

Endomicroscopy with Fluorescent CD105 Antibodies for "In Vivo" Imaging of Colorectal Cancer Angiogenesis

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ABSTRACT: The aim of this case report was to evaluate the feasibility of *in vivo* acquisition of microscopic images using fluorescent CD105 antibodies for molecular imaging in human colorectal cancer. After excluding the presence of tissue autofluorescence, the antibody solution was topically administered through a spray-catheter. The targeted area was analyzed by eCLE and images were recorded. The fractal dimension of tumor vessels and the vessel density were determined using ImageJ software. Immunohistochemistry was used as a gold standard. *In vivo* CLE analysis of CD105 expression enabled the study of tumor vascular network, revealing a chaotic structure.

KEYWORDS: CD105-FITC, colorectal cancer, confocal laser endomicroscopy (CLE), *in vivo* endoscopic imaging, neoangiogenesis

Introduction

Advances in angiogenesis-based biomarkers research as well as molecular imaging of gastrointestinal malignancies have provided important understanding of tumor progression and offered great promise for developing new strategies regarding antiangiogenic therapies used for improvement of patient outcomes. To date, determination of neoangiogenic status and its dynamic assessment in real-time has been challenging and, therefore, has made treatment optimization in colorectal cancers difficult. One strategy for antiangiogenic therapy is the long term suppression of forming new blood vessels [1]. Recent developments in endoscopic imaging technologies such as confocal laser endomicroscopy (CLE) have contributed to the progress from macroscopic evaluation to *ex vivo* molecular experiments and consequently to promising *in vivo* imaging by using fluorescently labeled antibodies [2,3].

Confocal laser endomicroscopy (CLE) provides real-time *in vivo* histological images ("virtual biopsies") of the gastrointestinal mucosa during endoscopy [4,5]. Since currently approved fluorescent dyes enabled more detailed assessment of the mucosal architecture and network vessels, the challenging idea that CLE combined with molecular-targeted fluorescent contrast agents could be used

with unlimited applications in various diseases has emerged [6]. Atreya et al [7] have recently demonstrated that *in vivo* molecular imaging with fluorescent antibodies to TNF is feasible in patients with Crohn's disease and that this could serve as a predictive biomarker for the therapeutic response to adalimumab therapy. Their study can serve as a cornerstone for *in vivo* applications of immunoendoscopy in evaluating responses to antiangiogenic therapy in colorectal cancers.

We had previously proposed CD105 in conjunction with CLE as a more reliable tool for real-time evaluation of the angiogenetic status of patients with colorectal cancer, demonstrating that specific imaging of tumor microvessels is feasible using *ex vivo* CLE examination and CD105 immunostaining on fresh tissue samples [8]. The presented case illustrates the feasibility of *in vivo* application of fluorescent antibodies for molecular imaging on human patients with colorectal cancer, before further adaptation of the method to targeted therapy.

Case report

The patient (65 years old) was recruited from Department of Surgery, Emergency County Hospital of Craiova, immediately before undergoing surgical intervention for rectal cancer. The patient read and accepted the written

informed consent prior to study entry. Ethics approval for this investigation was obtained from local Scientific and University Ethics and Deontology Committee. The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

In vivo imaging with FITC-CD105 antibodies

The patient underwent standard colonoscopy (CFQ160ZL, Olympus, Tokyo, Japan) followed by eCLE examination (Pentax EC-3870 CIFK, Tokyo, Japan) for the suspicious lesion identified before. Eight tumor biopsies were collected for immunohistochemistry and histopathological assessment. During endoscopic procedures, the patient has been anesthetized with i.v. narcotics (Propofol).

Before *in vivo* testing, multiple attempts were made at establishing the optimal antibody-tissue contact time using paraffin-embedded tissue sections under fluorescence microscopy conditions (FITC-CD105 incubated at 37°C, at 5 minutes, 10 minutes, 30 minutes and, respectively, 60 minutes time interval). We have chosen the concentration which provided the strongest signal without overexposure in order to avoid loss of details.

CLE device has been calibrated at the beginning of the experiment. Tumor was washed with saline solution (in order to cleanse the area of interest and to avoid diminishing of fluorescence signal related to osmotic pressure of other solutions). A spray catheter was fit to a syringe filled with 1 ml of the fluorescent labeled antibody solution (FITC-labeled anti-CD105/Endoglin antibody, Exbio, 1:5). The fluorescent antibody solution was topically administered through the spray-catheter after excluding the presence of tissue autofluorescence. After 10 min of incubation of the antibody solution and after handling the endoscope to achieve a stable position without motion artifacts, the targeted area was analyzed by eCLE and images were recorded. Images have been captured at a rate of 12 frames/second (7 µm thick, 0.7 µm lateral resolution and the field of view of 475 µm x 475 µm) up to a maximum depth of 250 µm.

Finally, at the end of the procedure, saline solution was injected into the colon lumen two times in order to wash out all unbound fluorescent dye.

Off-line analysis of CLE images

Further analysis of images obtained by CLE was performed offline by using ImageJ (National Institutes of Health, USA). The fractal dimension of tumor vessels was calculated using “fractal box count” tool on the confocal serial images converted to RGB stacks.

Fractal dimension of tumor microvessels represents the complexity of the vascular network and has been proposed as a parameter that can be used for the analysis of the therapeutic effect of an antiangiogenic therapy. For this purpose we used *fractal box count* tool to automatically obtain the fractal parameter, based on the protocol proposed by Waldner et al [6].

Vascular density, generally evaluated in immunochemistry by estimating the image area covered by vessels, was also estimated, as a non-fractal parameter. Grid method was applied on images combined into stacks to determine the vessel density.

Immunohistochemistry

Corresponding tumor biopsy samples underwent immunohistochemical staining for CD105 and CD31, assessing microvessel density (MVD) and vascular areas. Immunohistochemical results were interpreted by two experimented pathologists (DP, CVG) who were blinded to the subject and to the CLE data.

IHC staining was performed as previously described [3]. All the samples have been previously fixed in 10% buffered formalin and have been embedded in paraffin. Afterwards, 4µm thickness sections were prepared, deparaffinized in xylene, rehydrated in graded alcohol and washed with distilled water. Internal peroxidase activity and false antigenic sites were inhibited by 1% H₂O₂ (30 minutes) and by incubating the slides in 5% skimmed milk (Bio-rad, München, Germany). Tissue sections were incubated overnight at 4°C with the anti-CD105 monoclonal antibody (rabbit anti-human CD105 polyclonal antibody diluted as 1:50, LabVision, Fremont, CA, USA) at a 1/50 dilution and with the anti-CD31 monoclonal antibody (mouse anti-human CD31, IgG1, clone JC70A, Dako, Glostrup, Denmark), respectively. The sections were counterstained with Hematoxylin- Eozin and 3-4 hotspot high vessel density areas were captured using a Nikon Eclipse 55i microscope equipped with a 5 Megapixel CCD color camera (Nikon, Tokyo,

Japan). Brown staining was counted as CD105 and CD31 positive vessels (40x objective).

Fractal analysis of vascular network was assessed on binarized - skeletonized masks by using the fractal box counting method in ImageJ [9]. Microvascular density was assessed by vessel count and total vessel area on the images selected according to Weidner's "hot spot" method (Weidner et al., 1993).

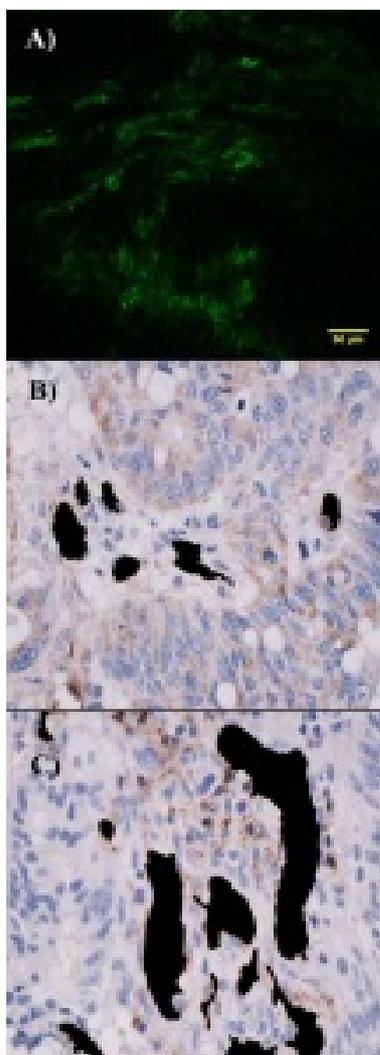


Figure 1. Representative in vivo CLE image of the rectal tumor, showing the vasculature network highlighted by CLE combined with FITC-CD105 (A); corresponding immunohistochemical staining for CD105 (B) and for CD31, with vascular areas marked (C) (40x)

In vivo imaging of tumor vascularization

In vivo CLE analysis of CD105 expression in human colorectal cancer enabled the study of vascular network, revealing a chaotic structure (Fig. 1A). Both good and medium quality images were eligible for clinical analysis (n=67 images). Fractal value was 1.46, indicating the chaotic architecture (with 2 being the fractal value for normal vessels) (Fig.2,

Fig.3). Tumor vessel density was 146.09 vessels/mm². No immunological side effects or other adverse events occurred when anti-CD105 antibodies conjugated with FITC have been administered topically in vivo.

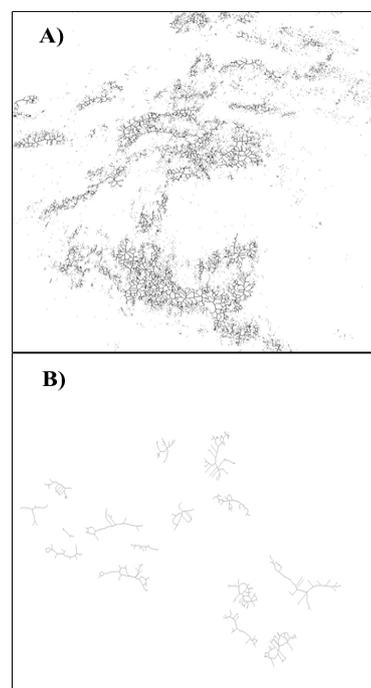


Figure 2. The skeletonized image of the tumor vessels for calculating the fractal dimension by using the ImageJ software –(A) CLE and (B) IHC

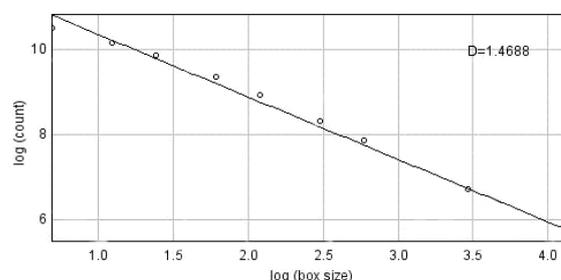


Figure 3. Depiction of the CLE vascular fractal dimension in a logarithmic scale [6]

Ex vivo assessment by using immunohistochemistry

Immunohistochemistry evaluation confirmed the presence of blood vessels stained with commonly used CD31 as well as with CD105, with less vessels detected by using CD105 than by using CD31 staining (Fig.1B, 1C). CD31 allowed a count of 294.3 tumor vessels/mm² and a vascular area of 21.4%. There was a count of 239.55 tumor vessels/mm² due to CD105 expression with a vascular area of 5.28%. Fractal dimension was 1.12 for CD105 stained images.

Standard histological assessment showed a moderately differentiated adenocarcinoma (G2) with mucinous areas. Postoperative stage of the tumor was T3N1M0.

Discussion

Acridflavine (landmark for cell nuclei-cytoplasm) [10] and SYTOX green (nucleic acid of dead cells) [11] are the only fluorescent dyes topically applied to an area of interest for use with CLE that have been reported to date. Otherwise, anti-VEGF antibodies [12] and fluorescently labeled RGD peptides [13] have also been proposed in tumor angiogenesis assessment for systemic administration. In colorectal cancer, *in vivo* studies on molecular imaging using endoscopic techniques have been applied on mice models [6,14,15].

Different markers with promising roles expressed by the vascular endothelial cells have been usually given by i.v. administration. Currently, no appropriate biomarkers which predict response to antiangiogenic treatment in colorectal cancer are validated. *To the best of our knowledge, this is the first report of in vivo CLE imaging of vascularization in human colorectal cancer using CD105.*

Most of CD105 functions are predominately associated with TGF- β signaling and only in some processes it is independent [16]. Dual blockade of both TGF- β and CD105 pathways leads to the inhibition of angiogenesis. Moreover, the anti-CD105 antibody as a single agent or CD105 as a target for oral DNA vaccine have also been reported to be effective for suppressing or preventing tumor progression and prolonging survival *in vitro* [17].

Because many studies have already reported the role of CD105 correlated with the vascular density in tumor aggressiveness and targeted therapy [18,19,20], CLE opens the possibility to apply CD105 targeted therapy (which until now was only tested *in vitro* and on animal models) to *in vivo* human subjects and to monitor recurrence or altered vessel morphology during therapeutic response.

In our previous study, the use of anti-CD105 antibodies was suitable for identifying microvessels especially in tumor tissue [8]. The presence of tumor vessels during *in vivo* examination was also confirmed with CLE by using the same endothelial marker.

Vessel morphology in tumors has a chaotic structure. Therefore, the fractal dimension differs from normal vessels to tumor vessels and can be used for the analysis of tumor development and morphological changes during antiangiogenic therapy [21,22]. Normal capillaries tend to have fractal dimensions that are nearly 2, whereas tumors usually have lower values [22]. Similarly to our case, in their protocol study on *in vivo* CLE on mouse model, Waldner et al [6] obtained a fractal value of 1.54, as a more chaotic architecture as seen in tumors.

Further *in vivo* use of CLE imaging with fluorescently marked antibodies for morphologic changes of newly-formed microvessels in colorectal neoplasm could be extended to detect changes in colon lesions vascularization as an indicator of early endoscopically detectable signs of cancer and to further selection of an appropriate therapeutic strategy.

Image accuracy could have been influenced by several factors. In our preliminary study, the time interval from the topic administration of antibodies to CLE examination was of 10 minutes. Given the fact that valid time interval of ideal antibody binding is dependent on the molecular target and the route of administration [6], further studies *in vivo* with various duration attempts are necessary for the standardization of the protocol. Other possible factors influencing image quality include CLE probe not placed properly, inadequate quantity of FITC-CD105, overexposure to laser power (e.g. the laser power was not turn off in time when it was not in use) the increased heat production of the laser light, embarrassing continuous observation of the same area for longer periods of time by phototoxic effect induced in the tissue. Therefore, by controlling these situations, CLE image quality could be improved so as to facilitate better *in vivo* detection of pathological changes.

Currently, the method is limited by the availability of a suitable fluorescent dye. In particular, when using fluorescent antibodies, the ability of an antibody to bind to its target *in vivo* might limit its overall usability in the absence of cell permeabilization (e.g. antibodies for intracellular targets) [6]. General technique-related limitations could imply CLE small field of view which could make this method prone to sampling error.

Conclusion

In vivo molecular imaging of human colorectal cancer neoangiogenesis using CLE and FITC-CD105 antibodies is feasible. Future *in vivo* applications of immunoendoscopy using CD105 could provide a more specifically analysis of tumor microvascular architecture in order to improve diagnosis, patient stratification and monitoring of antiangiogenic therapy.

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References

1. Zhang ZL, Wang JH, Liu XY. Current strategies and future directions of antiangiogenic tumor therapy. *Sheng wu hua xue yu sheng wu wu li xue bao Acta biochimica et biophysica Sinica* 2003;35(10):873-80.
2. Cartana T, Saftoiu A, Gruionu LG, et al. Confocal laser endomicroscopy for the morphometric evaluation of microvessels in human colorectal cancer using targeted anti-CD31 antibodies. *PLoS one* 2012;7(12):e52815.
3. Ciocalteu A, Saftoiu A, Cartana T, et al. Evaluation of new morphometric parameters of neoangiogenesis in human colorectal cancer using confocal laser endomicroscopy (CLE) and targeted panendothelial markers. *PLoS one* 2014;9(3):e91084.
4. De Palma GD. Confocal laser endomicroscopy in the "in vivo" histological diagnosis of the gastrointestinal tract. *World journal of gastroenterology : WJG* 2009;15(46):5770-5.
5. Nguyen NQ, Leong RW. Current application of confocal endomicroscopy in gastrointestinal disorders. *Journal of gastroenterology and hepatology* 2008;23(10):1483-91.
6. Waldner MJ, Wirtz S, Neufert C, et al. Confocal laser endomicroscopy and narrow-band imaging-aided endoscopy for in vivo imaging of colitis and colon cancer in mice. *Nature protocols* 2011;6(9):1471-81.
7. Atreya R, Neumann H, Neufert C, et al. In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nature medicine* 2014;20(3):313-8.
8. Ciocalteu A, Saftoiu A, Cartana T, Cherciu I, Gruionu L, Pirici D, Georgescu C, Gruionu G. Feasibility study for the evaluation of morphological pattern of neoangiogenesis in human colorectal cancer using confocal laser endomicroscopy and targeted anti- CD105 antibodies. *United European Gastroenterology Journal*; 2014;2 (Supplement 1)
9. Streba CT, Pirici D, Vere CC, et al. Fractal analysis differentiation of nuclear and vascular patterns in hepatocellular carcinomas and hepatic metastasis. *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie* 2011;52(3):845-54.
10. Neumann H, Kiesslich R, Wallace MB, et al. Confocal laser endomicroscopy: technical advances and clinical applications. *Gastroenterology* 2010;139(2):388-92, 92 e1-2.
11. Alencar H, Mahmood U, Kawano Y, et al. Novel multiwavelength microscopic scanner for mouse imaging. *Neoplasia* 2005;7(11):977-83.
12. Foersch S, Kiesslich R, Waldner MJ, et al. Molecular imaging of VEGF in gastrointestinal cancer in vivo using confocal laser endomicroscopy. *Gut* 2010;59(8):1046-55.
13. Beer AJ, Schwaiger M. Imaging of integrin $\alpha v \beta 3$ expression. *Cancer metastasis reviews* 2008;27(4):631-44.
14. Goetz M, Ziebart A, Foersch S, et al. In vivo molecular imaging of colorectal cancer with confocal endomicroscopy by targeting epidermal growth factor receptor. *Gastroenterology* 2010;138(2):435-46.
15. Becker C, Fantini MC, Wirtz S, et al. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut* 2005;54(7):950-4.
16. Levi B, Wan DC, Glotzbach JP, et al. CD105 protein depletion enhances human adipose-derived stromal cell osteogenesis through reduction of transforming growth factor $\beta 1$ (TGF- $\beta 1$) signaling. *The Journal of biological chemistry* 2011;286(45):39497-509.
17. Rau KM, Huang CC, Chiu TJ, et al. Neovascularization evaluated by CD105 correlates well with prognostic factors in breast cancers. *Experimental and therapeutic medicine* 2012;4(2):231-36.
18. Marioni G, D'Alessandro E, Giacomelli L, et al. CD105 is a marker of tumour vasculature and a potential target for the treatment of head and neck squamous cell carcinoma. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 2010;39(5):361-7.
19. Kleespies A. Re: the risk of developing metastatic disease in colorectal cancer is related to CD105-positive vessel count, by Romani AA, Borghetti AF, Del Rio P, et al. *Journal of surgical oncology* 2006;93(6):435-6.
20. Nassiri F, Cusimano MD, Scheithauer BW, et al. Endoglin (CD105): a review of its role in angiogenesis and tumor diagnosis, progression and therapy. *Anticancer research* 2011;31(6):2283-90.
21. Baish JW, Jain RK. Cancer, angiogenesis and fractals. *Nature medicine* 1998;4(9):984.
22. Baish JW, Jain RK. Fractals and cancer. *Cancer research* 2000;60(14):3683-8.

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