ANSWERING REVIEWERS

February 15, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: Mouse model revised.doc).

Title: Novel Diet-related Mouse Model of Colon Cancer Parallels Human Colon Cancer

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The manuscript has been improved according to the suggestions of reviewers:

1 The format has been updated by adding appropriate annotations to the legends to the figures.

2 Revisions have been made according to the suggestions of the reviewers.

Suggestions of Reviewer 02445067 and our responses

Abstract

(1) The aim is written as a sentence fragment and should be rephrased to clarify the Aim(s).

Revision (keeping Aim to 20 words) (page 2): We investigated the close parallels between our novel diet-related mouse model of colon cancer and human colon cancer.

(2) Specify the age range rather than stating "young" mice.

Revision (page 2): Twenty-two wild-type female mice (ages 6-8 weeks) were fed the standard control diet (AIN-93G) and an additional 22 female mice (ages 6-8 weeks) were fed the control diet…

Methods

(3) Specify source and collection of human tissues and experimental work conducted.

The following new text in red was added (pages 7-9)

Histopathology, gross and microscopic images of human tissue

Before any biopsy tissue samples were obtained during colonoscopy, informed consent was given by the patient, using a form approved by the University of Arizona Institutional Review Board. Biopsy specimens were completely fixed in 10% buffered formalin for 6 to 12 hours, followed by routine processing through graded alcohols and subsequent embedding into paraffin blocks. Tissue samples from colonic resections were obtained after

informed consent before surgery. Colonic segments were cut open and gross photographic images of colonic tumors and polyps were obtained. Adequate representative tissue samples were obtained from areas of tumors and adjacent colonic mucosa. Similar to the biopsy specimens, these tissue samples were fixed in 10% buffered formalin for 24 to 36 hours, transferred to graded alcohols, followed by paraffin embedment.

Three 4-micron tissue sections were cut from all retained paraffin-embedded tissues. The tissues were then placed on glass slides, stained with hematoxylin and eosin, and subjected to histopathologic analyses. Morphologic evaluation was performed using a brightfield digital light microscope (Motic BA300).

Histopathology, gross and microscopic images of mouse tissue

The GI tracts of mice, including rectum, colon, cecum, small intestine, stomach and lower esophagus, were removed, opened longitudinally, rinsed with phosphate-buffered saline (PBS) and divided into sections that could fit into paraffin blocks. All parts of the lower GI tract including rectum, colon and cecum were retained for fixation and paraffin embedment and any parts of the small intestine, stomach and esophagus that had a visible protrusion were retained. In addition, other organs including liver, pancreas, spleen, breasts and lymph nodes near breasts were examined, and if there were any potentially aberrant areas observed, sections of these organs were also retained. All retained sections were placed flat on Matricel membranes for good orientation. Segments of intestine with grossly visible mucosal nodules were photographed with a Sony Cybershot 7.2 megapixel camera. Sections were subsequently fixed in 10% formalin overnight at 4ºC, then transferred to 70% alcohol, and embedded in paraffin.

Three to six 4-micron tissue sections were cut (multiple sections were cut to ensure any tumors or aberrant areas were included in the sections) from all retained tissues. The tissues were then placed on slides, stained with hematoxylin and eosin, and assessed for histopathologic characteristics. Morphologic evaluation was made on all the tissues on slides, using a brightfield digital microscope (Motic BA300). There is currently no accurate substitute for histopathologic determination of colonic neoplasia[10].

Diagnosis of histopathology

Anil R. Prasad, MD, a surgical and cytopathologist with years of experience in GI pathology and immunohistochemistry diagnosed all of the tumors detected on the basis of histopathologic criteria. The mouse tumors were compared to human tumors at the same stages by histopathological analysis. Sections of the small and large intestines of mice and humans were evaluated for glandular architecture, cellular and nuclear morphology including cellular orientation, cellular and nuclear atypia, nuclear enlargement, hyperchromasia, chromatin clearing, pleomorphism, presence of nucleoli, atypical mitotic activity, frequency of goblet cells, crypt architecture, ulceration, invasion of malignant glands through the muscularis mucosa and submucosa and presence of infiltrating malignant glandular crypts in the muscularis propria. Digital photomicrographs of representative sections were obtained using Motic Images Plus 2.0 software.

Results

(4) Suggest omitting Figure 2 as it does not seem essential.

This Figure has been deleted.

(5) Figure 3 should show a corresponding proximal colon without tumours.

A corresponding proximal colon without tumors has been added (revised Figure 2, page13)

(6) Glare on the image should be removed to increase clarity of the image.

We are not able to remove the glare present in the original photo.

(7) Indicate tumours with arrow marker.

These are now indicated with arrow markers (Figure 2 panel A, page 13).

(8) The units on the ruler should be indicated or omit the ruler and insert a size bar.

Units of centimeters are now indicated (Figure 2 panel A, page 13)..

(9) Figure 4 The image is not of sufficient quality to observe the mucosal nodules specified in the legend for this figure.

The Figure (now Figure 3, page 14) was enlarged to make the mucosal nodules more evident.

(10) Table 2 Would be helpful to have the body weight and age at termination included.

The weights have been added to Table 2 (page 17). A note was also added to the text (page 15), giving the ages at termination as follows: “(Note that mice were 6 to 8 weeks old when received, acclimated to the control diet for 2 weeks, and then put on their diets for 10 months, so that the mice, at termination, were 12 to 12 ½ months old.)”

(11) Figures 6 - 12, 17 Legends should specify annotation on the images.

Annotations on the images (now labeled Figures 5-11, 16) have been added to the legends.

(12) The location of the images within the colon should also be indicated in legends.

The concentrations of bile acids in the proximal and distal colons of mice and humans are expected to differ, as described in the text on pages 32 and 33. Thus, the locations of tumors are expected to differ between mice and humans. Because we are describing the strikingly closely similar histopathological morphologies of tumors in mice and humans, we feel it would be distracting to also annotate images with possibly different locations, since different locations are not relevant to their histopathology.

(13) Sentence fragment "As reviewed by Scott et al.[25], 8-OH-dG is a carcinogenic DNA damage." should be revised.

This has been revised (page 24): “As reviewed by Scott et al.[25], the DNA damage 8-OH-dG carcinogenic."

(13) Different fonts used within the manuscript should be corrected.

The fonts are now Book Antiqua 12 point throughout the text.

(14) Use of gene symbols should be consistent e.g. PNS2 and Pms2 and gene symbols should be italicised.

We have now italicized gene symbols and they have all capital letters and we use non-italics with all capital letters for protein designations.

(15) Results of body weights associated with CGA fed mice should be incorporated.

The tissues from mice supplemented with CGA in addition to DOC were from a previously reported experiment, and the weights associated with the mice from which those formalin fixed paraffin embedded tissues were obtained are no longer available.

(16) Typo "egglant" requires correction.

Now spelled (page 34) “eggplant.”

Discussion

(17) The experimental mice exhibited a wide range of body weights. It appears that some mice were underweight and others obese. Is this correct? This should be discussed further. Is this due to differing genetics, age related factors? Further insight on the role of the genetics of these mice would be helpful in interpreting the results.

We added text [and an image of one heavy and one light mouse (figure 17, page 30)] to the Results section titled “Weight distributions” (page 30) as follows:

“Each mouse was weighed weekly, and no weight loss was detected for any of the mice during their 10 months on each diet. Mice with relatively low weights at the end of 10 months on their diets merely gained weight more slowly than heavier mice.

Each mouse, without respect to weight, appeared to be healthy and active (Fig. 17). The variation in mouse weights, like the variation in colors of these mice (Fig. 1), was likely due to the variation in their genetic constitutions. As pointed out in the Materials and Methods, the mice were the second generation (F2) of a cross between two well established, inbred, wild-type strains: C57BL/6J and 129S1/SvlmJ. The phenotypes of these F2 wild-type mice is expected to be varied, since the contribution of the two parental wild-type strains will be different in each F2 offspring. The varied weights of these mice may mimic the weight variations in the general human population.”

(18) What was the variation in gene expression of the biomarkers studied in the group of mice?

We added the language (page 23): “The examples in Fig.’s 12-16 were representative of the levels of biomarkers found, but with only three tissue samples, variation of the expression of each marker was not

quantitated."

(19) Are there marked differences in the expression of these markers in the parent strains?

The parent strains are maintained in the Jackson Laboratories (Bar Harbor, ME). We did not have any mice of the parent strains and could not evaluate expression of the markers in those strains.

Suggestions of Reviewer 01207047 and our responses

The significance of the study is limited by small number of cases in each groups of mouse and there is no statistical analysis.

We have now added material on statistical analyses (page 9 **Data Analysis** of Methods & pages 18 **Statistical analysis** and pages 30 [bottom] and 31 of Results).

(1) -Material & Methods- page 9, 8th line: It will be beter to use "invasion through the muscularis mucosa, submucosa and muscularis propria" instead of "penetration of crypts".

We have now used the language (page 9): "invasion of malignant glands through the muscularis mucosa and submucosa and presence of infiltrating malignant glandular crypts within the muscularis propria."

(2) -It will be better to use "AC" instead of "ADCA".

We now use AC throughout when abbreviating adenocarcinoma.

(3) - It will be better to use "preneoplastic and neoplastic lesions" instead of "colon tumors".

We now use "neoplastic lesions" on pages 12 [twice] and 15 (in Results section) and page 23 (at the start of the section on immunohistochemistry).

Immunohistochemical observations can expose molecular alterations that may indicate an area predisposed to formation of neoplastic lesions. We now use "preneoplastic areas" on page 23 where we describe "preneoplastic areas from which a neoplastic lesion might be expected to arise."

(4)-- It will be better to use "low and high grade dysplasia" instead of "lesser and higher malignant potential" .

The terms lesser and higher malignant potential have been removed and replaced with low and high grade dyslasia.

(5) - It will be better to give "magnification of the objectives"" instead of "scale barr".

The magnification of the objective was added to the legends of Figures. Scale bars were required in instructions to the authors, so they are also included.

(6) -Figure 11 B : Did large pale areas in the submucusa and muscularis propria represent large mucin pools?

Yes, they are mucin pools and this is now indicated in the legends of Figures 10 and 11 in which the mucin pools are evident.

(7) In Figure 12 B, It will be better to show this area (mucinous component) adjacent to invasive focus in the muscularis propria .

The presence of extravasated mucin, forming mucin pools adjacent to malignant glands has now been indicated in the text referring to Figure 11. (page 23).

Suggestions of Reviewer 00070919 and our responses

(1) ----AIM should be re-written, since it does not look like a full sentence. ----

Revision (keeping Aim to 20 words) (page 2): We investigated the close parallels between our novel diet-related mouse model of colon cancer and human colon cancer.

(2) The biggest drawback of this paper is the lack of statistical analysis. Methodological defect will greatly affect the persuasiveness of this article, though authors had done a fine job in morphological and molecular researchs.

We have now added material on statistical analyses (page 9 **Data Analysis** of Methods & pages 18 **Statistical analysis** and pages 30 [bottom] and 31 of Results).

(3) ----It is better to delete Fig. 2. ----

This figure has now been deleted.

(4) Additional factors such as weight loss, survival time could be included in the evaluation of mice fed diet+DOC.

We added text [and an image (Figure 17) of one heavy and one light mouse] to the Results section titled "Weight distributions" as follows (page 30):

"Each mouse was weighed weekly, and no weight loss was detected for any of the mice during their 10 months on each diet. Mice with relatively low weights at the end of 10 months on their diets merely gained weight more slowly than heavier mice."

In the section of results titled "Types and location of tumors" we now indicate that all mice studied survived to their time of termination, adding (page 15):

"(Note that mice were 6 to 8 weeks old when received, then acclimated to the control diet for 2 weeks and then put on their diets for 10 months, so that all mice, at termination, were 12 to 12 ½ months old.)"

In the Materials and Methods (page 7) we noted: "During the succeeding 10 months, 2 mice from each group died of unknown causes so that 22 mice in each group completed the experiment."

(5) In IHC study, it is better to use semi-quantitative data to demonstrate the difference of expression of antibodies.

Semi-quantitative data were used in reference to Fig. 12 for 8-OH-dG (page 24) and have now been added in reference to Fig. 15 (page 27) for beclin-1. For ERCC1 (Fig. 13) and for beta-catenin (Fig. 16), it is more the pattern of expression, rather than the level of expression that was of interest.

Suggestions of Reviewer 02549473 and our responses

(1a).- INTRODUCTION -- Most of the text presented in the Introduction section is indeed a discussion of results obtained either in the study itself and/or other studies. Therefore, it should be reallocated in the discussion section. Only the first two paragraphs would correspond to a formal Introduction.

We have now moved all the material after the first 2 paragraphs of the previous Introduction to the Discussion. We did add one further paragraph to the new Introduction (page 5) to smooth the transition to the Results.

(1b) In order to enhance the text quality, I suggest to rephrase more (i.e. avoiding to repeat phrases such as "we show here", "we observed here").

The phrases “we show here” and “we observed here” were removed throughout and the sentences reworded.

2.- RESULTS -- Page 13, line 8 -- Such information should not be part of the text body. It should be placed in the figure legend and the electronic address put in the reference list.

We put a reference in the legend of Fig. 3 (page 14) indicating that the Figure was part of a figure in Wikimedia, and put the electronic address in the REFERENCE list (reference 13).

3.- RESULTS -- Page 14, line 2 -- To the authors' minds, what is the reason why some mice in the control group developed tumors in the small intestine, whereas no mice in the study group (receiving the carcinogenic agent) showed these findings.

The difference in occurrence of small adenomas in the duodenum of 3 of the mice fed diet alone vs. no adenomas in the duodenum of mice fed diet+DOC was found to be statistically insignificant. This statistical analysis is now added to the results on page 18.

4.- RESULTS -- Comparison of human and mouse colonic tissues, again, looks more

like a discussion rather than a study result.

In the RESULTS we now briefly note similarities between mouse and human in the context of the specific characteristics (e.g. histopathologic features) or biomarkers considered. These similarities are commented on and evaluated more broadly in a general context in the DISCUSSION.

5.- RESULTS -- Pages 21-25 -- Immunohistochemistry evaluation of molecular markers contains a mixture of some sort of background, methodology and results for each of the marker assessed. The results section of the manuscript should be reserved to study results solely.

Sentences giving methods were removed from sections on immunohistochemical evaluation of molecular markers (pages 23-29).

6.- RESULTS -- Page 25, line 11 -- Weight distributions are outcome parameters not mentioned in the methods section.

We now note, in the MATERIALS AND METHODS section (page 6): "All mice were weighed and their weights recorded weekly."

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

Sincerely yours,

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