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In vivo molecular imaging of epidermal growth factor receptor in patients with colorectal neoplasia using confocal laser endomicroscopy

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ABSTRACT

Epidermal growth factor receptor (EGFR) plays an important role in tumorigenesis of colorectal cancer (CRC), and its *in vivo* molecular imaging in rodent models has become the subject of an increased number of studies using novel imaging techniques for gastrointestinal endoscopy. Current study aimed to evaluate the use of confocal endomicroscopy (CLE) for *in vivo* molecular imaging of EGFR in patients with colorectal neoplasia. Molecular imaging of colorectal neoplasia in patients was performed by CLE after topical application of a fluorescent-labeled molecular probe against EGFR. Representative images of CLE were chosen to calculate EGFR-specific fluorescence intensity. Targeted biopsy specimens were taken from each examined site during *in vivo* imaging for histology and immunohistochemistry (IHC). During *in vivo* molecular imaging in 37 patients, an EGFR-specific fluorescence signal was present in 18/19 CRC, and 12/18 colorectal adenomas. No or only weak fluorescence signal was observed *in vivo* in 10 cases of normal mucosa. CLE is a novel tool that could be used in molecular imaging with specific targeting of EGFR in patients with colorectal neoplasia. This technique demonstrates a promising imaging approach for targeted therapies of colorectal neoplasia.

1. Introduction

Colorectal cancer (CRC) is the world's fourth leading cause of cancer-related death in males and the third in females. An estimated 608,700 CRC-related deaths occurred globally in 2008 [1]. The well-accepted pathogenetic framework for sporadic colorectal tumorigenesis is an adenoma–carcinoma sequence, possibly with a series of genetic events and environmental alterations [2–4].

Epidermal growth factor receptor (EGFR) is a 170-kDa ligand-activated tyrosine kinase receptor [5]. Dysregulated activation of EGFR signaling pathway correlates strongly with the malignant behavior of colorectal tumors [6–8]. In premalignant colorectal adenoma, EGFR has been reported to be overexpressed in 24–80% of cases [9,10]. Overexpression of EGFR in CRC ranges between 25% and 97% [11–13], and seems to be related to a potential invasive risk and a poor outcome [14]. On the basis of these observations, EGFR has become a rational target in molecular therapeutic strategies for CRC [15]. However, the expression level of EGFR, as detected by current immunohistochemistry (IHC), does not seem to predict clinical response to treatment with monoclonal antibody therapies against this target [16]. The variation in EGFR expression and the inconsistency between EGFR expression and prediction of clinical response may, at least partially, be due to the technical pitfalls [17]. Different arbitrary cut-offs to define positive staining further complicate the observation. The desired goal in CRC therapy is to individualize treatment in accordance with the underlying predictive factors to minimize unnecessary adverse events [18]. Therefore, there is a need for better methods to guide patient selection for individualized therapy.

Endoscopy is the gold standard for diagnosis of colorectal neoplasia; however, up to 20% of precursor lesions may be missed using this procedure [19]. With the advances in optical instruments, especially the emergence of confocal laser endomicroscopy (CLE), the paradigm for detecting gastrointestinal tumors is changing from purely macroscopically structural imaging to include molecular imaging [20]. Currently, CLE can be performed using two devices, including an integrated CLE and a probe-based CLE (pCLE). Recent trials have reported *in vivo* molecular imaging using CLE, with the potential to significantly influence the diagnostic and therapeutic strategies in the field of gastrointestinal endoscopy [21,22]. Important breakthroughs in molecular imaging have been achieved in a first study targeting EGFR for *in vivo* imaging in a

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rodent model with human xenograft CRC using CLE [21]. The feasibility of *in vivo* molecular imaging using pCLE has also been reported with topically applied fluorescent-labeled heptapeptide in human [23] and fluorescent-labeled antibodies in porcine esophageal and gastric mucosa [24]. However, at present, no trial has used fluorescein-conjugated antibodies for labeling of neoplasia in humans *in vivo*. The aims of the current trial, therefore, were to evaluate the use of CLE for *in vivo* molecular imaging of EGFR in patients with colorectal carcinoma and adenoma and to perform a comparative analysis of *in vivo* EGFR molecular imaging using CLE and *ex vivo* detection of EGFR expression with IHC.

2. Materials and methods

2.1. Patients enrollment

From January 2011 to June 2011, 40 consecutive patients known to have large polypoid lesions in the colon or rectum from previous examinations as outpatients or inpatients at Qilu Hospital were enrolled in the study. Exclusion criteria were: tumor stenosis, acute lower gastrointestinal tract bleeding, familial adenomatous polyposis, metastatic carcinoma derived from organs other than the colorectum, impaired cardiac, liver or renal function, coagulopathy, fever, age <18 years, pregnancy, breastfeeding, or known allergy to fluorescein.

In all participants, surgical or endoscopic resection was planned in accordance with the evaluative results of the preoperative colonoscopy. Informed written consent was obtained from all participants prior to participation. The study protocol was approved by the Institutional Ethics Committee of Qilu Hospital and was conducted according to the revised Declaration of Helsinki (1989) (Trial Registration ID: NCT01372189).

All CLE procedures were conducted by three senior endoscopists (X.Z., X.G. and T.Y.) who were blinded to the histologic results of all participants. Each endoscopist had conducted more than 300 CLE procedures before embarking on the current study.

2.2. Confocal laser endomicroscopy system

The CLE system used in this study was a Pentax EC-3870K endomicroscope (Pentax, Tokyo, Japan) with a miniature laser scanning microscope integrated into the distal end of a conventional endoscope. Laser excitation was at 488 nm. The white-light endoscopy images and CLE images were simultaneously captured and stored.

2.3. Pilot study

Commercially available Alexa Fluor 488 (AF 488) conjugate-labeled anti-EGFR monoclonal mouse antibody (catalog number 16-246, Upstate Biotechnology, Billerica, MA, USA) was used to mark colorectal neoplasia *in vivo*. To establish the proper antibody concentration and incubation time, the first three participants with histologically confirmed colorectal carcinoma were recruited for a pilot study. After topical spray of 5 ml of AF 488 conjugate-labeled anti-EGFR antibody to the colorectal carcinoma at randomly assigned concentrations (1:500, 1:100, or 1:50 in 0.9% saline), CLE imaging was performed after a 10- and 15-min incubation.

A targeted biopsy was taken from the observed site which was located 5 mm immediately to the left of the mucosal erythema created by suction to stabilize the endomicroscope on the lesion. IHC of targeted biopsy samples was performed with a monoclonal mouse anti-EGFR antibody (catalogue number ab62, Abcam, Cambridge, UK). Consistency in fluorescence signal of CLE and immunostaining was achieved at a dilution of 1:50 and incubation time of 10 min. All three participants in the pilot study were not included in the prospective study.

2.4. In vivo molecular imaging of EGFR using CLE: prospective study

Thirty-seven patients were subsequently enrolled in the prospective part of the study. The bowel preparation with ingestion of hypertonic polyethylene glycol solution (SF-PGE, Staidson Biopharmaceuticals, Beijing, China) was performed 24 h before the study. First, all patients underwent conventional colonoscopy by CLE in white-light mode. The identified large polypoid lesions the in colon and rectum were examined by CLE microscopic view. The 1–2 cm^2 neoplastic region of interest (ROI) was rinsed at least three times with tap water to remove mucus and blood. CLE imaging was performed before applying the anti-EGFR antibody to check for autofluorescence. Using a standard colonoscopic spray catheter, approximately 5 ml of AF 488-labeled anti-EGFR antibody at a dilution of 1:50 was sprayed topically to the ROI. After a 10-min incubation, excess antibody was removed by gently rinsing the region with water. Confocal imaging of the 1–2-cm^2 colorectal neoplasia was then performed. Unspecific binding was excluded by confocal imaging of four cases of colorectal neoplasia after topical spray of an AF 488-labeled isotype control antibody (catalog number 16-240, Upstate Biotechnology, Billerica, MA, USA). Confocal imaging of normal mucosa adjacent to the neoplastic lesions from the same patients before and after antibody administration was also conducted in 10 cases of colorectal neoplasia. During CLE imaging of each observed site, the laser power and brightness were set at the same level. Then, targeted biopsy samples of the observed neoplastic lesions and normal mucosa were taken as described in the pilot study.

2.5. Ex vivo detection of EGFR expression

Targeted fresh biopsy samples from each examined site were fixed in 10%-pH neutral formalin, and embedded in paraffin. Slides of 5-μm sections were deparaffinized and hydrated. After antigen retrieval and blocking procedures, IHC staining for EGFR was performed by mouse monoclonal anti-EGFR antibody (catalog number ab62, Abcam, Cambridge, UK) and Dako Dual Envision + Detection Systems Peroxidase/DAB (Dako, Carpinteria, CA, USA) as per the manufacturers' instructions. Hematoxylin and eosin (H&E) staining according to the standard protocol served as the gold standard to classify the biopsy samples. In two cases (one CRC and one colorectal adenoma), an additional biopsy specimen was transferred to liquid nitrogen. Then, bench-top fluorescence microscopy (BX51, Olympus, Tokyo, Japan) of cryostatic sections was performed without EGFR re-staining (to visualize *in vivo* bound AF 488-labeled anti-EGFR). AF 488-labeled EGFR antibody was detected at 520 nm, nuclear counterstaining with Hoechst 33342 (C1026, Beyotime Institute of Biotechnology, Jinan, China) at 460 nm.

2.6. Evaluation

Fluorescence intensity of *in vivo* confocal imaging was quantified offline with Image J (NIH, Bethesda, MD, USA). For each observed neoplasia and normal region, a representative image with minimum motion artifact, and a strong fluorescence intensity was chosen by one evaluator (Z.L.) who was blinded to all the histologic diagnoses. Three different ROIs of 60 × 60 μm with the strongest fluorescence in the representative image were selected. The mean gray-scale value of the three ROIs was calculated within each image: black (0)–white (255), as described in detail previously [22].

The diagnosis and graduation of H&E staining results were performed by an experienced gastrointestinal pathologist (C.Z.) in a blinded manner in accordance with the modified Vienna classification [25].

EGFR staining intensity in IHC samples was evaluated as follows: EGFR expression was defined as positive if any membrane or cytoplasmic staining above background level was detected. Negative staining was reported when the absence of membrane or cytoplasmic staining was observed.

2.7. Human anti-mouse antibodies detection

Serum samples from four participants were taken 4–6 weeks after CLE imaging and were tested for human anti-mouse antibodies (HAMAs) by using a quantitative enzyme-linked immunosorbent assay plate (Medac, Hamburg, Germany), as described previously [26]. Before participating in the study, none of the four patients had received any type of mouse antibody application. Potential related side effects were defined and monitored based on a published study, which were classified into four grades: generalized skin symptoms, mild to moderate pulmonary and cardiovascular symptoms, unstable hemodynamics and cardiac or respiratory arrest [27].

2.8. Statistical analysis

Demographic and clinical data were collected by one investigator (J.L.) using a standardized record form. Data were analyzed using a statistical software package SPSS v11.0 (SPSS Inc., Chicago, IL, USA). Significance of comparison of the fluorescence signal intensity between CRC and colorectal adenoma, and between neoplasia and normal mucosa was calculated by a two-sided *t*-test. Kappa values were used to correlate *in vivo* confocal EGFR imaging and *ex vivo* immunostaining agreements: 0.01–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial, and 0.81–1.00, almost excellent. All tests of significance were two-tailed with the confidence interval of 95%. p values $\leqslant 0.05$ were defined as significant.

3. Results

Table 1 illustrates the demographic characteristics and final histologic diagnosis of the patients with colorectal neoplasia in the pilot and prospective studies.

3.1. Pilot study

Three participants (three men; age range 60–79 years, mean age 72 years) with previously confirmed colorectal carcinoma completed the study protocol (Table 1), and none of them

Table 1
Patient demographics and clinical characteristics of all colorectal neoplasia in this study.

	Pilot study	Prospective study
Patients (*n*)	3	37
Gender (*n*/*n*, male/female)	3/0	21/16
Mean age, years (range)	72 (60–79)	68 (40–83)
Histologic type (n)		
Adenoma (HGD/LGD)	0	18 (10/8)
Well-differentiated CRC	2	8
Moderately differentiated	1	11
Poorly differentiated	0	0

CRC, colorectal cancer; HGD, high-grade dysplasia; LGD, low-grade dysplasia.

experienced a severe adverse reaction during the entire imaging procedure. A specific fluorescence signal was observed in one participant after EGFR staining at the antibody dilution of 1:50 and incubation time of 10 min, whereas cancerous lesions incubated at 1:100 and 1:500 dilutions did not demonstrate a specific signal (Fig. 1). Imaging at the incubation time of 15 min yielded a decreased EGFR signal. One potential explanation is intestinal peristalsis and difficulty in locating the labeled antibodies. IHC performed in all of the three targeted biopsy samples showed positive staining correlating with the CLE imaging at the 1:50 antibody dilution.

3.2. In vivo molecular imaging of EGFR using CLE: prospective study

A total of 37 patients (21 men, 16 women; age range 40–83 years, mean age 68 years) with 37 neoplastic lesions in the colon or rectum were enrolled in prospective study (Table 1). After *in vivo* EGFR staining by topical application of the labeled anti-EGFR antibody to all 37 lesions, a specific fluorescence signal and EGFR accumulation could be detected in 18 (94.7%) of the 19 carcinomas (Fig. 2) and in 12 (66.7%) of the 18 adenomas (Fig. 3). The intensity of EGFR-specific fluorescence varied from only a weak fluorescence signal to heavy tissue staining in 30 neoplastic tissues (Figs. 2 and 3). A specific cellular signal could be observed by CLE imaging with 1000× magnifying power (Fig. 4). The transition zone could be observed as the region where the EGFR-specific fluorescence signal abruptly disappeared (Fig. 5). Eight fluorescence signal-positive carcinomas contained well-differentiated carcinomas (8/8), 10 moderately differentiated (10/11), and 0 poorly differentiated (0/0). Six out of 10 high-grade dysplasias (6/10) and six out of eight low-grade dysplasias (6/8) were CLE positive. A total of seven out of 37 (18.9%) colorectal lesions did not show an EGFR-specific signal. Quantitative assessments of the fluorescence intensity were measured in ROIs of colorectal carcinomas and adenomas. The mean fluorescence intensity was 52.84 ± 5.73 for carcinomas and 44.31 ± 4.86 for adenomas ($p = 0.329$). By contrast, normal mucosa sprayed with labeled antibody showed no specific fluorescence signal in seven cases and weak fluorescence signal in three cases (Fig. 6). The fluorescence signal intensity of normal mucosa was 36.23 ± 7.90 compared with 53.78 ± 7.06 in neoplastic tissues of the same ten patients ($p < 0.001$).

CLE of neoplasia before labeled anti-EGFR antibody application or imaging after spraying labeled isotype control antibody showed no specific signal.

3.3. Ex vivo EGFR immunohistochemistry and fluorescence microscopy

Ex vivo IHC performed on targeted biopsy samples revealed an increased EGFR staining in 18 of the 19 carcinomas and 13 of the 18 adenomas, correlating with the *in vivo* CLE findings (Figs. 2 and 3). The kappa value showed a substantial agreement between *in vivo* EGFR imaging using CLE and *ex vivo* EGFR staining by IHC ($\kappa = 0.788$). In fluorescence microscopy, cryostatic sections of one CRC and one adenoma confirmed the respective CLE fluorescence patterns after *in vivo* binding of AF 488-labeled EGFR antibodies (Fig. 7).

3.4. HAMAs detection

After topical application of the AF 488-labeled anti-EGFR antibody, no side effects were observed during *in vivo* CLE imaging and at the 4–6-week follow-up study by telephone. None of the four serum samples were found to be elevated for HAMAs titers.

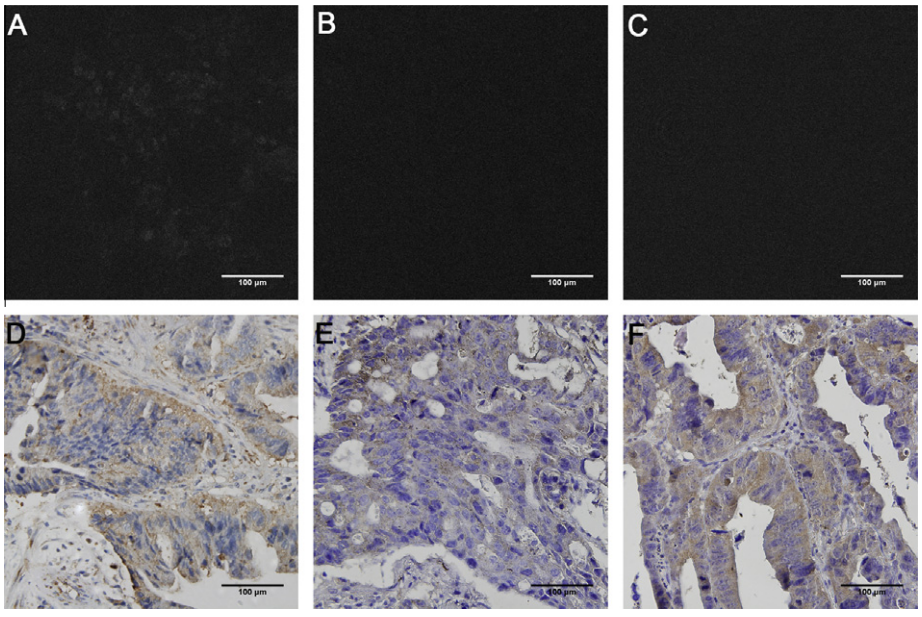

Fig. 1. Molecular imaging of colorectal cancer (CRC) in the pilot study. (A) An epidermal growth factor receptor (EGFR)-specific fluorescence signal is observed using confocal laser endomicroscopy at a probe dilution of 1:50. (B) and (C) No specific fluorescence is present at dilutions of 1:100 (B) and 1:500 (C). (D–F) Corresponding immunohistochemical staining demonstrated positive staining of EGFR in all three targeted biopsies of CRC (original magnification 400×).

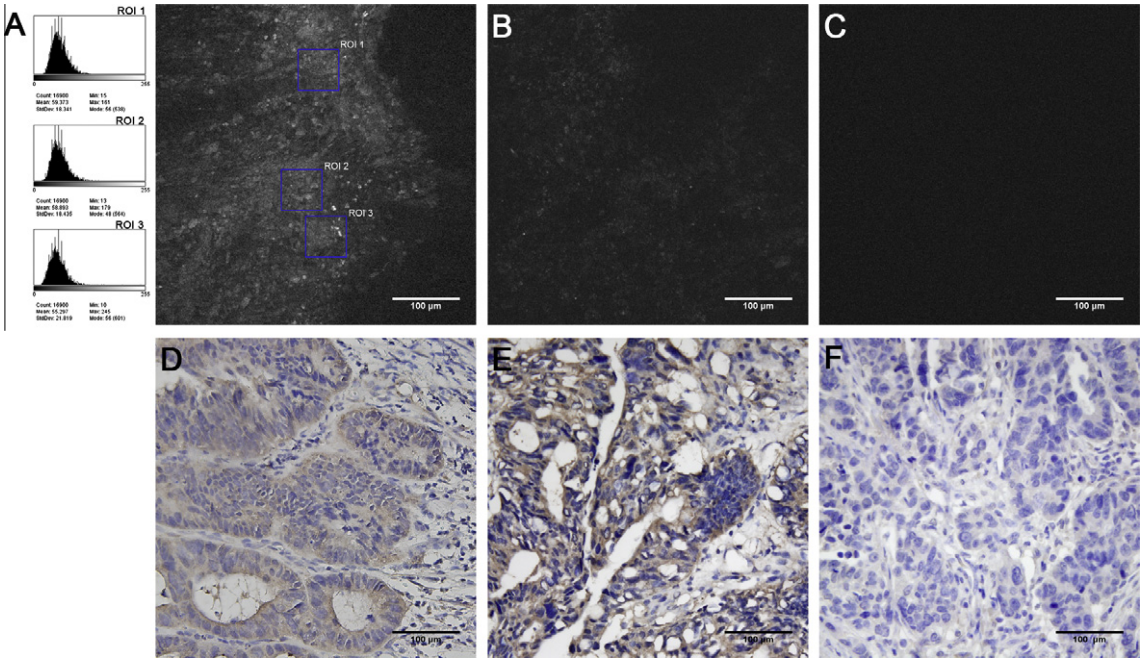

Fig. 2. Molecular imaging of epidermal growth factor receptor (EGFR) in patients with colorectal cancer (CRC). (A–C) *In vivo* confocal laser endomicroscopy views of three CRCs with different EGFR expression levels. Mean gray-scale value was 57.85 (A), 52.61 (B), and 39.41 (C), respectively. An example of signal intensity quantification on one confocal image (A) shows three regions of interest (ROI) (boxed in (A)) and the gray-scale values. (D–F) Corresponding *ex vivo* immunohistochemical staining of three CRCs with different EGFR expression levels (original magnification 400×).

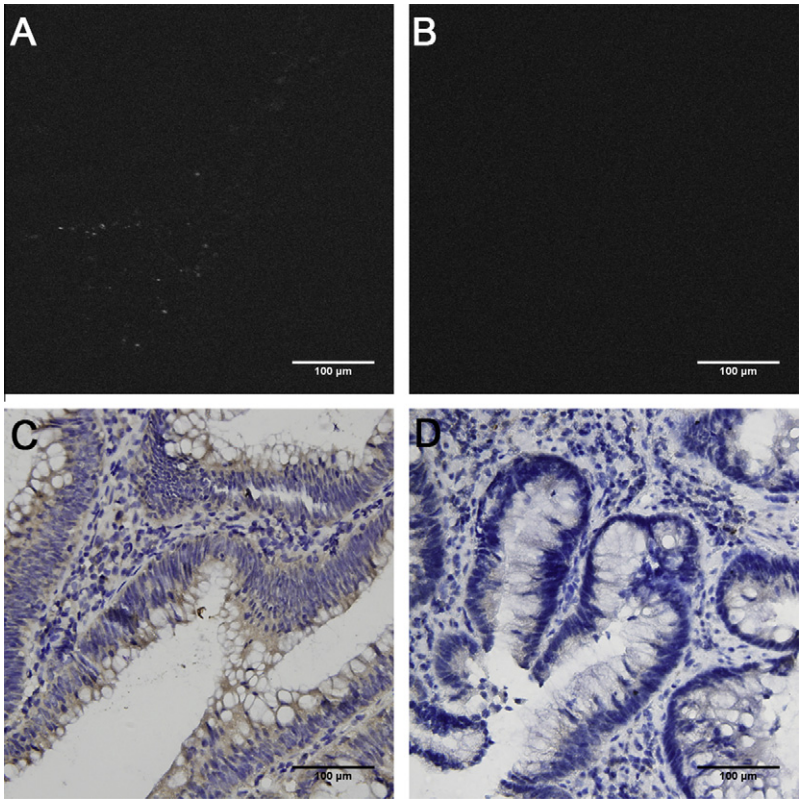

Fig. 3. Molecular imaging of epidermal growth factor receptor (EGFR) in patients with colorectal adenoma. (A) and (B) Confocal laser endomicroscopy imaging of colorectal adenomas after targeted molecular staining of EGFR reveals specific fluorescence (A) or no positive signal (B). (C) and (D) Immunohistochemistry of the respective biopsy specimens confirms the *in vivo* results (original magnification 400×).

4. Discussion

EGFR has been found to be an excellent epitope for molecular targeted CRC therapy. Therapeutic monoclonal antibodies targeting EGFR, such as cetuximab, have been approved by the US Food and Drugs Administration (FDA) [28]. Currently, patient selection for cetuximab targeted therapy mainly depends on IHC, which is influenced by various of factors, such as tissue sample

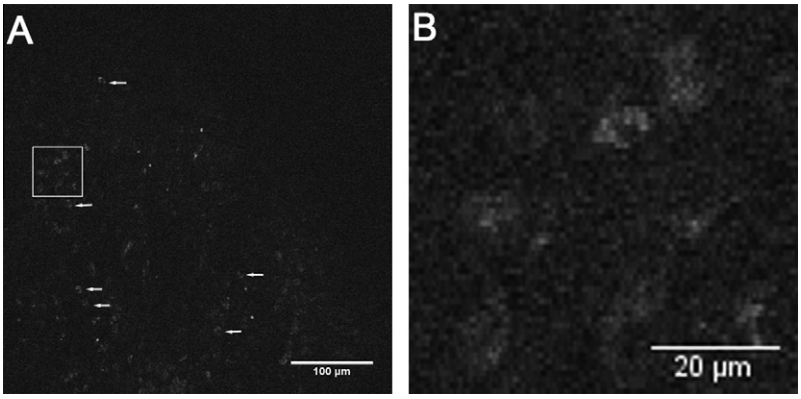

Fig. 4. High resolution of epidermal growth factor receptor (EGFR)–specific molecular imaging in colorectal cancer (CRC). (A) During *in vivo* confocal laser endomicroscopy imaging, a specific cellular signal of EGFR is observed in a CRC (arrows). (B) Magnification of a single cell (boxed in (A)) depicting an accumulation of EGFR-specific fluorescence.

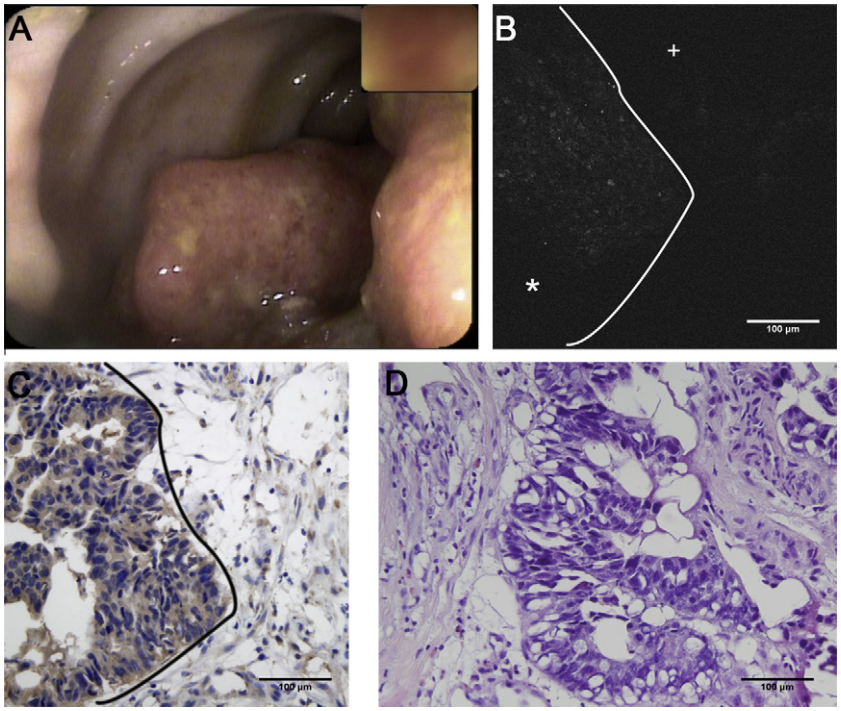

Fig. 5. Transitional zone. (A) Endoscopic view. (B) The transition zone could be seen which divides the images into positive-stained tumor (asterisk) and suspected healthy mucosa with no specific fluorescence signal (cross). (C) Immunohistochemistry of the transitional zone. Increased epidermal growth factor receptor (EGFR) expression is demonstrated in CRC compared with unstained healthy mucosa. (D) Corresponding hematoxylin and eosin staining ((C) and (D) original magnification 400×).

fixation, storage, and processing [29]. In addition, EGFR immunoreactivity is altered because the antigen is removed from its natural microenvironment. *Ex vivo* IHC only provides a momentary snapshot of the mucosa from where the tissue specimen has been taken. However, it has been reported that irregular EGFR expression may occur in a given tumor [30]. *In vivo* molecular imaging using CLE is closer to the natural situation than *ex vivo* procedures and may provide a more comprehensive state of EGFR expression by means of multiple optical biopsies. Furthermore, taking biopsy samples for IHC staining increases the risk of bleeding and other potential complications compared with the *in vivo* CLE method. This demonstrates the strong need for a molecular imaging method for CRC detection and surveillance [31].

Tremendous advancements in endoscopic techniques for imaging of the gastrointestinal tract, such as conventional endoscopy with narrow-band filters [26], near-infrared imaging [32], and capsule endoscopy [33], have been investigated for molecular imaging. The emergence of CLE, which permits real-time *in vivo* imaging of the gastrointestinal mucosa with a 1000-fold magnification, enables subsequent *in situ* immunofluorescence staining at the cellular and subcellular levels. In a landmark study, specific binding of topically applied fluorescent-labeled heptapeptide to colonic neoplastic cells could be shown by pCLE, with high sensitivity and specificity of 81% and 82%, respectively [23]. In further studies, molecular imaging has been proven to be feasible by targeting EGFR and vascular endothelial growth factor (VEGF) with fluorescent-labeled antibodies against EGFR and VEGF *in vivo* in rodent

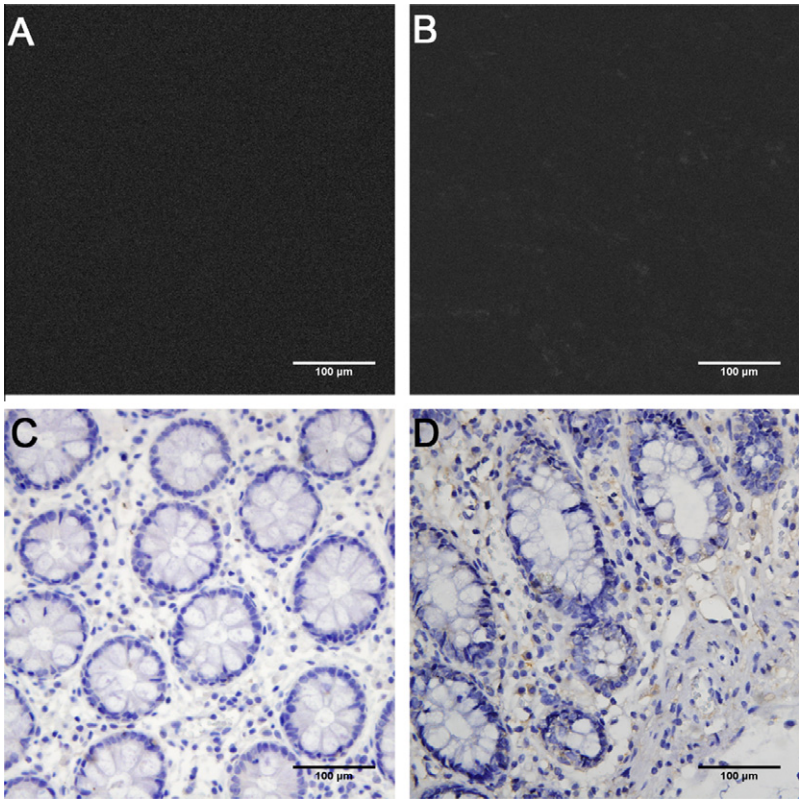

Fig. 6. Molecular imaging of epidermal growth factor receptor in normal mucosa. (A) and (B) No specific fluorescent signal (A) or only a slight fluorescence signal (B) was observed in normal mucosa of patients with colorectal neoplasia after topical application of fluorescent-labeled antibody by confocal laser endomicroscopy imaging. (C) and (D) Immunohistochemical staining of the respective biopsy specimens confirmed the intravital fluorescence patterns (original magnification 400×).

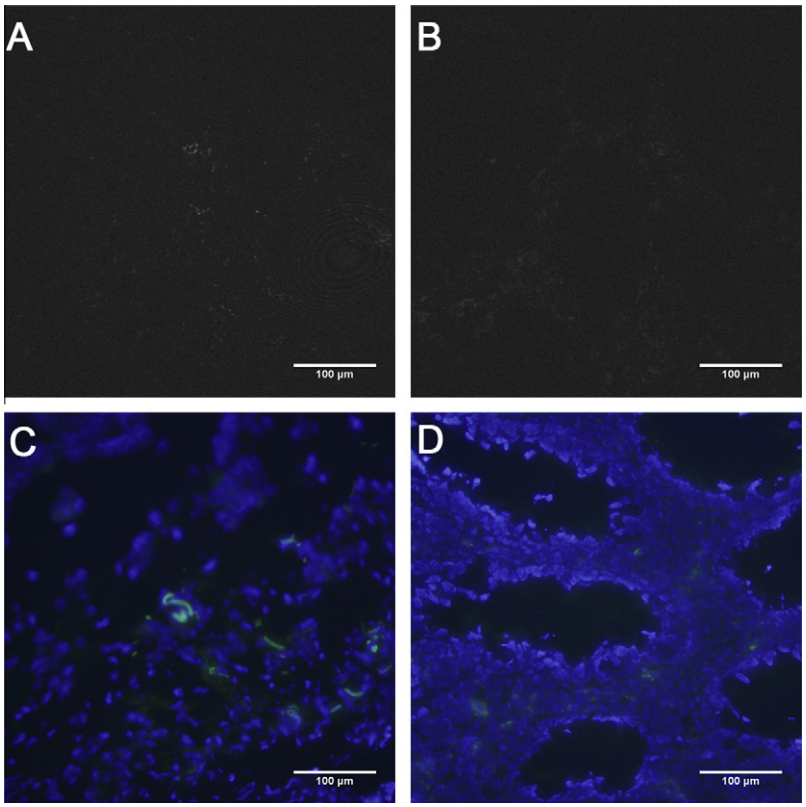

Fig. 7. *Ex vivo* fluorescence microscopy. (A) and (B) Molecular imaging of epidermal growth factor receptor (EGFR) in one colorectal cancer (CRC) (A) and one colorectal adenoma (B). (C) and (D) Corresponding *ex vivo* fluorescence microscopy of cryosections with *in vivo* bound AF 488-labeled anti-EGFR antibodies and *ex vivo* Hoechst 33342 blue nuclei counterstaining confirmed an intravital fluorescence pattern in one CRC (C) and one colorectal adenoma (D) ((C) and (D) original magnification 400×).

models and *ex vivo* in human tissue specimens using CLE [21,22]. These feasibility studies demonstrate that sufficient fluorescence intensity and an adequate contrast could be displayed by CLE in future investigations, by targeting an appropriate biomarker *in vivo*.

In this study, it has been proven, for the first time, that *in vivo* endomicroscopic molecular imaging of EGFR in patients with colorectal neoplasia is possible using CLE by targeting EGFR with a fluorescent-labeled antibody. Our pilot *in vivo* study demonstrated that specific EGFR staining was achieved at an antibody dilution of 1:50 and incubation time of 10 min. By topical application of fluorescent-labeled anti-EGFR antibody, CLE could observe and differentiate EGFR staining types based on the fluorescence intensity in patients with CRC and colorectal adenoma *in vivo*. The normal mucosa surrounding the neoplastic lesions showed no or only a weak fluorescence signal, and topical application of fluorescent-labeled isotype control antibody did not show a specific EGFR signal. These results corroborated the specificity of EGFR staining in colorectal neoplasia *in vivo*.

Currently, there are two possible routes of application for the molecular probes to gain access to the ROI. Topical application of the molecular probe onto the mucosal surface, used in current study, has been explored in many molecular imaging studies since it has many advantages compared with intravenous application [21,23,26]. With topical application using a spray catheter during colonoscopy, molecular imaging of an ROI can be carried out within a time frame for the endoscopic procedure, usually a few minutes, and local concentration may be higher than that used for systemic administration, whereas intravenous application needs a lead time for the probe to be distributed throughout the body. Furthermore, immunogenicity may be less and potential side effects fewer with topical application than with intravenous application. In accordance with this, we could not demonstrate the formation of HAMAs in a subset of our patients. All patients had been admitted to hospital for surgical or endoscopic resection. On the other hand, intravenous application is efficient for use in complete tumor tissue, while topical application is not a reasonable option for a large mucosal area.

There are some limitations to our approach of molecular imaging in patients with colorectal neoplasia using CLE. First, *in vivo* antibody labeling of the colorectal neoplasia may interfere with the *in vitro* IHC staining protocol. However, *in vitro* IHC correlated well with the *in vivo* findings. Topical application of a molecular probe in a local ROI might reduce the effect of *in vivo* labeling compared with systemic administration. Second, molecular imaging of EGFR by topical application of the probe in the local ROI could not demonstrate the expression status of the entire tumor, since EGFR expression is only captured at the tissue surface [34]. In the future, the development of a safe, intravenously applied molecular probe might overcome the drawback of topical application in detecting irregular biomarker expression. Third, the total number of patients in the prospective study was limited. Future study is to be performed to validate the current findings and correlate the progression features of neoplasia with *in vivo* molecular imaging in a larger study population. Fourth, the limited infiltration depth of CLE and the limited permeation of the antibody after topical administration might be possible disadvantages in clinical use. However, deeper tissue imaging can be achieved by near-infrared probes or use of small molecules [23,35,36]. Novel confocal systems with multiple excitation wavelengths may emerge with the perspective to facilitate clinical translation of the concept of molecular imaging in the field of gastrointestinal endoscopy. Ideally, such approaches would be combined with a macroscopic mode of molecular detection of lesions of interest (“molecular chromoendoscopy”). Finally, although the level of fluorescence was quantified, the fact that normal mucosa had a weak fluorescence in some cases suggests the limited applicability of this model in its present form. More selective biomarkers may prove valuable in the future.

In conclusion, our study shows, for the first time, that molecular imaging of EGFR is feasible *in vivo* using a fluorescent-labeled antibody against EGFR in combination with CLE in patients with colorectal neoplasia. More researches should be performed before clinical application. This technique shows a promising imaging approach for targeted individual therapies of colorectal neoplasia.

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None.

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