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Editor, Dr. Jin-Lei Wang
Director
World Journal of Gastroenterology

Copenhagen, March 15th, 2013

Dear Dr. Jin-Lei Wang,

Thank you very much for your e-mail of February 28th, 2013 and the positive criticism of the first manuscript version (ESPS Manuscript NO: 1877).

The manuscript has been revised according to the comments of the editor and the three external reviewers, and we hope you will find the revised manuscript suitable for publication in *World Journal of Gastroenterology*.

Please find enclosed the revised manuscript in Word format (file name: 1877-edited.doc) and the point-to-point outline listed below.

Title: miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis

Author: Mehmet Coskun, Jacob Tveiten Bjerrum, Jakob Benedict Seidelin, Jesper Thorvald Troelsen, Jørgen Olsen, and Ole Haagen Nielsen

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 1877

Point-to-point outline:
1. The format has been updated according to the suggestions of the editor.
   - Methods and Results part in the “Abstract” section has been extended to fulfil the required number of words.
   - A “Comments” section is now included in the revised manuscript.

2. Revision has been made according to the suggestions of the reviewers and the point-to-point response to the comments of the reviewers is listed below. Author responses are stated in italics.

Reviewer no. 1:

1. How do the authors explain the inability to detect differences between active Crohn's disease and controls, especially in light of other published data?
The initial sample size in cohort 1 is quite small--what were the estimates of variability in the datasets and could this have contributed to the inability to discriminate among groups?

Response: We appreciate the reviewers comment, but currently we have no obvious explanation for the inability to detect differences between Crohn's disease and controls as compared to previous findings. However, to the best of our knowledge, only Wu et al. (Inflamm Bowel Dis. 2010,16:1729-38) have previously used microarrays on intestinal tissue samples to differentiate between active Crohn's disease and controls, whereas other studies have examined the expression of miRNAs by qRT-PCR analysis, which is inherently a much more sensitive technique than microarray. When comparing our study with the study by Wu et al. there is, however, a striking difference in the miRNA microarray platforms used; Wu and colleagues used a miRNA microarray platform from NCode (Invitrogen) detecting ~470 unique human miRNAs, while we used a platform from Geniom Biochip miRNA (Febit) detecting almost 850 unique human miRNAs. Thus, it is possible that the overall miRNA expression panel may not follow the same pattern due to the dissimilar number of detected miRNAs in different arrays. Furthermore, the stringent significance and fold change criteria set by the current study might very well exclude subtle differences present in the expression profiles of patients with Crohn's disease and controls. Finally, we agree with the reviewers' comment regarding the sample size of cohort 1, and we cannot exclude that the limited size of the microarray cohort contributes to the inability to discriminate among groups. These details and limitations have, however, been added and discussed into the revised manuscript.

2. Given the nature of Crohn's inflammation in the bowel, how did the authors standardize their biopsy collection to make sure they were sampling inflamed Crohn's colitis and not skip areas?

Response: Pinch biopsies were obtained from endoscopically non-inflamed or inflamed areas of the descending colon. Non-inflamed samples originated from CD patients with endoscopically quiescent Crohn's colitis in the descending colon, and no other segments of the colon or ileum was inflamed. Inflamed samples originated from CD patients with endoscopically active Crohn's colitis in the descending colon. The endoscopic diagnosis of active or inactive CD was confirmed by histopathology conducted on parallel biopsies taken within an inch of the 1st biopsy. These details have been added to the "Materials and Methods" section of the revised manuscript.

3. Does the verification of differences of the candidate miRNAs take into account significance levels for multiple comparisons?

Response: We thank the reviewer for this comment. However, we have not corrected the significance levels for multiple comparisons as the selection of tested miRNAs was not done at random, but was based on the highest correlations coefficients and fold changes. This circumstance would not be considered if we corrected the results using Bonferroni or Dunn's test. Thus, correction for multiple comparisons is usually not performed when the comparisons are made as validation procedures of microarray results, e.g. Wu et al. (Inflamm Bowel Dis. 2010,16:1729-38).

<u>Reviewer no. 2:</u>

The manuscript written by Coskun et al. reports microarray-based miRNA profiling of colonic mucosal biopsies from patients with ulcerative colitis, Crohn's disease, and controls,

and found four new miRNAs are specifically upreulated in patients with ulcerative colitis. They also predicted pro-inflammatory target genes for those miRNA. The data are quite important and help understanding of the pathogenesis of ulcerative colitis. However, there are some concerns that need to be addressed.

Minor points

1. Ischemic colitis, nonspecific colitis or bacterial colitis might be more suitable controls for ulcerative colitis than Crohn's disease or normal controls in terms of disease specificity of the findings.

Response: The reviewer is acknowledged for the suggestion that ischemic colitis, nonspecific colitis or bacterial colitis might be more suitable controls for ulcerative colitis than Crohn's disease or normal controls. However, we do not believe that these conditions will be rational controls for UC as i) ischemic colitis mainly occurs in the elderly people, ii) nonspecific colitis is an unclassified inflammation with a wide range of unknown causes, and iii) bacterial colitis is due to infections caused by microbes with a wide range of intestinal impact. Thus, these three different forms of colitis may not characterize the same type of inflammatory condition as seen in ulcerative colitis. Consequently, being able to identify differentially expressed miRNAs in IBD vs. controls, and in ulcerative colitis vs. Crohn's disease is of outmost importance for our pathophysiological understanding of these diseases and for the development of a potential diagnostic tool.

2. Did all the patients with Crohn's disease have colitis? The pattern of miRNA expression could be different depending on the site at which the biopsy was performed. Were the biopsies taken from all patients according to the same criteria?

Response: This comment is similar to the comment raised by Reviewer# 1 in point 2 (please see comments above). Accordingly, we have added more information regarding patients and tissue collection in the "Materials and Methods" section of the revised manuscript.

3. The pattern of miRNA expression could be also different depending on the treatment that each patient received. How did the author exclude the effect of treatment on the analysis?

Response: This is obviously an insightful observation. However, it is very difficult to recruit IBD patients receiving identical treatments or no treatments at all - especially in a relatively large cohort of patients. Thus, it has not been possible to exclude the effect of treatment on the analysis. This statement or limitation has, however, been clarified in the revised manuscript.

4. Are there any data on the longitudinal analysis of miRNA expression in the same patient? If a patient shows any changes in the pattern of miRNA depending on the activity of ulcerative colitis, the data may have additional information on the pathogenesis of the disease.

Response: We agree with the reviewer that it could be interesting to examine the longitudinal analysis of miRNA expression in the same patient, which, however, could form the basis for another study.

Reviewer no. 3:

The aim of the paper entitled “miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis” is to analyze the miRNA expression in IBD patients and healthy individuals in order to identify new potential miRNA biomarkers in IBD using miRNA microarray profiling. The experiments performed in colonic mucosal biopsies. The results of the study miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with UC, so the level of these miRNAs may serve as new potential biomarkers for this disease. Please include a detailed written commentary below:

Major comments:
1. It is hard to understand that, why the researchers performed the experiments in colonic biopsies instead of blood samples. Because endoscopical interferences are much more invasive and expensive.

Response: We would like to thank the reviewer for this thoughtful comment. We agree that blood samples provide opportunities for minimally invasive approaches; however, we believe that differential miRNA expression in the tissues might also yield insight into the complex mechanisms underlying the disease pathogenesis.

2. The experimental groups of the study are contrary to expectations. It seems that adding a “quiescent CD group” will make the study worthwhile.

Response: We appreciate the comment. However, we are confused by the suggestion to add a quiescent CD group. In fact, we have performed miRNA microarray analysis on endoscopic pinch biopsies obtained from active UC, inactive UC, active CD, inactive CD, and controls (cohort 1). Meanwhile, we agree that the description regarding the experimental groups was unclear in the former version of the manuscript. This matter has now been dealt with in the revised manuscript.

3. The sample size of cohort data is not clearly explained in “material and methods” part of the study. If the size of groups is 4, 4 and 2, in UC, CD and control groups, then it is hard to validate the data in such small groups.

Response: The aim of this study was to use the miRNA microarray expression profiles as a hypothesis generating tool to identify new IBD-associated miRNAs. Therefore, we used a small miRNA microarray cohort (cohort 1) and a large validation cohort (cohort 2) for the qRT-PCR validation analysis. Thus, the validation analyses were performed in an independent validation cohort on samples from patients with active UC (n = 20), inactive UC (n = 19), and controls (n = 20). In order to clarify this matter, we have rephrased the details regarding the sample size of cohort data in “Materials and Methods” of the revised manuscript.

4. The authors mentioned that miRNAs-20b levels are increased in both active and quiescent disease, and colorectal cancers related with oxidative stress but not disease itself. It decreases the biomarker value of these miRNAs. It needs an explanation.

Response: Our results illustrate that miR-20b levels are increased in both active and quiescent UC. We believe this makes miR-20b a potential biomarker to differentiate between controls and UC as it is not a disease activity-dependent upregulation. miR-20b expression has been reported to be increased in human cancers, including lung cancer, gastric cancer and leukemias. Furthermore, miR-20b facilitates cellular adaption to normoxia and hypoxia in vitro by regulating the transcription factor hypoxia-inducible factor 1-alpha (HIF-1-alpha). Therefore, it is likely that miR-20b might be a part of the pathophysiology in colitis-associated cancer. These considerations have been added to the Discussion section of the revised manuscript.

And also please revise:
The introduction parts: The second paragraph of the introduction is inessential.

Response: As suggested by the reviewer, the second paragraph of the "Introduction" section has been rephrased.

The "Materials and methods" part: Please explain the sample size of groups clearly.

Response: As suggested, the sample size of groups is more clearly described in the revised manuscript.

Sincerely,

Mehmet Coskun
PhD, MSc

Ole Haagen Nielsen
Professor, MD, DMSc