In Vivo Molecular Imaging of Colorectal Cancer With Confocal Endomicroscopy by Targeting Epidermal Growth Factor Receptor

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BACKGROUND & AIMS: Epidermal growth factor receptor (EGFR) is a therapeutic target for colorectal cancer (CRC). However, technical challenges have limited in vivo imaging of EGFR in CRCs. Confocal laser endomicroscopy (CLE) enables accurate microscopic visualization of CRC in patients during endoscopy. We evaluated the ability to use CLE in vivo for instantaneous molecular imaging of EGFR in CRC models. METHODS: Tumors were grown in mice (n = 68) from human CRC cell lines that expressed high (SW480 cells) or low (SW620 cells) levels of EGFR. Tumors were visualized in vivo with a handheld CLE probe after injection of fluorescently labeled EGFR antibodies. EGFR-specific fluorescence was graded from 0 to 3. Neoplastic and non-neoplastic specimens from human colorectal mucosa were examined. In vivo findings were correlated with histopathology, immunohistochemistry, and fluorescence microscopy analyses. RESULTS: CLE analysis of cell cultures confirmed the different expression levels of EGFR between cell lines. In living animals, CLE differentiated EGFR expression levels between tumor cell lines (mean fluorescence, 1.92 ± 0.22 [SW480] and 0.59 ± 0.21 [SW620], P = .0004). CLE analysis of EGFR expression in human specimens allowed distinction of neoplastic from non-neoplastic tissues (mean fluorescence, 2.0 ± 0.37 vs 0.25 ± 0.16, respectively, P = .0035). CONCLUSIONS: CLE can be used for in vivo, molecular analysis of CRC and to differentiate EGFR expression patterns in xenograft tumors and human tissue samples. Because CLE can be performed during endoscopy, in vivo molecular imaging might be used in diagnosis of CRC and to predict response to targeted therapies.

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in Western countries. Early detection during colonoscopy has been associated with a significantly improved survival. Autonomous cancer cell growth occurs through a multistep progress that includes up-regulation of growth factors and their receptors such as epidermal growth factor receptor (EGFR). EGFR is a transmembrane glycoprotein tyrosine kinase receptor that promotes tumor proliferation, invasion, and metastasis formation and neovascularization.

In CRC, EGFR overexpression has been reported in 25%–94% of cases and has been associated with a poor prognosis and early recurrence. The central role of the EGFR-mediated pathway has made it an attractive target for anticancer therapy. Cetuximab, a chimeric monoclonal antibody targeting the extracellular domain of EGFR, competitively prevents binding of the natural ligand and triggers the internalization of the receptor. It shows clinically beneficial antitumor activity. EGFR overexpression on the tumor was mandatory for early trials evaluating anti-EGFR therapies. This view has recently been challenged by demonstrating kras mutations in the downstream signaling cascade as more significant and by postulating a lack of relationship between EGFR expression and response to targeted therapy. Together, all these trials demonstrate efforts to provide an individualized therapy to ensure optimal antitumor efficacy while at the same time minimizing adverse events and the financial burden on the health care system.

This requires accurate selection of patients who could benefit from such a targeted therapy. Numerous optical technologies have been mandated for intravital noninvasive functional detection of (pre-)neoplastic lesions, such as spectroscopy, autofluorescence imaging, and induced fluorescence imaging. Recently,
confocal laser endomicroscopy (CLE) has been introduced, providing immediate microscopy during the ongoing endoscopy. With this novel technique, an intravital differentiation among normal, regenerative, and neoplastic mucosal changes is possible with high accuracy based on validated and reproducible morphologic criteria. Although a first study has recently reported molecular imaging with CLE, no trial has used labeled antibodies against a well-defined tumor-associated target, and, until today, analysis of receptor expression is a domain of ex vivo immunohistochemistry. The aim of the current study was to evaluate fluorescent targeting of EGFR for in vivo molecular imaging using CLE in a rodent model of human CRC and in human colon tissue.

**Materials and Methods**

**Tumor Cell Lines**

SW480 and SW620 cells were cultured in RPMI (with 10% fetal calf serum, 1% glutamine, 1% streptomycin, and 1% penicillin) at 37°C (5% CO2). Cells were harvested after 24–48 hours. For fluorescence-activated cell sorting (FACS), 10^5 tumor cells were incubated with 2.5 μL of fluorescein isothiocyanate (FITC)-labeled anti-EGFR antibody (CBL416F; Chemicon/Millipore, Schwalbach, Germany), isotype control (36875K; Pharmingen, Germany), or no antibody (negative control). Immunohistochemistry (IHC) of cytospins of single cell suspensions was performed with EGFR pharmDx (DakoCytomation, Carpinteria, CA). In short, slides were incubated with proteinase K and blocked with peroxidase. After incubation with the primary antibody, labeled polymer was added, and specific staining was visualized with 3,3’-diamino-benzidine.

Tumor cells cultured in chamber slides (Nalge Nunc International, Naperville, NY) were incubated with FITC-labeled anti-EGFR-antibody without fixation. Cell suspensions were first evaluated by confocal microscopy (FIVE1; Optiscan, Notting Hill, Australia), using exactly the same settings as for in vivo mouse imaging. Next, bench top fluorescence microscopy of cell suspensions was performed (IX70; Olympus, Hamburg, Germany). FITC-labeled anti-EGFR was detected at 516 nm, nuclear counterstaining with DAPI (Vector Laboratories, Burlingame, CA) at 470 nm.

**Animal Models**

Tumors were induced by injection of 10^5 SW480 or SW620 cells in 68 Balb/c nu/nu mice (Charles River Laboratories, Inc, Sulzfeld, Germany). Subcutaneous tumors were induced in the groin of 55 mice, cells were injected into the spleen after a small left lateral incision in 6 mice, and a 5-mm vital xenograft from a subcutaneously induced tumor was implanted into a cecal pouch via a small abdominal incision in 7 mice. Imaging was performed after 3–6 weeks when tumor size approximated 5–10 mm or wasting occurred. For confocal imaging, mice were deeply anesthetized using ketamine-xylazine (120 mg/kg and 16 mg/kg intraperitoneally [IP], respectively). After the examination, mice were killed by a ketamine-xylazine overdose. Animal procedures were approved by the local review board (Landesuntersuchungsamt Rheinland-Pfalz: 1.5 177-07-04/051-59).

**CLE and Staining Protocol**

In the rigid confocal probe (FIVE1; Optiscan), an excitation wavelength of 488 nm was delivered, and light emission was detected at 505–585 nm. Serial en face optical sections of 475 × 475 μm were obtained with a lateral resolution of 0.7 μm (1024 × 1024 pixels) at variable imaging depth (adjustable from surface to 250 μm) but with otherwise predefined and fixed instrument settings throughout molecular imaging experiments including laser power of 1000 μW and standardized brightness and γ control. For tumor morphology, Fluorescein (Alcon Pharma, Freiburg, Germany) and acriflavine (Sigma Pharmaceuticals, South Croydon, Victoria, Australia) were injected intracardially at 100 μg/g and 10 μg/g body weight, respectively, as previously established. 0.1 μg/g Of FITC-labeled anti-EGFR antibodies (Chemicon/Millipore) in 200 μL phosphate-buffered saline were injected 45 minutes prior to confocal imaging after a run-in phase demonstrated that this time frame permits antibody binding to target sites. For intravital confocal imaging, the tumor was exposed by a small skin or abdominal incision, and the handheld confocal probe was positioned in a way to achieve full contact of the confocal imaging window with the tissue. The complete tumor tissue was screened with the confocal probe for fluorescent signal by carefully moving the probe across the tumor surface while at the same time adapting imaging plane depth.

Confocal imaging of the tumor before the injection of anti-EGFR-antibody or after injection of a FITC-labeled isotype control antibody served as negative controls to exclude interference by autofluorescence or unspecific binding. In addition, fluorescence in kidney, spleen, and liver was evaluated after antibody injection.

**Ex Vivo Correlation of EGFR Expression**

From the examined sites, tissue specimens were fixed in 4% buffered formalin and embedded in paraffin. Serial sections of 4 μm were stained with H&E. Additional specimens from 21 mice were snap frozen in liquid nitrogen. Bench top fluorescence microscopy of tumor crossections was performed without EGFR restaining (to visualize in vivo bound FITC-labeled EGFR antibodies) and with ex vivo nuclear counterstain as specified above.
EGFR immunostaining using the immunohistochemical system kit EGFR pharmDx was performed according to the EGFR pharmDx scoring guidelines. It included the front of invasion from the mean of 3 high-power fields because this region was shown to contain the greatest density of EGFR-positive cells. IHC of tumor cell membranes was scored as follows: 1+, weak; 2+, moderate; and 3+, strong. The absence of membrane staining or cytoplasmic staining was reported as negative (0).

**Imaging of Human Tissue**

Colonoscopic biopsy or surgical specimens of non-neoplastic (normal, n = 5; unspecific colitis, n = 1; ischemia, n = 2) and neoplastic colon tissue (CRC, n = 6; intraepithelial neoplasia, n = 2) were incubated with FITC-labeled anti-EGFR-antibody (Chemicon/Millipore) for 30 minutes at 1:50 dilution at room temperature shielded from light. Confocal imaging was performed without further tissue processing using the FIVE1 probe (n = 14) or the confocal endomicroscope (n = 2) that has the same optical properties (Pentax EC-3870CIFK; Pentax, Tokyo, Japan). IHC was performed with EGFR pharmDx. Final histopathology served as the gold standard to classify specimens. The study protocol was approved by the Ethics Committee of Rheinland-Pfalz, Germany (No. 837.321.03).

**Statistical Analysis**

From confocal images captured in vivo, EGFR-specific fluorescence intensity was graded as absent (0), weak (1+), moderate (2+), or strong (3+). This semiquantitative grading was used to match in vivo staining with scoring in IHC and to provide a scale that is easily transferable to clinical practice. Confocal images were ranked independently by 2 investigators. If differences in the grading occurred, consensus was obtained by again jointly evaluating the images (n = 3 tumors). Fluorescence from bench top microscopy of cryosections was graded accordingly.

Statistical analysis was performed using the statistical software package GraphPad Prism (v5.00; GraphPad, Inc, La Jolla, CA). Fisher exact test was used to examine the statistical significance of differences of the fluorescence signal strength between SW480 and SW620 tumors and between neoplastic and non-neoplastic human tissue samples. All P values were generated using 2-sided tests. For the primary hypotheses of differences in the fluorescence signal strength between SW480 and SW620 tumors and between neoplastic and non-neoplastic human tissue samples, the global significance level was set at 5%. Because a second analysis investigated the semiquantitative assessment of fluorescence strength, a Bonferroni correction was used to adjust for these 2 tests in both groups;

![Figure 1](image-url)
a difference was considered statistically significant if $P$ was $\leq .025$. The comparison of means of the fluorescent signal strength was regarded as explorative, and the $P$ values of the corresponding tests are presented for descriptive reasons only. Spearman rank correlation coefficient was used to correlate in vivo confocal endomicroscopy and ex vivo bench top fluorescence microscopy on cryosections.

**Results**

**Differential EGFR Expression by Tumor Cell Lines**

FACS analysis confirmed the high EGFR expression of SW480 cells, whereas SW620 cells only showed an absent-to-mild EGFR staining. These patterns were paralleled by a strong specific staining of SW480 cells in IHC of cytospins in contrast to an almost absent specific staining of SW620 cells (Figure 1).

The ability of handheld CLE to discriminate EGFR expression was corroborated by visualizing unfixed tumor cells in cell culture after incubation with FITC-labeled antibody: A strong cellular signal was seen in SW480 cell culture, whereas SW620 cells did not show a specific signal. EGFR-positive tumor cells showed a superficial, cytoplasmic, or (peri-)nuclear signal. Bench top fluorescence microscopy of SW480 or SW620 cells confirmed the staining patterns from CLE (Figure 2).

**Tumor Morphology in Vivo**

Tumor morphology was readily available using the FIVE1 confocal probe in both SW480 and SW620 tumors. After acriflavine injection, the densely packed tumor cells with an altered nuclear-to-cytoplasmic ratio were visualized. Fluorescein depicted the disorganized overall tumor tissue structure. In vivo findings correlated well with ex vivo H&E staining, as demonstrated previously.35

**In Vivo Molecular Imaging of Human CRC in Rodent Models**

With the handheld confocal probe, large areas were screened for specific fluorescence by moving the probe gently across the tumor surface. In most SW480 tumors, CLE found a clear specific signal within the malignant cells after systemic antibody application. The high resolution of the confocal system permitted the observation of cytoplasmic, cell surface, or perinuclear signal, similar to confocal microscopy of cell cultures (Figure 3). In bench top fluorescence microscopy, cryosections of SW480 tumors confirmed the intravital fluorescence patterns after in vivo binding of FITC-labeled antibody.
Figure 3. High-resolution molecular imaging in vivo. Within a SW480 tumor, a membranous staining pattern is visualized in vivo (arrows, A). Magnification of a single cell (boxed area in A) shows accumulation of the fluorescence signal at the level of the cell surface (arrows, B).

Figure 4. Molecular imaging of SW480 tumors in live mice. CLE showed a clear specific signal within the tumor tissue after systemic application of FITC-labeled anti-EGFR antibody (arrows, A). Specificity of this signal was confirmed by ex vivo IHC of tumor sections (arrows, B). Ex vivo bench-top fluorescence microscopy confirmed the FITC signal (arrows; green) within the tumor after intravitral staining (C; overlay with ex vivo nuclear counterstaining [blue], D).
anti-EGFR-antibodies (Figure 4). In most SW620 tumors, no such specific staining was observed in vivo with the CLE or in cryosections after intravital staining (Figure 5). IHC was performed in a subset of tumors that had not been visualized previously in vivo because intravital antibody labeling of the human-mouse xenograft interfered with IHC protocols in both paraffin-embedded and cryopreserved tissue. In SW480 tumors, a specific signal was found in contrast to no such signal in SW620 tumors.

To corroborate the specificity of in vivo molecular imaging, 3 types of negative controls were performed in addition to imaging EGFR-negative SW620 tumors: First, no fluorescent signal was seen in tumors before the application of FITC-labeled antibody. Second, the injection of FITC-labeled isotype control antibody did not result in tumor-specific fluorescence. Third, a nontumor-associated specific fluorescence after the EGFR-antibody injection in tissue other than tumor was not observed.

However, metastases demonstrated a specific fluorescent pattern according to the cell type of the primary tumor. An EGFR-positive liver metastasis (after intrasplenic injection of SW480 cells) showed a strong signal after in vivo labeling (Figure 6). The metastasis was only found by screening the macroscopically normal liver with the confocal probe in vivo. A targeted biopsy from this region correlated this finding to the invasion front of the tumor tissue ex vivo.

Quantification of EGFR Expression by Fluorescence Intensity

The EGFR-specific signal from confocal images captured in vivo was graded from 0 to 3+ (Figure 7A) with a substantial interobserver agreement of 93%. Importantly, the complete tumor tissue was screened for the
site showing the strongest fluorescence, which was then included in the evaluation. If tumor tissue was assessed accordingly, a significantly higher EGFR expression was observed in SW480 tumors and SW620 tumors \((P < .01, \text{Figure 7B})\). This discrimination was also significant \((P = .002)\) if only the presence of any fluorescent signal was evaluated \((0 \text{ vs } 1+ \text{ to } 3+)\). In detail, SW480 tumors stained positive in 21 (87.5%) and negative in 3 (12.5%) tumors. SW620 showed a specific fluorescence in 7 (41.8%) tumors, whereas no such signal was observed in 10 (58.8%) tumors. Five of the 7 positive SW620 tumors showed only weak fluorescence \((1+)\). Here, unspecific staining could not be completely excluded. Mean fluorescence intensity and SEM was 1.92 ± 0.22 in SW480 tumors and 0.59 ± 0.21 in SW620 tumors \((P < .001, \text{Figure 7C})\). These results were confirmed in bench top fluorescence microscopy (fluorescence intensity \[\text{mean ± SEM}\], 2.60 ± 0.22 in SW480 tumors and 0.36 ± 0.24 in SW620 tumors, \(P < .001\)). The correlation between bench top fluorescence microscopy on frozen sections and in vivo confocal microscopy was substantial \((\text{Spearman}, \text{ } r = 0.81)\).

**EGFR Expression on Human Colorectal Tissue**

To anticipate a potential use of EGFR targeted imaging in colonoscopy with CLE, the topical application of labeled antibody was simulated by incubation of fresh tissue specimens from healthy colon or neoplastic lesions in antibody solution. All specimens from neoplastic tissue showed a specific signal after EGFR staining \((8/8 \text{ lesions})\), whereas healthy tissue and samples from unspecific colitis did not show specific fluorescence. Weak fluorescence \((1+)\) was found in only 2 samples of resection specimens after ischemia. Differences between neoplastic and non-neoplastic specimens were statistically significant, if only the absence or presence of any specific
fluorescence was evaluated \( (P = .003) \) or if differences were assessed semiquantitatively \( (P = .007) \). Mean specific fluorescence of human tissue samples was \( 2.13 \pm 0.30 \) for neoplasia and \( 0.25 \pm 0.16 \) for normal mucosa \( (P < .002) \). IHC performed in a subset of surgical resection specimens showed strong \( (3+) \) staining correlating with CLE findings (Figure 8).

**Discussion**

In this study, the first evidence is provided that in vivo endomicroscopic molecular imaging of human CRC is possible by specifically targeting EGFR with a fluorescently labeled antibody. This approach successfully visualized and differentiated human CRC tumor types based on their EGFR expression in a rodent xenograft model in vivo. To mimic a potential topical use of such molecular imaging in CLE in humans, labeled antibody was applied to unfixed human tissue specimens, and successful molecular imaging was achieved. Both intravenous application in rodents and topical application in fresh human tissue provided adequate contrast for confocal imaging.

Intravenous application of labeled anti-EGFR in the current study resulted in a quick delivery to the tumor without the necessity to clear the tissue from adherent

**Figure 7.** Quantification of specific in vivo fluorescence. Signal strength was graded from absent \((0)\) to weak \((+\), moderate \((+ +)\), and strong \((+ + +)\) \((A)\). A significantly stronger expression was demonstrated in SW480 tumors \((light\ columns)\) than in SW620 tumors \((dark\ columns)\), \( P < .01 \) \((B)\). Mean specific fluorescence in vivo after EGFR labeling was \( 0.59 \pm 0.21 \) in SW620 tumors and \( 1.92 \pm 0.22 \) in SW480 tumors \( (P = .0004) \). Bars indicate SEM \((C)\).
tissue or mucus. Thus, the complete tumor tissue could be rapidly screened for the strongest EGFR expression exploiting the full variable imaging plane depth of the confocal probe. Topical application may prove more practical for clinical use during colonoscopy and is probably associated with less immunogenicity and adverse events, although the incubation period may still have to be optimized for clinical application. Spraying catheters and even enemas have been advocated for topical delivery of targeted dyes. However, in most studies using CLE so far, systemic contrast has been preferred to superficial staining usually obtained with topical application.

The percentage of EGFR positivity in human CRC has been reported with surprisingly high variability ranging from 25% to 94%. Technical pitfalls might account at least in part for this observation and might explain reports on the inconsistent influence of EGFR expression in response to targeted therapy. Irregular EGFR expression within a given tumor has been observed, and even multiple random thin tissue sections for routine IHC may therefore be prone to sampling error. Screening the tumor tissue in vivo by taking multiple optical biopsy specimens as performed in our approach may render EGFR patterns more comprehensively, although confocal endomicroscopy is still confined to superficial tumor areas by its limited imaging plane depth. Only 78% concordance between EGFR expression in primary CRC and synchronous metastases has been reported. This change in receptor expression could explain for the finding that some SW620 tumors in the current trial demonstrated a specific fluorescence signal, whereas some SW480 tumors were EGFR negative. In fact, these 2 cell lines are derived from the primary tumor (SW480) and metastases (SW620) of the same patient.

Interestingly, EGFR immunoreactivity has been found to be inversely correlated to times of fixation and storage of tissue, and the fixative itself has modified EGFR immunoreactivity. These problems might be tackled by an in vivo approach. IHC is not universally standardized.

**Figure 8.** Molecular imaging in humans. Tissue samples from a neoplastic lesion were incubated in labeled EGFR antibody solution to mimic topical application during colonoscopy (A). CLE showed a specific signal within the lesion (arrows, B). Mean specific fluorescence of human tissue samples was 0.25 ± 0.16 for normal mucosa vs 2.12 ± 0.30 for neoplasia (P < .002, C). Bars indicate SEM. Histopathology confirmed malignancy, and IHC showed EGFR expression in multiple tumor cells (arrows, D).
nor is the antibody used or its target site,\textsuperscript{39,45,46} and IHC is therefore challenged by different new modalities such as in situ hybridization or ligand binding assays.\textsuperscript{47} Reproducibility among different trials has been further complicated by the use of different arbitrary cut offs to define EGFR positivity, ranging from 1% to 10% of cells. Therefore, in the current study, images were captured at standardized instrument settings, and a semicontinuous assessment scheme was applied. An automated calculation of the specific fluorescence intensity relative to a background region may help to further objectify such fluorescence measurements at real time in the future. Cellular staining patterns such as cytoplasmic or membrane activity\textsuperscript{40} could be reproduced in vivo.

In vivo molecular imaging, ie, the combination of novel imaging technologies with targeted staining, has rapidly emerged with the perspective to significantly impact on both basic research and clinical practice. Labeling of EGFR for molecular imaging in whole tissue has been examined before in multilayer constructs or slices of squamous cell dysplasia ex vivo. In this study, a bench top confocal microscope was used, and permeability enhancing agents were needed prior to application of antibody.\textsuperscript{48} In rodent models, tumors were macroscopically located by luminescence 1–5 days after the injection of light emitting live bacteria accumulating within the tumor tissue.\textsuperscript{49} Specific targeting of EGFR with a near infrared probe permitted intravital fluorescence microscopy in breast cancer xenografts in rodents.\textsuperscript{50} However, these approaches still relied on bulky bench top devices and staining protocols not appropriate for use in humans. Our group has recently reported on the use of a miniaturized prototype confocal probe for in vivo fluorescent imaging of neuroendocrine tumors in rodents with carboxyfluorescein-labeled, high-affinity ligands to somatostatin receptors.\textsuperscript{35}

In humans, both detection and characterization of lesions by molecular endoscopy have been studied. A pilot colonoscopy trial used labeled monoclonal antibodies against carcinoembryonic antigen with a prototype fluorescence endoscope for macroscopic tumor detection. Specific fluorescence after topical administration was seen in 19 of 25 carcinomas\textsuperscript{51} but were, however, not correlated to in vivo microscopy. A different approach was followed in a recent trial in which a heptapeptide sequence identified from a phage library was conjugated with fluorescein. The molecular target of this sequence is not yet elucidated, but, after topical administration during colonoscopy, dysplastic lesions were visualized by confocal microscopy with high sensitivity and specificity (81% and 82%, respectively).\textsuperscript{31} Inherently, the problem of a receptor- or antigen-negative tumor has not been addressed in the trials mentioned above. However, an antibody “cocktail” targeting multiple receptors\textsuperscript{52} or multichannel imaging may prove valuable in the future.

Many studies on molecular imaging used excitation and emission wavelengths in the near infrared spectrum to achieve deeper tissue imaging. In the current trial, fluorescein-based detection was preferred because the FIVE1 probe used has identical optical properties as the endomicroscope registered for endoscopy in patients, and fluorescein has been in routine clinical use for decades with a favorable safety profile.\textsuperscript{53,54} In addition, specific binding of anti-EGFR-antibodies to tumor tissue occurred rapidly within a time frame still acceptable for a colonoscopy setting. Although meticulous evaluation of potential clinical applications is mandated, this ensures that an immediate transfer from bench to bedside is feasible from a technical point of view even today.

In summary, the current trial demonstrates that molecular imaging is feasible in vivo by targeting EGFR in a xenograft model of human CRC and ex vivo on human tissue. Discrimination of tumor cells was possible based on their molecular signature. As a perspective for further clinical use, this approach could ideally be combined with a high-resolution macroscopic fluorescent imaging instrument. Such a combined approach would then offer a red-flag technique for macroscopic detection (a “molecular chromoendoscopy”) and simultaneous confocal endomicroscopy to detect immediately the expression patterns of a lesion during ongoing endoscopy. With this, targeted individualized therapies could be based on an enhanced diagnosis and accurate selection of patients.

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