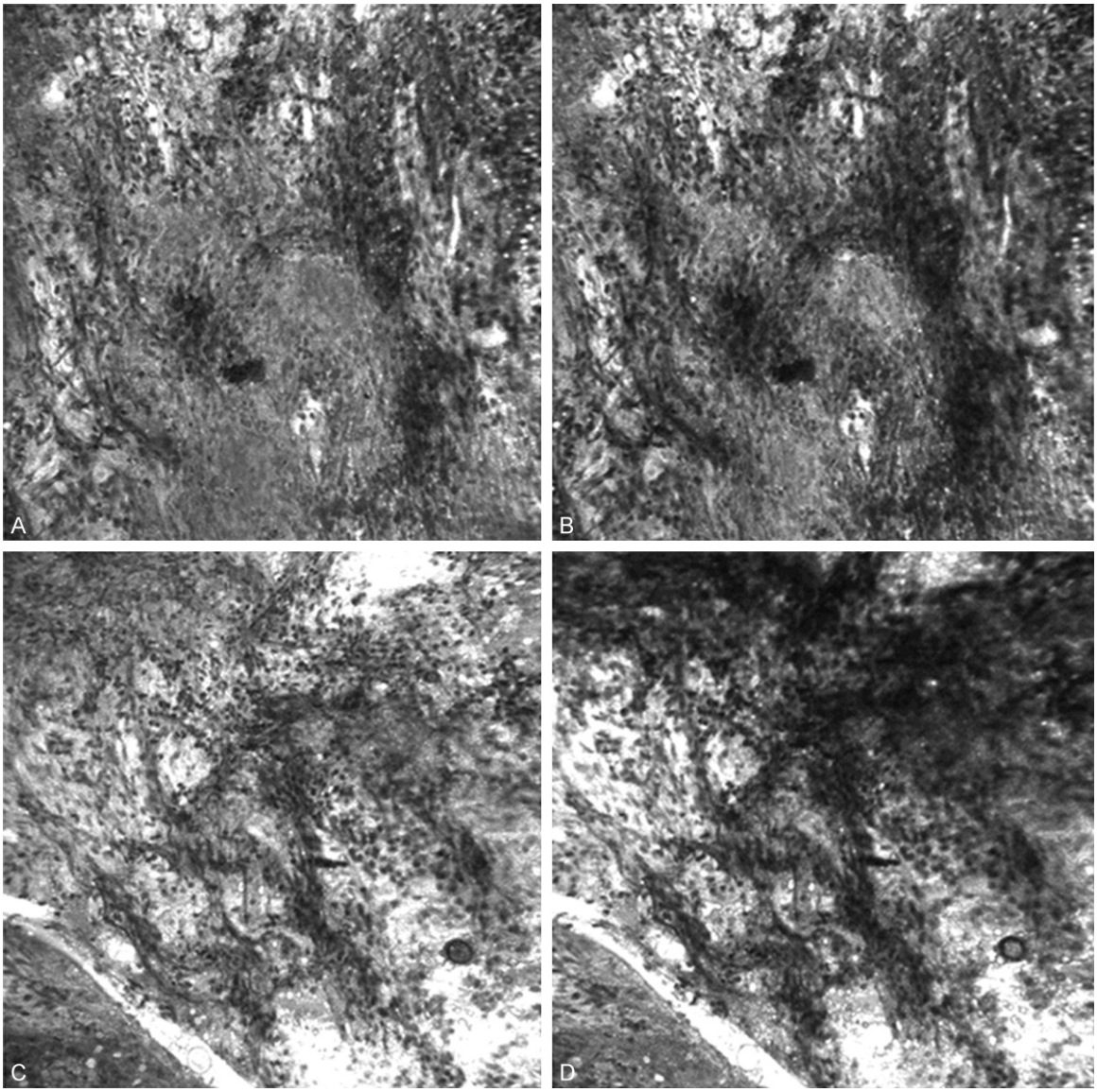

Figure 2. Confocal laser endomicroscopy sectional scanning. A, B: Visible internal structures of metastasized lymph node (cortex, lymphoid follicles, and small blood vessels); C, D: The tissue cells and lymphocytes located in metastasized lymph node.

CLE diagnostic criteria for lymphatic metastasis

Based on sectional images captured in Phage I, internal structures of metastasized lymph node were clearly visible, such as cortex, lymphoid follicles, small blood vessels, tissue cells, lymphocytes, etc. (**Figure 2**). After comparing them with the normal pathological images under different magnifications (**Figure 3**), manifestations and features of tumor cells in CLE images involved diffuse enlargement of alien cells with large and hyperchromatic nuclei while the nucleoplasm ratio was disordered. In addition, medulla and cortex were in mutual integration forms (**Figure 4A**). And parts of the heterocyst were observed to assemble in a nest or cluster form without any regular arrangement (**Figure 4B**). The characteristics mentioned above were similar with those examined by H&E staining method (**Figure 4C** and **4D**). Combining with the pathological diagnostic standard, the CLE detection criteria of lymph node metastasis could be summarized as follows: The abnormal large heterocyst appeared in lymph nodes with irregular nucleus and disordered nucleoplasm

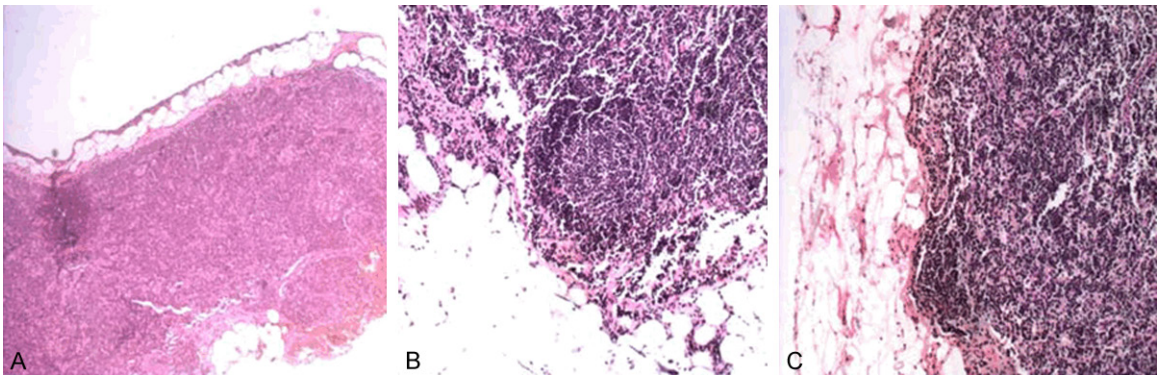

Figure 3. Normal pathological image of lymph nodes in different magnifications under white-light endoscopy. A: ×40 times; B: ×100 times; C: ×400 times

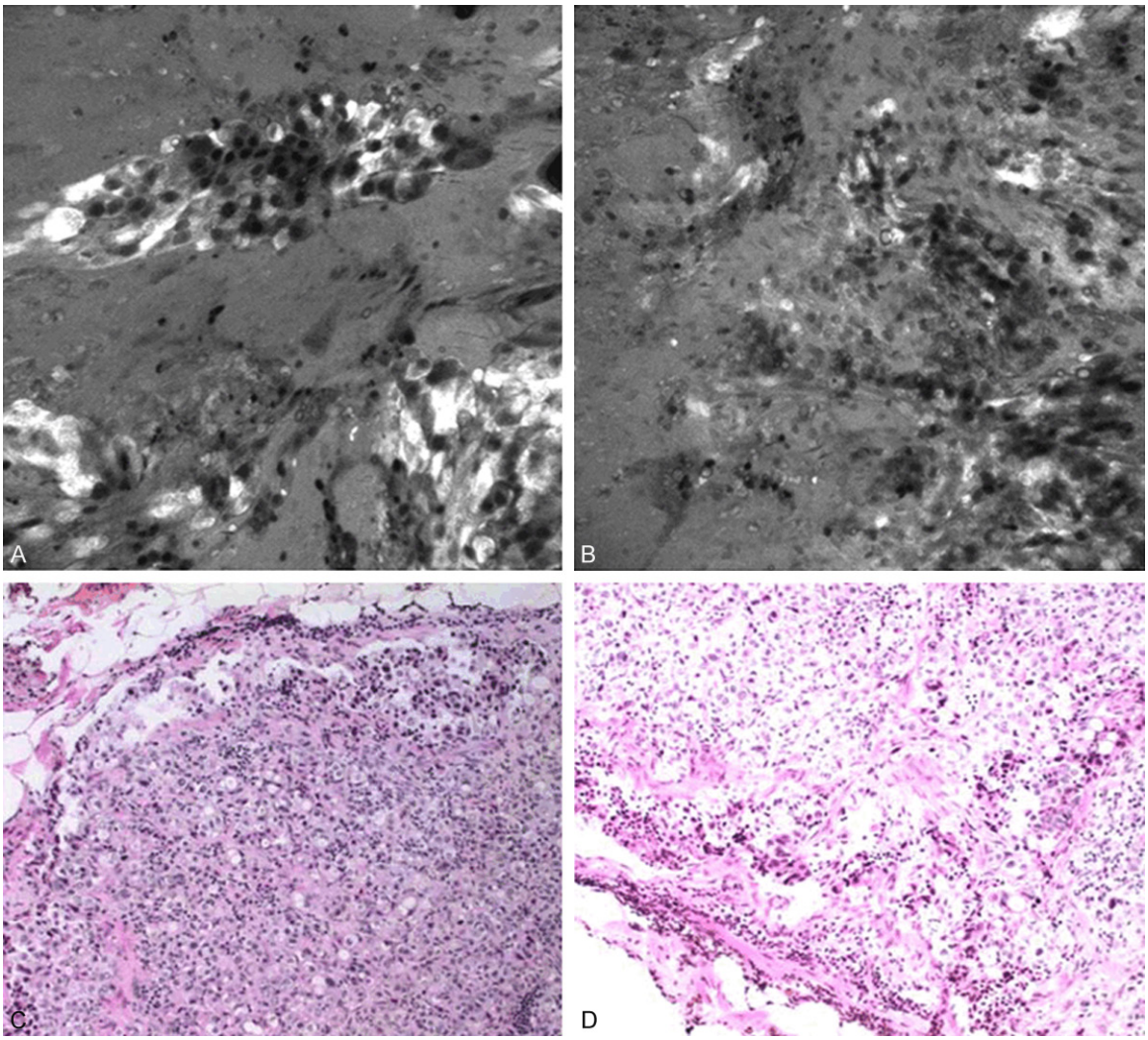

Figure 4. Comparison of confocal laser endomicroscopy detection and pathological examination for tumor cells in metastasized lymph nodes. A: Boundaries between medulla and cortex disappeared; B: Parts of the heterocyst were observed to assemble in a nest or cluster form without any regular arrangements; C, D: Metastasized lymph node in pathological images, where the medulla and cortex were in mutual integration forms and abnormal large heterocysts were arranged irregularly.

Table 2. Sensitivity, specificity, accuracy and positive predictive value and negative predictive value of CLE in lymph nodes metastasis diagnosis

		Positive (metastasis, case)	Negative (non-metastasis, case)
Results of pathological diagnosis		80	44-
Results of CLE diagnosis	Positive (case)	71	14
	Negative (case)	9	30
Indication for CLE			
Sensitivity (%)		88.75	
Specificity (%)		68.18	
Accuracy (%)		81.45	
Positive predictive value (%)		83.53	
Negative predictive value (%)		76.92	

Table 3. Sensitivity, specificity and accuracy of CLE diagnosis in lymph nodes with different sizes

For lymph nodes	Grouping	Group A	Group B	Group C
	Numbers	37	60	27
For CLE diagnosis	Sensitivity (%)	82.21	86.51	89.34
	Specificity (%)	60.10	68.92	70.32
	Accuracy (%)	85.29	77.78	88.89

ratio; The heterocyst arranged irregularly and aggregated in clusters;·Those aberrant tumor cells had special structural changes, and the boundaries between medulla and cortex disappeared, even with gastric pits or glandular tubes formed.

Evaluations of CLE diagnosis

With reference to the pathological diagnosis, sensitivity, specificity and accuracy of CLE diagnosis in Phage II were evaluated, respectively. Positive predictive value and negative predictive value were also determined (**Table 2**).

By comparing the size of lymph node with the standard parameter

$$\left(\frac{\text{maximum diameter} + \text{minimum diameter}}{2}\right),$$

the lymph nodes studied in Phage II can be divided into three groups that were Group A (< 0.5 cm), Group B (0.5 cm-1.0 cm) and Group C (> 1.0 cm), respectively. And those evaluations were calculated again to analyze the relationship between the size and the lymphatic metastasis (**Table 3**). Accuracies of CLE diagnosis for Group A, Group B and Group C were 85.29%, 77.78% and 88.89%, respectively, with no significant difference between groups ($P > 0.05$).

CLE scanning time for lymphatic metastasis detection

CLE examination times for 124 lymph nodes in Phage II were shown in **Table 4**. Individual lymph node was sliced *in vitro* and stained with acridine yellow before CLE scanning. And the total CLE detection time varied from 2 min to 14 min with (8±3.2) min on average. 6361 CLE images were retained and 51 images per lymph node was calculated.

Discussion

Results in the present study showed that the metastasized and non-metastasized lymph nodes can be identified by CLE imaging *in vitro*. It is beneficial to use fluorescent contrast agent in CLE observation of cell morphology. When 0.05% acriflavine hydrochloride is well distributed by local spraying to observe the lymph node *in vitro*, it can combine with the nucleic acid to display structures of the cytoplasm and nuclei clearly, offering great advantages for judging the cell proliferation [18]. Under contrast of the acridine yellow, the nucleus and parts of the cytoplasm in lymph node can be clearly highlighted in this study, providing detailed virtual histology images for lymph nodes. Thus it is possible for clinicians to detect whether the removed lymph nodes have metastasized in the abdominal cavity instantly and accurately. Researches have revealed that the joint application of fluorescein sodium and acriflavine hydrochloride can achieve advantage complementation with each other to improve observation of the mucosal morphology of digestive tract [19-22].

CLE imaging quality that resulted from the image artifacts [23-25] was the main factor affecting accurate judgment of lesions in this

Table 4. CLE sectional scanning time for single lymph node

Tag	Examination time (min)	Tag	Examination time (min)	Tag	Examination time (min)
1	2	43	6.5	85	8
2	3.5	44	8	86	9
3	3	45	3	87	12
4	2.5	46	5.5	88	10
5	4	47	11.5	89	7.5
6	6	48	7	90	11
7	11	49	4	91	8
8	10.5	50	6.5	92	10.5
9	9	51	3.5	93	14
10	3	52	8	94	8.5
11	8	53	2	95	11
12	6	54	11	96	4
13	9.5	55	9.5	97	7.5
14	8	56	12	98	13
15	5	57	8.5	99	5.5
16	8.5	58	10	100	10
17	6	59	3	101	6.5
18	7.5	60	10	102	14
19	12	61	9	103	10.5
20	5.5	62	3.5	104	9
21	4	63	8	105	7.5
22	7	64	9.5	106	4
23	6.5	65	7	107	12
24	11	66	10	108	10
25	13	67	11	109	4
26	10.5	68	3	110	8.5
27	4	69	6	111	13
28	5.5	70	8	112	11.5
29	6	71	10.5	113	4
30	9.5	72	4	114	5.5
31	8	73	7.5	115	12
32	10.5	74	10	116	8
33	6.5	75	8	117	10.5
34	8	76	12	118	11
35	12	77	2	119	4
36	5	78	11	120	6
37	7.5	79	13	121	8.5
38	6	80	9	122	2
39	9	81	9.5	123	14
40	11	82	14	124	14
41	4	83	3.5		
42	2	84	11		

study. The causes of artifact had been analyzed in previous investigations, including the non-flat contact between the front of endoscope and the mucosal surface, breathing, pulses, gastrointestinal peristalsis and other factors [24]. Hence the spasmolysis agent and proper attraction were applied to make mucous membrane surface flat contact with the front of endoscope and reduce the artifacts when participants in this study were conducted the CLE scanning. Current clinical practice has proved that no serious adverse effect was reported in the application of fluorescein sodium. Only a few people showed minor skin rashes and itchings, all of which can be cured by allergy treatments [19-21]. But the clinical risk requires further evaluation. Due to *in vitro* experiment of this study and the avoidance from effects of breathing and the mucous of tissue surface on the CLE observation, the retained CLE images were in higher quality than those in previous studies.

The study was initially designed to establish cytological relationship between gastric carcinoma lesions and lymph node metastasis, but during the research, we discovered that the current clinical pathology only can determine parts of the cell types for the primary lesion, such as gastric signet ring cell carcinoma, tubular adenocarcinoma and papillary adenocarcinoma. Other lesions, such as mucous adenocarcinoma and medullary carcinoma need to be further evaluated by immunohistochemical method. Moreover, there are still lack of cytological typing standards on lymphatic metastasis and diagnostic methods related with pathologic differentiation degree. Therefore no in-depth CLE research was released upon the correlation between gastric carcinoma primary lesions and lymph node metastasis in Phage I and only the status of lymph node metastasis was considered as the main research objective.

CLE diagnostic value for complete lymph node was poor, and the observable rate was only 40% in this study, which had great correlations with the CLE technology itself and the morphologic characters of the lymph node. Results revealed that sensitivity, accuracy and positive predictive value of CLE in diagnosing metastasis have reached more than 80%, which were closely related with the cellular and molecular levels observed by CLE. It is effective and accurate for CLE to detect positive lymph nodes and provide the specified number of the metasta-

sized lymph node, superior to researches on CT and endoscopic ultrasonography imaging technology [29-34]. CLE can perform biopsies, but it has certain restrictions in detecting negative lymph node metastasis, for the specificity was only 68%. Cells arranged in the pathological image are denser than those in the CLE image. For pathological images, the nuclear morphology can be distinguished by the chroma difference and complete lymph node structures can be observed under low magnification microscope, including medulla, cortex, fibrous trabeculae, lymphoid follicles and so on. But in CLE images, cells arranged relatively widespread with no distinct histological morphology. The nucleus can be clearly observed, and which is not as good as pathological imaging due to the gray-scale imaging. And personal experience of the clinician may also be the cause of low specificity. In this paper, nearly a third of negative lymph nodes (14/44) were misdiagnosed as positive lymph nodes, exaggerating the nodal metastasis.

The traditional examinations during operation, such as the routine frozen section (FS) and paraffin section, are time-consuming and tedious and need extensive workload. But CLE examination time for individual lymph node was 2 min to 14 min, for 8 min on average, which will reduce waiting times for the surgery and work burdens for the clinical pathologists. Compared with the traditional examinations, CLE can make an instant and accurate diagnosis for the resected abdominal lymph node, the new technology substitutable or auxiliary for the histologic diagnosis. With the continuous improvement and perfection of CLE technology and the development and application of new contrast agent [12, 30, 34], CLE will make greater breakthroughs in the fields of disease diagnosis and treatment, comprehensively replace the traditional histologic diagnosis, realize the real-time *in vitro* "endoscopic biopsy", and benefit the patients ultimately.

Disclosure of conflict of interest

None.

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