In vivo molecular imaging of gastric cancer by targeting MG7 antigen with confocal laser endomicroscopy

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Background and study aims: In vivo molecular imaging represents a powerful tool for the immediate diagnosis of gastric cancer. In this study, the monoclonal antibody MG7, which is a specific molecular marker against gastric cancer, was labeled with fluorescent agents to enable in vivo real-time imaging by confocal laser endomicroscopy (CLE).

Patients and methods: In vivo molecular imaging was performed in tumor-bearing mice from two kinds of human gastric cancer cell lines. Xenograft tumors were visualized in vivo first with a whole-body fluorescent imaging device and then by CLE using fluorescently labeled MG7 antibody. Gastric cancerous tissue and noncancerous mucosa from human biopsies or surgical specimens were also examined ex vivo by CLE.

Results: Intravital imaging of xenograft tumors revealed a specific cellular signal, whereas no specific signal was observed in control tissue or in mice injected with irrelevant antibodies. An ex vivo experiment on human specimens using a rigid confocal probe showed positive fluorescent staining in 22/23 samples diagnosed as gastric cancer and weak signals in 5/23 noncancerous tissue samples. CLE evaluation correlated well with immunohistochemical findings.

Conclusions: Screening tumors in vivo by CLE may help to detect MG7-Ag-positive tissues, decrease the sampling error by screening the large tumor surface not routinely screened by biopsy or conventional immunohistochemistry, and facilitate early detection of gastric carcinoma.

Introduction

Gastric cancer remains the second leading cause of cancer-related death across the world, with an increasing mortality rate reported every year [1]. Endoscopy is the gold standard procedure for early detection of gastric neoplasia, and the use of modalities such as chromoendoscopy, narrow band imaging, and magnifying endoscopy has resulted in further progress [2–6]. Recent studies have investigated the application of confocal laser endomicroscopy (CLE) for the in vivo microscopic diagnosis of gastric carcinoma and this technology has proved to be useful in differentiating between non-neoplastic and neoplastic gastric lesions based on validated morphological criteria [7–9]. However, most of these studies were performed using fluorescein as the contrast agent for nonspecific fluorescent imaging, which only provided anatomical and not functional or molecular data of gastrointestinal diseases.

Recently, several researchers have applied CLE in the field of molecular imaging with fluorescently labeled antibodies or peptides [10–12]. The visualization of specific molecular targets achieved by CLE allows a dynamic intravital perspective of a much larger tissue than conventional immunohistochemistry (IHC). Thus, it can enable real-time selection of the most appropriate tumor or lesional areas from which to obtain biopsy for standard histology. Moreover, although other devices for molecular imaging of the gastrointestinal tract such as immunoscopy [13], fluorescence endoscopy [14,15], immunoscintigraphy [16], and near-infrared imaging [17,18] have also proved to be effective, all of these approaches have served as wide-field, red-flag techniques rather than the microscopic molecular observation provided by CLE.

MG7-Ag, a novel tumor-associated antigen discovered by Fan et al. [19], is expressed in 94% of gastric cancerous tissue and in 83.6% of the serum of patients with gastric carcinoma [20]. The monoclonal antibody MG7 was originally produced by immunizing the BALB/c mice with the gastric cancer cell line MKN-46–9 [10]. By using an IHC method, MG7-Ag was confirmed to be located mainly in the cellular membrane, cyto-

plasm, and the glandular lumen, but not in the nucleus [21]. There was no MG7-Ag expression found in normal gastric mucosa [21]. Moreover, in patients with gastric cancer positive for MG7-Ag there was a weaker T cell immune response and more proinflammatory cytokine secretion [22], which may contribute to the worse prognosis of MG7-Ag-positive gastric carcinoma. Thus, we reasoned that if visualization of fluorescently labeled MG7 antibody can be achieved using CLE, large gastric cancerous tissues could be screened in vivo during the endoscopic process.

The aim of the present study, therefore, was to show the general feasibility in live human gastric cancer cell lines, and then to evaluate in vivo molecular imaging in tumor-bearing mice. Based on these findings, further studies were carried out on viable human cancerous and noncancerous tissue.

Materials and methods

Antibody preparation

Human gastric cancer cells, MKN-46 – 9 (1×10^7) were injected intraperitoneally (i.p.) with Freund complete adjuvant into 6-week-old BALB/c mice and boosted four times at 2-week intervals. The spleens were then taken for hybridoma preparation by fusion with mouse SP2 /0 myeloma cells as described previously [23]. Enzyme-linked immunosorbent assay (ELISA) was used to select antibodies binding to MKN-46 – 9 cells. Briefly, cells were grown in 96-well plates and fixed in pre-chilled methanol/acetone (1:1). Plates were then blocked with 5 % fat-free milk and incubated with hybridoma culture supernatants, followed by washings and incubation with horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St Louis, Missouri, USA). The color reaction, which developed by adding the substrate tetramethylbenzidine dihydrochloride (Sigma-Aldrich), was measured by a BioRad ELISA reader at 450 nm. The hybridoma with the strongest affinity was selected and 1×10^6 cells were intraperitoneally injected into 10-week-old BALB/c mice. The ascites containing MG7 antibodies was then collected, desalted, and purified through Protein A/G. Then, 10 µg of purified MG7 antibody was prepared for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) test, and coomassie blue staining showed two separate bands. The examination of murine viruses was negative. The procedures were in accordance with sterility test regulations for biological agents of the Pharmacopoeia of the People's Republic of China. Alexa Fluor (AF) 488 fluorescent dye was purchased from Invitrogen (Carlsbad, California, USA), and labeled according to the manufacturer's protocol.

Tumor cell line characterization

The gastric cancer cell lines BGC-823 and SGC-7901 were cultured in RPMI-1640 medium (Invitrogen) containing 10 % fetal bovine serum, 1 % streptomycin, and 1 % penicillin at 37 °C with 5 % CO_2. For flow cytometry, cells were harvested at the log-growth phase. Then, 106 tumor cells were incubated with 4 µL of AF488-labeled MG7 antibody, irrelevant antibody (normal mouse IgG), or phosphate-buffered saline (PBS) for 20 minutes. IHC staining of tumor cells was performed to verify the presence of MG7-Ag using the same protocol as that used for tissue specimens (see *Histological examination,* below) [22].

In addition, 10^5 tumor cells cultured in chamber slides were incubated with AF488-labeled MG7 antibody (2 µL in each well), irrelevant antibody (AF488-labeled normal mouse IgG), or no antibody (PBS as negative control). Then, CLE (FIVE1; Optiscan, Notting Hill, Australia) was performed on unfixed cells using the same settings as for in vivo mouse experiments. Immediately after CLE imaging, cell cultures were examined using bench-top fluorescence microscopy (BX51; Olympus, Hamburg, Germany) without further staining or amplification of fluorescence signals. In addition, nuclear counterstaining was achieved using Hoechst 33342 (Beyotime Biotechnology Inc., Nantong, China).

Tumor-bearing mice

To further investigate the feasibility of intravital molecular imaging of MG7-Ag using CLE, the tumor-bearing mouse model was examined. Four-week-old BALB/c nu/nu mice weighing 15 – 20 g were used in this animal study. Solitary tumors were induced by injection of 2×10^6 tumor cells at a site above the right hind leg of the mice (15 using BGC-823 cells and 15 using SGC-7901 cells). About 2 – 4 weeks after inoculation, imaging was performed when the tumor size was 5 – 10 mm. All mice were deeply anesthetized using 10 % chloral hydrate (3 µL/kg i.p.) and sacrificed after the examination by a chloral hydrate overdose. The procedures were approved by the local committee of Animal Care and Use and in accordance with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines for animal studies [24].

Macroscopic fluorescent imaging

After intracardiac administration of AF680-labeled MG7 antibody (1 µg/g body weight), a total of six tumor-bearing mice were imaged at various time points using a cooled charge-coupled device camera (Kodak 2000MM Image Station; Eastman Kodak Company, New Haven, Connecticut, USA). The excitation wavelength of AF680 was 679 nm, and fluorescence was detected at 702 nm. Three mice bearing BGC-823 tumors and three bearing SGC-7901 tumors were examined to explore the optimal time point and dosage for targeted molecular imaging of MG7-Ag. In addition, two mice in each group were set as controls by the injection of AF680-labeled irrelevant antibody (mouse IgG, 1 µg/g body weight) and undergoing the same procedure as the others. Briefly, anesthetized mice were placed in a light-tight chamber and fluorescence images were taken using 60 seconds of exposure time with 700 nm emission filters.

CLE imaging

According to the results of full-body fluorescent imaging of tumor-bearing mice, AF488-labeled MG7 antibody (1 µg/g body weight) was injected intracardially and mice (10 in each group) were shielded from light for 48 hours. After anesthesia, the xenograft tumor was exposed by making a small superficial incision. Confocal images were obtained using a novel rigid confocal mini-microscope (FIVE1; Optiscan), which is equivalent to the integrated flexible CLE [25]. A blue laser delivered an excitation wavelength of 488 nm, and light emission was detected at 505 – 585 nm. Images were collected from the surface down to 250 µm within the tumor with a lateral resolution of 0.7 µm (1024 × 1024 pixels). The field of view was 475 × 475 µm and the laser power was set at 1000 µW.

For in vivo CLE imaging, the handheld confocal probe was placed directly onto the tumor surface, and the whole tumor tissue was screened for specifically bound fluorescent signals. The hepatic tissue was also evaluated to serve as a negative control. In addition, in order to visualize tumor morphology, fluorescein sodium (100 µg/g body weight, n = 2) or acriflavine (100 µL of 0.05 %, n = 2) was injected intravenously or sprayed onto the tumor surface, respectively.

After in vivo imaging, mice were sacrificed by chloral hydrate overdose and tissue specimens were collected for histopathological examination.

Human study

The study protocol was approved by the ethics committee of Qilu Hospital and was conducted in accordance with the revised Declaration of Helsinki (2008). Surgical specimens (5 – 10 mm) or endoscopic biopsies of gastric cancerous tissue and noncancerous gastric tissue were incubated with AF488-labeled MG7 antibody (1:50 dilution) for 10 minutes at room temperature and shielded from light. The tissue was then washed three times with PBS and examined using CLE (FIVE1). CLE findings of in vitro human fresh tissue were analyzed immediately after the CLE procedure. Histopathological evaluation and IHC were then performed for all specimens and served as the gold standard.

Semi-quantitative analysis of CLE images was performed immediately after CLE imaging. One experienced gastrointestinal pathologist (C.J.Z) made all histopathological diagnoses and was blinded to the results of the endomicroscopic observation.

Histological examination

Tissue specimens were fixed in 10 % formalin and embedded in paraffin or immediately frozen in liquid nitrogen for cryostatic sections. Hematoxylin and eosin (H&E) staining was performed on serial sections of 4-µm intervals for histopathological analysis. In order to confirm the intravital fluorescence patterns, cryosections were immediately examined by bench-top fluorescence microscopy without MG7-Ag re-staining, and ex vivo nuclear counterstaining was achieved using Hoechst 33342.

IHC was performed as described previously [22]. Briefly, the slides were incubated in a 1:10 dilution of normal goat serum for 20 minutes to block unspecific binding, and were then incubated with the primary monoclonal antibody MG7 (1:200) at 4 °C overnight. Tissue sections were then washed and incubated with a goat anti-mouse secondary antibody, and MG7 localization was revealed using DAB (3,3'-diaminobenzidine tetrahydrochloride). For negative controls, irrelevant antibody or PBS was added instead of the primary antibody. Unspecific staining to nonspecific mouse antigens would be excluded with reference to IHC results of negative controls in this study.

Statistical analysis

For the endomicroscopic images of human specimens, MG7-specific fluorescence signal was ranked as negative (0), weakly positive (+), moderately positive (++) or strong expression (+++), similar to conventional IHC. One CLE image of good quality with the strongest fluorescent signal (if present) was chosen from each electronic folder by one investigator (X.L.Z) who was blinded to patients' clinical data and IHC results. Three experienced CLE investigators (C.Q.L, J.L, Y.Q.L) who were not involved in data collection were then invited to independently give the semi-quantitative grading of MG7-Ag expression of all human specimens observed by the CLE probe.

IHC results were evaluated according to intensity (score 1, light brown; score 2, brown; score 3, deep brown) and amount of positive DAB staining (score 1, stained area > 0 and < 30 %; score 2, 30 % – 70 %; score 3, > 70 %) [11]. The comprehensive score was calculated as the sum of these two indices. A comprehensive score of 0 was defined as negative, scores 2 – 3 as weakly positive (+), score 4 as moderately positive (++), and those above 4 were defined as strong (+++).

Statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS, Chicago, Illinois, USA). Demographic data of patients were expressed using mean ± SD. A nonparametric test was applied to compare the statistical significance between cancerous and noncancerous tissue. All tests of significance were two-sided and a *P* value of less than 0.05 was considered to be statistically significant. When the outcome was defined as the presence of specific fluorescent signal (from + to +++), the Kappa coefficient with 95 % confidence intervals (CIs) was used to evaluate the agreement between CLE images and IHC results on MG7-Ag targeting. A Kappa value of < 0.20 indicated poor agreement, 0.20 – 0.40 fair, 0.41 – 0.60 moderate, 0.61 – 0.80 good, and 0.81 – 1.0 excellent. Spearman's correlation coefficient was also applied to assess the semi-quantitative results between CLE and IHC findings. Interobserver agreement in determining the semi-quantitative grading of confocal images was expressed as the intraclass correlation coefficient (ICC) [26]. The rating criteria of ICC were similar to those of the Kappa value.

Results

In vitro cell experiments

The MG7-Ag expression was confirmed in the two gastric cancer cell lines BGC-823 and SGC-7901 by using flow cytometry (**Fig. 1 a, b**). IHC results also demonstrated specific staining of both tumor cell lines in the cell membrane and cytoplasm (**Fig. 1 c, d**).

The hand-held rigid CLE (FIVE1) enabled the visualization of MG7-Ag of unfixed tumor cell cultures after incubation with AF488-labeled MG7 antibody, which also showed membranous and cytoplasmic staining patterns (**Fig. 1 e, f**). The immediate bench-top fluorescence microscopic observation confirmed the CLE results (**Fig. 1 g, h**). Cells incubated with AF488-labeled irrelevant antibody (normal mouse IgG) or PBS revealed no specific fluorescent signal following the same experimental protocol.

In vivo animal experiments

In the preliminary study of full-body imaging of tumor-bearing mice, specific fluorescent signals were observed at 24 hours after intracardiac injection of AF680-labeled MG7 antibody (1 µg/g body weight). In addition, the tumor fluorescence intensity reached its maximum at 48 hours after antibody administration, whereas for the AF680-labeled irrelevant antibody-injected mice, no significant fluorescence signal was observed at the tumor sites. Successful tumor imaging demonstrated the specificity of in vivo targeting of MG7-Ag using AF680-labeled MG7 antibody (**Fig. 2**).

With reference to the data obtained by full-body imaging, in vivo tumor imaging was achieved in 20 tumor-bearing mice (ten for each cell line) using the rigid confocal probe. At 48 hours after AF488-labeled MG7 antibody injection, a cellular and membranous signal could be visualized in both BGC-823 and SGC-7901 tumors (**Fig. 3 a**). The hepatic tissue, which served as the negative control, revealed no specific fluorescent signal at the time of in vivo CLE imaging. Ex vivo IHC results and fluorescent microscopic imaging of cryosections resembled in vivo endomicroscopic findings (**Fig. 3 b, c**).

In addition, tumor morphology was available after intravenous administration of fluorescein sodium or surface application of acriflavine, which showed either irregular dark malignant cells or

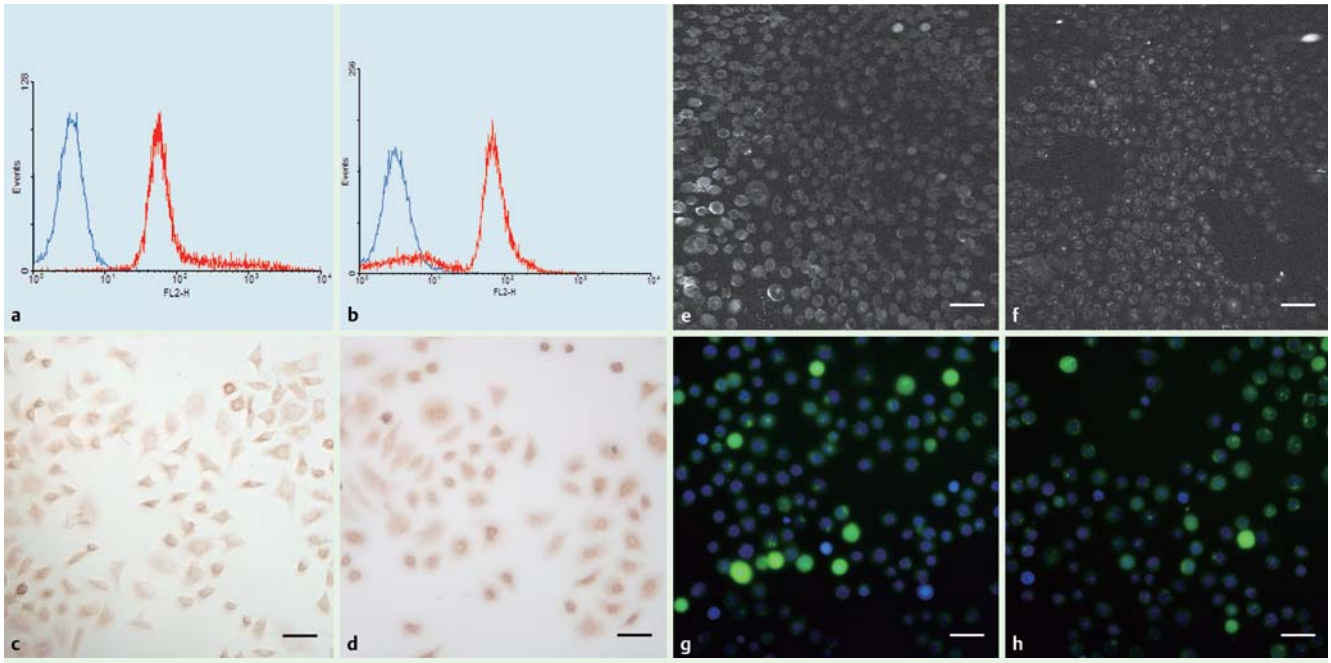

Fig. 1 Gastric cancer cell line studies. **a, b** Flow cytometry results for BGC-823 and SGC-7901 tumor cell lines 20 minutes after incubation with AF488-labeled MG7 antibody (red) or irrelevant antibody (blue). **c, d** Immunohistochemical staining of BGC-823 and SGC-7901 tumor cell lines showed the expression of MG7-Ag on the cell membrane and cytoplasm. **e, f** Confocal laser endomicroscopy imaging of BGC-823 and SGC-7901 tumor cell lines after incubation with AF488-labeled MG7 antibody showed membranous and cytoplasmic staining patterns. **g, h** Bench-top fluorescence microscopy of BGC-823 and SGC-7901 tumor cell lines after nuclear counterstaining (Hoechst 33342) demonstrated similar findings. Scale bars, 50 µm.

large, brightly stained nuclei. H&E staining of tumor specimens correlated well with in vivo observations (▶ **Fig. 3 d, f**).

MG7-Ag expression on human tissue in vitro

In order to evaluate the feasibility of MG7-Ag targeted imaging during CLE examination, fresh ex vivo specimens were incubated with AF488-labeled MG7 antibody. A total of 46 samples, including 18 surgical specimens and 28 biopsy specimens from 23 patients with gastric cancer, were analyzed prospectively. Histopathology results confirmed 23 cancerous tissues and 23 noncancerous tissues (▶ **Table 1**). Specific signals (+ to +++) were found in 22/23 samples diagnosed as gastric cancer, whereas noncancerous samples revealed no (0; n=18) or only weak (+; n=5) fluorescent signals (▶ **Fig. 4 a–d**). There was a significant difference on confocal fluorescent imaging between cancerous and noncancerous gastric tissue (nonparametric test, $P<0.001$). IHC analysis showed findings similar to those of in vivo CLE (nonparametric test, $P<0.001$) (▶ **Fig. 4 e–h**). In addition, the kappa coefficient showed a good agreement between endomicroscopic MG7-Ag visualization and IHC results (Kappa = 0.72; 95 %CI 0.62 – 0.83).

The correlation between different grades of IHC results of human samples and semi-quantitative assessment of in vivo MG7-Ag expression using CLE was also evaluated. A significant correlation with a Spearman's r of 0.87 could also be observed ($P<0.001$).

For interobserver assessment of the semi-quantitative assessment scheme of CLE images, 46 confocal pictures from 46 human specimens were blindly evaluated by three independent investi-

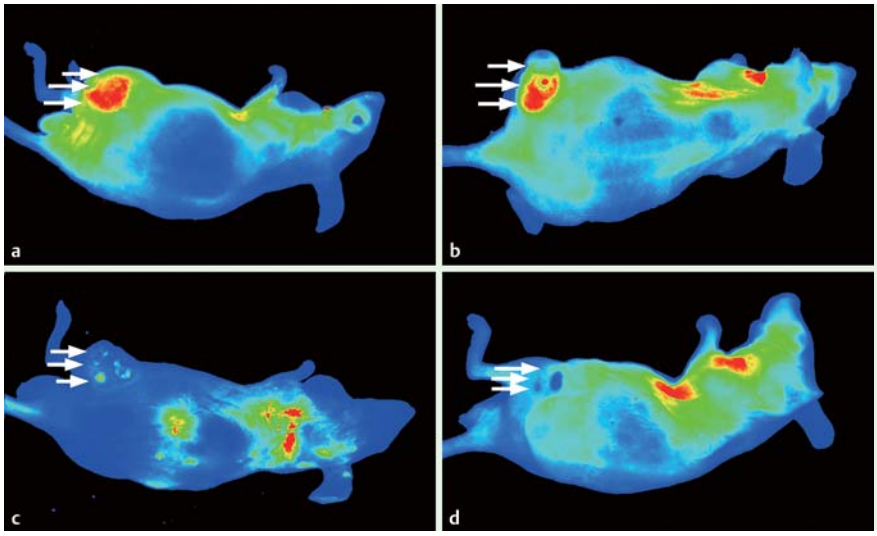

Fig. 2 Whole-body imaging of tumor-bearing mice. **a, b** The strongest fluorescent signal was observed in both BGC-823and SGC-7901 tumors (arrows) at 48 hours after intracardiac injection of AF488-labeled MG7 antibody. **c, d** No significant fluorescence signal was observed in either BGC-823 or SGC-7901 tumors (arrows) after injection of AF680-labeled irrelevant antibody.

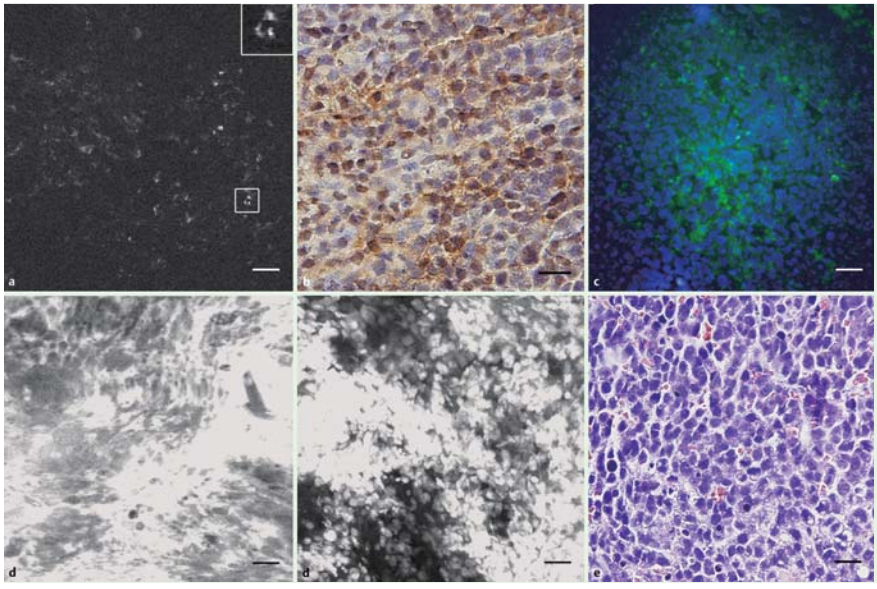

Fig. 3 In vivo microscopic imaging of tumor-bearing mice using confocal laser endomicroscopy (CLE). **a** CLE of a human xenograft after intracardiac injection of AF488-labeled MG7. Magnification of a single cell (boxed in **Fig. 3a**, upper right) demonstrates fluorescence accumulation on the cell membrane and cytoplasm. **b** Ex vivo immunohistochemical MG7-Ag expression patterns in CLE-scanned cancer tissues. **c** Bench-top fluorescence microscopy of cryosections with in vivo bound MG7 antibody and ex vivo nuclear counterstaining (Hoechst 33342) confirmed the expression of MG7-Ag in the tumor. **d, e** Tumor morphology could be displayed after intravenous administration of fluorescein sodium and topical acriflavin. **f** Hematoxylin and eosin staining of tumor specimen showed findings similar to those of CLE. Scale bars, 30 µm.

Table 1 Patients with gastric carcinoma examined with confocal laser endomicroscopy.

Patients, n	23
Sex, male/female, n	16/7
Age, mean (range), years	58 (32–80)
Gastric carcinoma, n	23
Histological differentiation, n	
Poorly differentiated	12
Moderately differentiated	7
Well differentiated	4

gators, and the results showed an excellent ICC value (ICC = 0.883; 95 %CI 0.819–0.929).

Ex vivo CLE imaging of fresh human tissue added a median procedure time of 15 minutes (range 14–17 minutes) compared with conventional endoscopy. This time period was from the incubation of fresh tissue with AF488-labeled MG7 antibody to the time of diagnosis from CLE images.

Discussion

The combination of fluorescently labeled antibody probes and visualization using CLE provides a novel method for real-time in vivo microscopic imaging of patients with gastric cancer. In this study, specific molecular imaging of gastric cancerous tissue was possible using CLE both in the mouse xenograft tumors and the human gastrointestinal mucosa using the MG7 antibody as a specific biomarker.

The MG7 antigen has been proved to be a specific molecular marker of gastric cancer [19,21]. In order to achieve targeted imaging of MG7-Ag in the present study, AF488 was used as the fluorescence agent to label the mouse anti-human MG7-Ag antibody. AF488 is a superior alternative fluorescent dye to fluorescein as it has an absorbance maximum of 495 nm and an emission maximum of 519 nm with a high extinction coefficient of 71 000 mol/L-1 cm-1, which is ideal for CLE imaging. In addition, the Alexa Fluor conjugate was reported to be extremely bright and more photo-stable than other conventional fluorescent tracers [27]. By using AF488-labeled MG7 antibodies, MG7-Ag was successfully displayed in gastric cancer cell lines, tumor-bearing mouse model, and human tissue. Initial in vitro experiments established binding of the AF488-labeled MG7 antibody conjugates to two kinds of gastric cancer cell lines. Flow cytometry studies showed a clear increase in binding of AF488-labeled MG7 antibody to both tumor cell lines and this was further validated using IHC of cell cultures. Subsequent CLE imaging of tumor cell slides showed the general feasibility and facilitated the assessment of incubation time and antibody concentration in vitro. After systematic application of the fluorescent antibody, in vivo specific staining of the xenograft gastric cancer cells was achieved in tumor-bearing mice, first using a whole-body imaging device (Kodak 2000MM) and then followed by successful microscopic real-time imaging with the rigid CLE probe. Finally, targeted fluorescence imaging was demonstrated on fresh human tissue from gastric carcinoma after topical application of labeled antibody.

In order to verify the specificity of these findings, healthy tissue from the same mouse or patient were also examined using the fluorescently labeled antibody, and labeled irrelevant antibody was applied in four mice, all of which revealed no specific signal, or only weak fluorescence signal in five noncancerous human specimens. There was an excellent correlation between the semi-quantitative grading criteria of CLE imaging and IHC results. In addition, to test the reliability of the CLE evaluations, interobserver agreement analysis of semi-quantitative grading of CLE images among three observers also revealed an excellent agreement.

Previous studies have reported that MG7-Ag can be detected by IHC with a sensitivity of 94 % and a false-positive rate of 51.3 %, and its expression level in gastric carcinoma was correlated with tumor differentiation, which is about 98.1 %, 97.6 %, and 75 % in poorly, moderately, and well differentiated gastric cancers, respectively [20]. The data from the present study were consistent with previous findings and demonstrated a sensitivity of 100 % and a false-positive rate of 34.78 % using IHC, and a sensitivity of 95.65 % and a false-positive rate of 21.74 % using CLE. However, as there were only 23 patients involved in the present human ex vivo study, positive IHC results were observed in all cancerous tissue and could not be correlated with level of tumor differentiation. Yet the proportions of weakly positive specimens in well differentiated gastric cancers vs. those of poorly to moderately differentiated ones were 50 % and 10.53 %, respectively, sug-

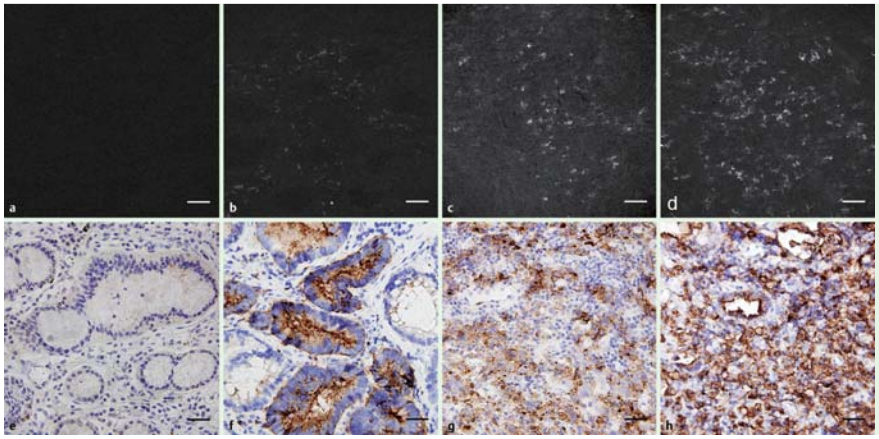

Fig. 4 Semi-quantitative evaluation of endomicroscopic images of human specimens and immunohistopathology. **a – d** Specific signals of endomicroscopic images were graded from negative to weakly, moderately, and strongly positive. **e – h** Corresponding immunohistochemical MG7-Ag expression patterns revealed a good correlation with in vivo microscopic findings. Scale bars, 50 µm.

gesting a trend of stronger MG7-Ag expression in poorer differentiated cancerous tissue, although without statistical significance in the present data set (Fisher's exact test, P = 0.125). Statistical analysis of endomicroscopic fluorescent imaging revealed findings similar to those of IHC.

Among the noncancerous human tissue examined by rigid confocal probe, five out of 23 specimens displayed weak fluorescence signals; corresponding H&E examination confirmed one specimen with high-grade intraepithelial neoplasia, two with intestinal metaplasia, and two with inflammation. This was also in accordance with other studies which showed that the positive rate of MG7-Ag in gastric precancerous lesions (including intraepithelial neoplasia, intestinal metaplasia, and atrophic gastritis) was about 51.3 % [20]. Therefore, although in vivo CLE imaging helped to detect MG7-Ag-positive tissues, diagnostic histology is still needed after in vivo and ex vivo IHC visualization of MG7-Ag in order to avoid over-treatment of these patients in the future.

According to previous studies, MG7-Ag-positive gastric cancer patients may suffer from a poorer prognosis [22]. Thus, screening the tumor tissue in vivo by CLE may help to detect MG7-Ag-positive tissues, decrease the sampling error by screening the large tumor surface not routinely screened by biopsy or conventional IHC, and facilitate early detection of gastric carcinoma. Although currently there is no Food and Drug Administration-approved drug against MG7-Ag, research has led to the development of MG7 scFv/SEB fusion protein which can specifically target gastric cancer cells, enhance the activity of T cells, and induce tumor cell apoptosis to exert an antitumor effect on gastric cancer [28]. Thus, in vivo simultaneous MG7-Ag imaging using CLE might also help to select patients who could benefit from MG7 targeted therapy in the future.

There are some limitations to the present study. First, only xenograft animal models were evaluated in this study. Surgical orthotopic implantations would provide a more accurate clinical model [29]. However, because the primary outcome of this study was to evaluate the feasibility of fluorescence imaging of MG7-Ag in human gastric cancer, surgical orthotopic implantation was not carried out. Second, only semi-quantitative assessment of CLE images was applied, which may suffer from potential inaccuracy compared with quantitative analysis. However, a semi-quantitative scheme in this setting may enable easier real-time judgment upon CLE images in clinical practice given the excellent interobserver agreement of (ICC = 0.883; 95 %CI 0.819 – 0.929), and semi-quantitative assessment is a common evaluation tool for IHC. Third, earlier studies have shown that MG7-Ag is mainly located in the cellular membrane and cytoplasm, but not in the nucleus [21]. The membranous distribution of MG7-Ag ensured its combination with topically applied AF488-labeled MG7 antibody. In addition, previous research has revealed the mode of internalization of MG series of monoclonal antibodies [30]. Another possible explanation is that cancer cells often suffer from impaired membranous integrity, which may allow part penetration of topical antibody molecules. However, more definite mechanisms of how topically applied antibody enters the cell have yet to be investigated. Fourth, although the incubation of fresh human tissue simulated in vivo topical application of fluorescently labeled MG7 antibody, in vivo human endomicroscopy was not performed in the present study. To clarify this issue, an in vivo study for patients with gastric cancer is being planned. Finally, limitations also relate to the availability of this new technology and availability of trained endoscopists who are able to perform the investigation and interpret the data. Potential side effects of this method and its cost-effectiveness are also still to be investigated in future studies.

In conclusion, the present study demonstrated that targeted imaging using a fluorescently labeled MG7 antibody is possible with CLE both in a xenograft model of human gastric cancer in vivo or on fresh human tissue. This technique may have provided us with enhanced diagnosis of gastric cancer and accurate selection of MG7-Ag-positive patients. Although in vivo human studies are needed to further investigate its clinical role, the data from the present study have verified the feasibility of CLE-mediated immunodiagnosis of gastric cancer from bench to bedside.

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Competing interests: None.

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