Stony Brook Medicine

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Re: WJG manuscript 62541

Dear Dr. Yan**,**

We were pleased to learn that the editors of World Journal of Gastroenterology have made the preliminary decision that our manuscript is acceptable for publication after our appropriate revision. We thank the reviewers for their thoughtful and thorough comments and respond point by point as follows:

Round-1

Reviewer #1:

- "The authors hypothesize that the antibiotics will impact analysis of multi-omic datasets generated from resection samples to investigate biological CRC risk factors. The aim of the manuscript was to assess the impact of preoperative antibiotics on integrated microbiome and human transcriptomic data generated from archived frozen CRC resection samples. The authors "explored the effect of five variables (tumor histology, preoperative antibiotics, laterality of CRC location, diabetes mellitus, Black/AA race) on analysis of microbiome and host transcriptome among archived frozen CRC resection samples. As the authors did not only assess the impact of the use of antibiotics on the profile of the intestinal microbiota, I would like to suggest to add these other outcomes in the objectives of the study."

Although limited by word count, the Abstract/Aim now states "assess the impact of preoperative antibiotics and other variables on integrated microbiome and human transcriptomic data." (Page 3, line 7)

- "The authors studied 51 pairs of frozen sporadic CRC tumor and adjacent non-tumor mucosal samples from 50 CRC patients. As results, it was observed a significant effects of histology ($p = 0.002$) and antibiotics ($p = 0.001$) on microbial β-diversity, increased Fusobacterium abundance in tumor vs. nontumor groups and detected significantly reduced bacterial load in the +antibiotics group. The authors emphasized that "there is a measurable effect not only on the tissue-associated microbial communities but also on the host colonic transcriptomic profiles" regarding the use of the antibiotics. But the authors should discuss the clinical importance of this finding, whether if this effect on gut microbiota is permanent or transient since the patient used antibiotics just before surgery.

Can we assume that the microbiota will recover some time after using the antibiotic? Or not? Cite some articles about this topic."

The Discussion now states: "Though the effect of the antibiotics is transient, and the baseline microbiome is shown to recover within 1.5 months with the most potent antibiotics[35], the impact of preoperative antibiotics is still significant since downstream experiments are performed on tissue specimens collected perioperatively." (Page 27 Line 8-11)

- "In conclusion, is an interesting article with original findings that studied the interaction among gut microbiota, use of antibiotics and CRC. I would like to suggest to the authors that they discuss more about the effects of the use of antibiotics on the intestinal microbiota and its implications for the treatment of CRC.

We have inserted the following text: "The use of antibiotics can shift the microbiome depending on the dosage and duration of the antibiotic exposure. Several studies have shown that tumorigenesis and tumor growth can be attenuated with different antibiotic cocktails and timing of antibiotic exposure with duration of inflammation. On the other hand, early exposure to antibiotics increased risk of CRC and interfered with chemotherapy efficacies due to microbial dysbiosis.[10] With these conflicting findings and this change in protocol at our institution, it allowed us to examine how differential use of antibiotics, along with other clinical/demographic factors influences integrative, multi-omic analyses of CRC."(Page 5 Line 26-Page 6 Line 3)

- In addition, the authors could discuss the role of the intestinal microbiota in CRC.

*We have inserted the following text: **"**According to the driver and passenger hypothesis, there are key pathogens that can drive tumorigenesis and support other bacteria as passengers to proliferate and exacerbate disease in sporadic colorectal cancer.[3] Similarly, the keystone hypothesis states that certain low abundance pathogens can promote inflammation by altering a normal microbiota into a dysbiotic one.[4] There are certain pathogens that are associated with CRC including increased abundances of Peptostreptococcus, Bacteroides fragilis, Fusobacterium nucleatum, and Escherichia coli and decrease abundances of Clostridium, Bifidobacterium, Faecalibacterium and Roseburia.[1,10] The potential mechanisms at which these particular bacteria may affect the adenoma-carcinoma sequence including gene expression alternations, promotion of chronic inflammation and release of carcinogenic metabolites.[8] " (Page 5, Line 6-15)*

Reviewer #2:

- In the present original article Malik et al showed that preoperative antibiotics, given in patients undergoing surgery for colorectal cancer (CRC), change microbiota composition, diversity and transcriptomic profile. Main comments: 1) The main drawback of this manuscript is that the key finding (change in microbiota after antibiotics) is an expected and well known result that does not add any novelty to present knowledge. Authors should have rather investigated another endpoint, for instance whether change in microbiota was effective to prevent peri-operative infections.

Because this study is a prospective observational study, it is not designed to measure the effect of reducing surgical site infections. That was addressed in a multicenter, single blind randomized controlled study recently reported by (Basany et al. 2020). Our 16S total bacteria PCR results indicate that short term exposure to antibiotics (24h oral neomycin and metronidazole) reduces

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mucosal associated bacterial abundance. In addition, though this finding of "change of microbiota after antibiotics" is expected, this finding more importantly highlights how this form of specimen collection (collecting tissue or stool or effluent after colorectal surgeries) may impact omics including host transcriptional profiling in addition to changes in microbial composition in future discovery studies on CRC. The following novel findings are included in the Core Tip: "Utilizing FFPE samples as a source of DNA retains polarity of the observed trends but impair signal strengths. Expression of VBP1, which is implicated in suppressing CRC metastasis, was significantly decreased in expression in both Black/AA tumor and nontumor samples. " (Page 4 Line 13).

- A real control group of patients without CRC is absent. Normal tissue of patients with CRC is not as reliable as control.

Although we agree in principle, patients only undergo colonic resection for clinical care so collection of specimens from truly healthy controls is not ethical or feasible. We have collected and analyzed samples from subjects with other pathologies, e.g. gastrointestinal bleeding from nontumor sources, diverticulitis, colonic inertia, colonic obstruction, inflammatory bowel diseases. We acknowledge that the microbiome and transcriptional profiles are potentially altered in all of these conditions. We have begun shifting collection to additional research colonoscopic biopsies of subjects undergoing index screening colonoscopy but we anticipate that it will take some time before we can accumulate enough "normal" biopsies to analyze.

- What do Authors mean for "tumor histology"?

Tumor histology refers to tissue with abnormal cells, more specifically, cancerous cells. Normal histology refers to tissue in which cells are normal on histological level as determined by clinical pathological review by board certified pathologists.

- A minor linguistic revision is necessary.

In addition to the content revision we have made additional checks to correct spelling and grammatical errors.

Reviewer #3:

- This complex and original research resulted in an elegant manuscript, written in a nicely manner, containing a lot of very important data from applying an integrative multi-omic analysis of colorectal cancer resection specimens. The overall structure of the manuscript is well respected. The references are pertinent and up-to-date. This original work deserves to be published, after some minor modifications. 1. The title refers only to antibiotic administration before resection, however this research analysed many more aspects. It would not hurt to emphasize these aspects.

*We thank the reviewer for the kind remarks and have modified the title **"**Impact of preoperative antibiotics and other variables on integrated microbiome-host transcriptomic data generated from colorectal cancer resections."*

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- The Abstract should present the fact that it was a prospective study. Please also insert the period the study was conducted.

*In response to the reviewer's comments we revised the abstract as follows: **"Methods**: Genomic DNA (gDNA) and RNA were extracted from prospectively collected 51 pairs of frozen sporadic CRC tumor and adjacent non-tumor mucosal samples from 50 CRC patients archived at a single medical center from 2010-2020. 16S rRNA gene sequencing (V3V4 region, paired end (PE), 300 bp) and confirmatory quantitative polymerase chain reaction (qPCR) assays were conducted on gDNA. RNA sequencing IPE, 125bp) was performed on parallel tumor and non-tumor RNA samples with RNA Integrity Numbers (RIN) scores ≥ 6. " (Page 3 Line 10)*

- Core tip presents more explicitly the essence of this research. Maybe data could be harmonized with the Abstract, so that the Abstract appears clearer. It is not only analysing the effect of antibiotic administration before resection (however, this appears as the aim of the study). Maybe the conclusion of the Abstract should be reformulated, given the complex results.

We made revisions but the word limit for the conclusion (30 words) limits our ability to expound on our other findings

- Introduction contains the adequate background and it demonstrates why the following five aspects were chosen: tumor histology, preoperative antibiotics, laterality of colorectal cancer location, presence of diabetes mellitus and of Black/African Ancestry race. Please insert the aim of the study by the end of Introduction. Please consider that this research studied much more than the effects of antibiotics (and only 16 [31.4%] of 51 samples were exposed to oral antibiotics, as it appears later in Table 2).

We revised the introduction - *"We report here the results of this pilot study to investigate the effects of the tumor histology, preoperative antibiotics, laterality of CRC location, diabetes mellitus status and Black/African Ancestry on microbiome and human transcriptomic data of colorectal cancer patients." (Page 7, Line 2-5)*

- Material and methods: please clarify the period the study was performed. Was it 2010-2020?

We revised Material and Methods"All the archived samples were collected prospectively from CRC patients scheduled between 2010-2020 for surgical resection for clinical care, who had given their informed consent for banking of surgical remnant for possible genomic sequencing, collection of longitudinal clinical metadata and access to clinical formalin-fixed paraffin embedded (FFPE) tissues for possible genomic sequencing under a protocol approved by the Stony Brook IRB (#163184). "(page 7 Line 12)

- Please also insert here the protocol of using antibiotics (after 2017 - it appears in Introduction – oral neomycin and metronidazole 24 hours in advance of the procedure).

We included the following statement in the revision -"*Participants who enrolled in the study after 2017 were automatically assumed to be exposed to preoperative antibiotics as the new protocol*

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prescribes oral neomycin (1 gram x 3 doses) and metronidazole (500 mg x 3 doses) 24 hours pre-procedure to the patient. " (page 7 line 24-27)

- In Results (page 11) and Discussion (page 29), however, it appears that antibiotics were also given intravenously 30 minutes before the surgery. Please clarify.

*We **included the following statement in the revision** "Prior to that time, the standard of care was to administer only intravenous antibiotics within 30 minutes of incision, and only a few CRC resection patients were placed on short-term oral antibiotics within a month of the surgery, for various clinical indications." (Page 5 Line 21)*

- Page 7, line 7: Please insert a period after (0,1,2,3,4). Page 7, line 9 – please replace "was" with "were. Otherwise, this paragraph contains all the necessary data and it is presented in detail, including Statistical analysis.

We corrected these grammatical and punctuation errors in the revised manuscript.

- Results are explained clearly, also in tables (3-9) and figures (1-3). Just please remove in the title of Table 3 "between the following groups" – written twice. And please add respective colors to "antibiotics" – Figure 3. Also, please remove (Page 23 – lines 1-2): "Lower detection rates of F. nucleatum nusG gene by PCR were previously reported on archived FFPE CRC tissues, ranging from 13%-45% [31,32]" and add it to Discussion. Same for "Expression of the von Hippel-Landau binding protein 1 or VBP1 in Black/AA CRC tumors (n = 64) was also observed to be significantly lower compared to White/EA CRC tumors (n = 284, p = 0.026) in The Cancer Genome Atlas (TCGA) database and in a recently published transcriptomic profiling study[20]." – page 27, lines 6-9.

We corrected these grammatical and punctuation errors in the revised manuscript. New figure with correct colors for figure 3 is incorporated. In addition, "Lower detection rates of F. nucleatum nusG gene by PCR..." and "Expression of the von Hippel-Landau binding protein..." were moved to the Discussion. (Page 26 Line 24-27 and Page 27 Line 8-15 respectively).

- Discussion paragraph could be expanded a bit. Please add "showed/showing" after report – page 29, line 4.

We made the following revisions in response to this suggestion:
"showing" was added on what is now page 26 line 23.

***"**We are particularly intrigued by the results indicating reduced VBP1 expression in Black/AA tumor vs not Black/AA tumor and Black/AA nontumor vs. not Black/AA nontumor, because of a recent report showing that VBP1 suppresses HIF-1α-induced epithelial-mesenchymal transition in vitro and tumor metastasis in vivo[40]." (Page 27 Line 15-19)*

"Going forward, probably the best solution is to perform targeted PCR assays on archived FFPE CRC and advanced adenoma tissues collected prior to the change in preoperative antibiotics protocol in order to sufficiently power the analysis of evaluating the effect of laterality, diabetes status and race on microbiome and transcriptomic data of colorectal cancer patients. In particular,

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utilizing the targeted PCR to detect Fusobacterium, it can possibly be used as a clinical prognostic biomarker for diabetic colorectal cancer patients. " (Page 28 Line 24-29)

- Please add Conclusion and Study Highlights.

We added these two sections in the revised manuscript.

- There are no Conflict-of-Interest Disclosure Form and Copyright License Agreement files. Please add.

These documents are uploaded with the submission of the revised manuscript.

- Please also add the STROBE Statement—checklist.

This document has been uploaded with submission of the revised manuscript.

Round-2

We thank the reviewers for their comments and have responded to each reviewer as follows:

Reviewer 1. "The answer regarding negative control is not satisfactory. Indeed, this study lacks a reliable control group that limits the reproducibility "

We are interpreting this comment as the reviewer asking for additional data from colonic specimens that have no adjacent colorectal cancer. We understand that the reviewer has concerns that the adjacent colorectal tumor may have field effects on the nontumor region. Please note that we are not claiming that the nontumor regions are "normal". While the biobank has collected surgical resection specimens from some non colorectal cancer subjects, the problem is that these specimens also represent pathological states which may also be associated with altered microbiome composition and transcriptional profiles and therefore also do not represent true "controls". In fact this investigative team has previously published reported microbiome and transcriptional changes in disease unaffected ileal regions of patients with ileal Crohn's disease compared to nontumor ileal specimens from colorectal cancer resection specimens as a "control" for Crohn's disease. Going forward, as discussed in our revised discussion, we plan to prospectively collect colonoscopic research biopsy specimens to obtain a control group, since we will be able to document that the colon is normal and the subjects are not administered preprocedure antibiotics. We also discussed further confirming our findings with archived formalin fixed paraffin embedded tissue specimens where additional preoperative antibiotics were not administered, in order to confirm the reproducibility of our findings with frozen archived specimens.

Reviewer 2 "The authors followed the reviewers' recommendations and made the necessary adjustments to the article."

No further revisions requested by this reviewer.

Reviewer 3 I am very pleased after attentively reading the new version of this manuscript and the authors' reply. I highly appreciate that the authors performed the corrections and considered my comments, as well as those of the other reviewers (except for the very good remark of the

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Reviewer nr. 2 – “a real control group of patients without CRC is absent”, but I also agree with the Authors’ point of view). Just some comments please: Due to an unknown reason, Tables do not display properly and Figures do not appear at all in the new Word file. Also, there is no STROBE Statement - checklist. The format of the Conflict-of-Interest Disclosure Form is not the requested one (as I can see after downloading). Please double-check and correct. Otherwise, I consider this manuscript having strong points and findings, deserving to be published.

I have attached the Word document that I cut an paste the Tables and Figures from to the online form. Thus far I have not been able to upload the manuscript online so that it formats correctly. I have my students currently working on trying to upload so that the tables and figures come out. I will also attach the Strobe checklist and the desired forms for the disclosure of conflict of interest both my email directly to the editor.

I hope these revisions meet with your approval.

Sincerely,

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