

ANSWERS TO REVIEWER'S COMMENTS

June 8th, 2015



Dear Editor,

Thank you very much for reviewing our manuscript. We found the comments and suggestions very helpful and constructive and we have addressed all reviewers' comments. Please see below our answers to specific comments, and the page and line references to the changes in the text of the manuscript (highlighted in yellow). We are confident that you will find the new version of the manuscript much improved, and consider it for publication in your journal.

Please find enclosed the edited manuscript in Word format (file name: 18045-review.doc).

Title: Tumor Neoangiogenesis Detection by Confocal Laser Endomicroscopy and Anti-CD105 Antibody: Pilot Study

Author: Adriana Ciocâlțeu, Adrian Săftoiu, Daniel Pirici, Claudia-Valentina Georgescu, Tatiana Cârțână, Dan Ionuț Gheonea, Lucian Gheorghe Gruionu, Cosmin Gabriel Cristea, Gabriel Gruionu

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 18045

The manuscript has been revised according to the reviewer's suggestions:

1 Format has been updated

- (1) Please provide language a certificate letter from a professional English language editing company (Classification of the manuscript language quality evaluation is B). For manuscripts submitted by non-native speakers of English, please provide a language certificate from one of the professional English language editing companies mentioned in **'The Revision Policies of BPG for Article.'**

The author's answer: The manuscript has been thoroughly revised by two of the authors (GG and AS) who have near-native English language skills and an extensive experience with scientific publications in English. There are several stylistic and language corrections throughout the text of the revised manuscript.

The title must be informative, specific, and brief (Title should be no more than 10~12 words/60 bytes. Please revise it). Words should be chosen carefully for retrieval purposes. All nonfunctional words should be deleted, such as 'the', 'studies on', 'observations of', and 'roles of', etc.)

The author's answer: We revised the title of our manuscript as suggested by scientific editor and by reviewer no 40529: "Tumor Neoangiogenesis Detection by Confocal Laser Endomicroscopy and Anti-CD105 Antibody: Pilot Study"

- (2) Author names should be given first, then the complete name of institution, city, province and postcode.

The author's answer: We added the postcodes.

- (3) Ethics approval:

Clinical trial registration:

Informed consent:

Conflict-of-interest:

Data sharing:

The author's answer: We added the requested statements in the text and we attached the documents.

According to clinicaltrials.gov, our study does not meet the criteria for "applicable device clinical trial" ("Small clinical trials to determine the feasibility of a device or a clinical trial to test prototype devices, where the primary outcome measure relates to feasibility and not to health outcomes").

- (4) In order to attract readers to read your full-text article, we request that the first author make an audio file describing your final core tip. This audio file will be published online, along with your

article. Please submit audio files according to the following specifications: **Acceptable file formats:** .mp3, .wav, or .aiff. **Maximum file size:** 10 MB. To achieve the best quality, when saving audio files as an mp3, use a setting of 256 kbps or higher for stereo or 128 kbps or higher for mono. Sampling rate should be either 44.1 kHz or 48 kHz. Bit rate should be either 16 or 24 bit. To avoid audible clipping noise, please make sure that audio levels do not exceed 0 dBFS.

The author's answer: We added the audio file.

- (5) Please put the reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. Please check across the text.

The author's answer: We added the reference numbers.

- (6) *Writing requirements for each subsection*

The author's answer: A Comments Section is now attached to the submission:

COMMENTS

(1) Case characteristics

The main clinical signs the patients showed were alternating diarrhea and constipation, accelerated intestinal transit, recent constipation, unintended weight loss, rectal bleeding, abdominal pain or discomfort.

(2) Clinical diagnosis

Only three patients accused rectal bleeding as a single symptom, also confirmed by the physical examination (digital rectal examination).

(3) Differential diagnosis

Other common digestive diseases such as hemorrhoidal disease, inflammatory bowel disease or irritable bowel syndrome were excluded.

(4) Laboratory diagnosis

Seven patients presented nonspecific laboratory tests findings such as moderate elevated hematological

values of erythrocyte sedimentation rate (three patients), slightly elevated white blood cells count (two patients) and moderate anemia (two patients); two patients presented slightly elevated values of both tumor markers CEA and CA19-9, while three of them had only slightly elevated CEA values.

(5) Imaging diagnosis

Computed tomography scan excluded the presence of metastases in all ten patients and described rectal wall thickening in four cases.

(6) Pathological diagnosis

Histological examination of endoscopic samples revealed moderately differentiated adenocarcinoma (G2) in five cases, well differentiated adenocarcinoma in two cases (G1), mixed subtypes in three cases (G1/G2- two cases, G1 with mucinous areas - one case) (**Table 1**).

(7) Treatment

Tissue samples from patients with histological diagnosis of rectal cancer were collected during colonoscopy before undergoing surgical resection or neoadjuvant therapy (**Table 1**).

(8) Related reports

We have extended our study on *immunoendoscopy* from two previous studies where we demonstrated that CLE allows specific imaging of vessels in human fresh colonic biopsies by using fluorescently labeled antibodies directed against a *panendothelial marker* (CD31). This method allowed us to estimate vascular network parameters, which are difficult to evaluate from thin cross-sectional images generated with conventional immunohistochemistry [12,13]. The feasibility of endoglin targeting for diagnostic applications was addressed in two distinct animal models and the results indicated that it is a useful and safe procedure for tumor imaging [8,26]. To our knowledge, no previous studies using fluorescently-labeled CD105 with CLE in patients with rectal cancer have yet been performed.

(9) Term explanation

Immunoendoscopy: Targeting markers of angiogenesis in association with confocal laser endomicroscopy examination

Panendothelial markers: present equal staining intensity in both small and large vessels and comparable

reactivity in both frozen and paraffin sections, with obvious disadvantages regarding antigen specificity and sensitivity. They can identify all types of blood vessels in a given tissue sample, irrespective of being mature or immature.

(9) Experiences and lessons

Specific imaging and quantification of tumor microvessels are feasible in human rectal cancer using CLE examination and CD105 immunostaining of fresh tissue samples. A larger number of patients is needed to study the correlation between MVD and tumor differentiation grade and staging, with great potential for CD105 staining combined with CLE analysis to provide a more reliable evaluation of the angiogenetic status of patients with colorectal cancer. CLE monitoring of the relationship between endothelial presence of CD105 and survival of patients would be of great interest.

(10) Peer- review

The manuscript has original results. This is an interesting study on “Tumor Neoangiogenesis Detection by Confocal Laser Endomicroscopy and Anti-CD105 Antibody”. The research is limited to a small number of patients and, for this reason, this study should be considered PILOT.

For the figures, the fonts and lines can be edited or moved. It can be made by ppt.

The author's answer: We have edited the figures as suggested by the scientific editor.

2 Revision has been made according to the suggestions of the reviewers

(1) Reviewer no 36825:

The manuscript has original results. The only question requires precision is the number of investigated regions and pictures by CLE in the samples obtained from the tumor and from the normal colonic mucosa.

The author's answer:

We have added more details about the image processing and analysis in the Material and Methods Section at page 8, lines 176-179: “This software was used to obtain the Z projection of the confocal serial image stacks from each biopsy sample (60-250 images per biopsy sample). The vascular density and the vessel diameters were measured from the Z projections within two 50x475 μm rectangular regions of interest centered in the middle of each image in the horizontal and vertical direction as before [13]”

We have also explained in more detail the selection process for the image data in the previous study which is cited in the text [13].

(2) Reviewer no 40529:

This is an interesting study on "Neoangiogenesis evaluation of rectal cancer using confocal laser microscopy and anti-CD105 antibodies". The research is limited to five patients and for this reason, this study should be considered PILOT, and the definition reported in the TITLE.

The author's answer:

We considered the recommendation of the reviewer and we changed the title to include the word "pilot": "Tumor Neoangiogenesis Detection by Confocal Laser Endomicroscopy and Anti-CD105 Antibody: Pilot Study". An additional number of five patients were added to the study.

The authors should provide:

1) All the details regarding the five patients, ages and not rangem sex, definitive staging, definitive histopathological report regarding the grading.

The author's answer:

We have added the details about the patients in the Material and Methods section at page 7, line 137 and lines 144-154, and in Table 1, page 22:

"Tissue specimens from ten patients 47-80 years old (mean age of 65.2 ± 9.9 years),..."

"The main clinical signs the patients presented at admission in the hospital were alternating diarrhea and constipation, accelerated intestinal transit, recent constipation), unintended weight loss, rectal bleeding, abdominal pain or discomfort. Only three patients accused rectal bleeding as a single symptom, also confirmed by the physical examination (digital rectal examination). Seven patients had nonspecific findings for the laboratory tests such as moderate elevated hematological values of erythrocyte sedimentation rate (three patients), slightly elevated white blood cells count (two patients) and moderate anemia (two patients). Two patients presented slightly elevated values of both tumor markers CEA and CA19-9, while three of them had only slightly elevated CEA value. Computed tomography scan excluded the presence of metastases in all ten patients and described rectal wall thickening in four cases. Histological examination findings from endoscopic samples are summarized in Table 1."

2) Why in advanced stage it was not performed preoperative RT-CMT?

The author's answer:

In our study, we included patients prior to undergoing any therapy (neoadjuvant RT-CMT or surgery). We consider that preoperative treatment is not relevant for our CLE results. To the reviewer suggestion, we highlighted the inclusion criteria in the Material and Methods section, *Subjects* Subsection (page 7, lines 138-140) and we inserted Table 1 with patient characteristics (page 22), including if they underwent neoadjuvant/adjuvant therapy after our evaluation.

"... before undergoing surgical resection or neoadjuvant therapy to avoid artifacts (e.g. false positive resulted from fibrosis or inflammation increased in case of radio-chemotherapy)."

3) Why the tissues were collected during colonoscopy and not on the surgical specimens?

The author's answer:

We analysed CD105 expression using CLE on fresh biopsy from patients during colonoscopy before they underwent any therapy, including neoadjuvant RT-CMT, in order to increase the accuracy of the study by avoiding artifacts (e.g. false positive results from fibrosis or inflammation increase in case of RT-CMT), and inconsistent results. Another reason was to ensure the shortest time from biopsy sampling to CLE imaging in order to prevent tissue degradation and loss of fluorescence signal.

Page 7, lines 138-140: "...before undergoing surgical resection or neoadjuvant therapy to avoid artifacts (e.g. false positive resulted from fibrosis or inflammation increased in case of radio-chemotherapy)"

4) How many biopsies were taken for each patient from tumor area and normal surrounding tissues? The term "several" is not acceptable in a scientific paper

The author's answer:

We inserted in the Material and Methods Section, *Confocal laser endomicroscopy* Subsection, the number of biopsies per patient (page 7, line 153-161): "six biopsies were taken from tumor, avoiding the ulcerated areas (paired biopsies for CLE assessment, standard immunohistochemistry and histopathological examination, respectively), as well as four biopsies from macroscopically normal surrounding tissue samples (paired biopsies for both CLE processing and standard immunohistochemistry)."

5) Why did the authors choose patients with CRC? Could they comment in the discussion if tumors arising from other organs would have the same expression of CD105?

The author's answer:

We have improved the discussion of the potential utility of the method in colorectal cancer, providing more examples to demonstrate the expression of CD105 in tumors.

Page 12, lines 252-258: "Rectal cancer is one of the solid cancers which benefits of antiangiogenic therapy with high chances of curability when the treatment is applied at an early stage. To date, no appropriate tissue biomarker exists for staging, prediction or monitoring of the clinical response to a therapeutic intervention (e.g. antiangiogenic therapy). Beyond its already presumed roles (higher affinity for microvascularization, prognostic role), recent *in vitro* studies suggested that targeting endoglin could improve treatment and could reverse resistance to bevacizumab in some refractory cancer patients [18]."

Page 13, lines 299-300: "Other studies are needed to investigate if the same method could be applied to other tumor types."

(3) Reviewer no 505502:

The authors stated that specific imaging and quantification of tumor microvessels is feasible in human rectal cancer using Confocal Laser Endomicroscopy (CLE) examination and CD105 immunostaining of fresh tissue samples. It may be very useful, however, there are a few points to be solved;

1. You evaluated only five cases. It is too small to evaluate the usefulness of your examination. You should increase cases.

The author's answer: We specified in the title that this is a pilot study, and included five more patients, which were recruited to the study subsequent to the preparation of the manuscript (ten patients in total).

2. In your methods, you perform 3D reconstruction of images acquired by using Image J. But you didn't show the images of 3D reconstruction. You should show the images.

The author's answer:

We have used the Z-projection of the stack of images to make the measurements. Representative images of Z-projections of the images obtained in a single CLE scan are shown in figure 1A, 1B, when using CD31 and in figure 1C, 1D, for CD105. In Material and Methods, we specified: "This software was used to obtain the Z projection of the confocal serial image stacks from each biopsy sample (60-250 images per biopsy sample). The vascular density and the vessel diameters were measured from the Z projections within two 50x475 μ m rectangular regions of interest centered in the middle of each image in the horizontal and vertical direction as before [13]." (page 8, line 176-179).

3. You evaluate neoangiogenesis by using fluorescently labeled antibodies with CLE and immunohistochemistry, but the association between the two ways is hard to understand. Why did you perform fluorescently labeled staining with CLE? You should state the benefits of fluorescently labeled CD105 with CLE and the differences between the two ways.

The author's answer:

As suggested by the reviewer, we have added details in the Discussion Section about benefits of the method and differences in comparison with IHC on page 12, lines 271-275: "Endoglin, as a specific marker for activated endothelium, mainly reacts with fresh or frozen tissue, while its activity in paraffin-embedded specimens is dependent on fixation [16]. In the present study, a qualitative comparison between the two methods (CLE *versus* IHC) lead to similar results. The major advantage of the CLE method is time efficacy and less artifacts in comparison to common IHC regarding the processing techniques [13]."

(4) Reviewer no 2440486:

More data would be essential to convince readers that C105 expression (staining) is unique for tumor vessels, rather than mixed with inflammatory conditions or fibrosis. Furthermore, some of the data in the paper needs to be improved, like Figure 3, the error bar was so large, that the repeatability of this experiments was questionable.

The author's answer:

At the reviewer's suggestion we have revised and corrected the data in Figure 3 according to the immunohistochemistry data provided in the manuscript.

Additionally, we have increased the number of patients to ten and we have also improved the discussion with more examples and references to demonstrate the roles of CD105 expression in colorectal cancer (Page 12, lines 252-258 and lines 271-275).

Page 12, lines 252-258: "Rectal cancer is one of the cancers which can benefit from antiangiogenic therapy with high chances of curability when the treatment is applied at an early stage. To date, no appropriate tissue biomarkers exist for staging, prediction or monitoring of the clinical response to a therapeutic intervention (e.g. antiangiogenic therapy). Beyond its already presumed roles (higher affinity for microvascularization, prognostic role), recent in vitro studies suggested that endoglin targeting could improve treatment and could reverse resistance to bevacizumab in some refractory cancer patients [15]."

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3 References and typesetting were corrected. The number of references was reduced to 26.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

Adrian Săftoiu MD, PhD, MSc, FASGE



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