Author's response to reviews

Title: Molecular Pathways Undergoing Dramatic Transcriptomic Changes During Tumor Development in the Human Colon

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Version: 3 Date: 8 October 2012

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The Biomed Central Editorial Team

Object: MS: 3241921196862096 - Molecular Pathways Undergoing Dramatic Transcriptomic Changes During Tumor Development in the Human Colon. Rosalia Maglietta et al.

Thank you for being interested in our manuscript. Encouraged by the positive assessment of the reviewers and according to your suggestion of resubmitting the manuscript to BMC Cancer journal, we have prepared a new version of the manuscript in which we have addressed all the remarks and doubts raised by the referees.

Response to Reviewer #1 (Dr. Iris Simon)

In general, we agree that our response to Dr. Simon’s previous comments was less than exhaustive. We will try to answer with more precision in this letter.

Point 1.

The major criticism for this paper is (a) the small sample size and (b) the lacking independent validation.

This issue has been partially addressed by using publically available datasets and by using a different analysis method. The small samples size obviously remains an issue.

It is true that our sample of tumor tissues is small. However, collection of high-quality human tissues is no simple task. We joined forces from two institutions in an attempt to maximize the number of samples in our series. Unfortunately, some of the collected tissues failed to meet our stringent quality criteria for microarray analysis. Others were excluded after analysis because quality control of the microarray data identified them as outliers. In the end, we managed to obtain reliable samples of 59 tumors, each with a paired sample of normal mucosa. A total of 118 exon arrays were used to analyze this sample. The cost was substantial since, at that time the samples were analyzed, the cost of each exon array was very high.

Point 2.

Although it is very interesting to analyze the pathways that are differentially up and down regulated between during cancer progression, there is no definitive proof for any of the hypothesis made in the paper. Many of the results might be in agreement with published observation but one wonders if the opposite could be supported just the same. It should therefore be clearly stated that this report is describing a hypothesis-generating analysis rather than results. This has been partially addressed by change of wording.
As the reviewer notes, we have already partially addressed this point. We have made a second attempt here with the new version of the Conclusions section (see also Point 5). However, we believe that our hypotheses are solid: not only are they based on rigorous transcriptomic analysis of “real” human tissue samples (i.e., they are experiment-driven hypotheses), they are also in agreement with data obtained with many other approaches in the last 20 years or so. Could these previously published data also support the opposite hypotheses? Not unless most of what has been published thus far has been found to be untrue.

Finally, it is important to stress that testing our hypotheses with bench work was beyond the scope of this study. It would require the planning of a new study or several new studies.

Point 3.

1.) Do have patients follow-up? With other words, are there indications that some tissue samples came from aggressive or less aggressive cancer that formed metastasis or new tumors in short time and are in these samples cancer-driving pathways stronger?
   a. This has not been addressed and is a major limitation since we know that tumors that have fast growth and likely metastasise must have a different biology than tumors that stay confined to the colon.

Our previous reply to this observation was indeed too brief. All of the patients whose tumors were analyzed in this study had relatively early-stage colorectal cancers (mainly because our objective was to analyze the transitions from normal mucosa to precancerous lesions of different size, and to cancer). Indeed, as shown in Table 1 (this version and earlier versions as well), none of the patients had metastases to the liver or to other organs at the time of operation, and in most cases there was no microscopic evidence of lymph node metastases either (N0). To characterize these tumors in terms of relative aggressiveness would have required 2-5 years of follow-up.

Point 4.

2.) Results and Discussion section is difficult to read as it jumps between different unrelated pathways. Figure 4 is very helpful and maybe can be used as a guide for this section.
   a. For me the result and discussion section is still too long. A lot of results are described in the figures. They do not need to be described again in the text.
Suggestions for improvement:

1.) Focus the result section on those pathways that are well understood (as it has been done for example for the cell-cycle genes/ Figure 2.
   a) Has been partially addressed but still has not shortened the manuscript.
2.) Validate results in publically available datasets (this is possible at least for results in cancer patients) Has been done.
In our previous revision, we shortened the Results & Discussion section by almost one page. We have now re-edited this section so that it focuses solely on those pathways that were found to be upregulated in tumor tissues. The pathways displaying tumor-related downregulation are now discussed in Additional File 1 for readers who might be interested.

The current Results & Discussion section is now about 3.5 pages shorter than the last version, with a word count (2601) that is similar to that of other articles published in BMC Cancer.
The English has been extensively revised by a medical editor from the United States. Hopefully, this intervention has improved the readability of the Discussion.

Point 5.
3.) Sharpen the conclusion section. At the moment it is more a discussion. What are the major new results? How does this analysis helps a patient or a physician to better deal with the disease?
   a. This has not been addressed. The conclusion is even longer. I would appreciate a conclusion of one paragraph with major take-home messages. That the study can be “launchpad” for even more systemic biology analysis doesn’t seem as an achievement to me. Which of the pathways would be the most interesting to follow up on?

We have revised the Conclusions section to render it more concise (the length has been reduced from 484 to 123 words). However, the results of our study do not have any immediate implications for the management of patients with colorectal tumors. They are basic research findings, and as such the fact that they open new roads to an improved understanding of colorectal tumorigenesis may not be a major achievement, but it is certainly a valid one.

Response to Reviewer #2 (Dr. Pablo Conesa-Zamora)

Point 1.
It is commonly known that there are several pathogenic sequences leading to colon carcinoma. The model proposed by the authors could be sustainable if the preinvasive lesions belonged to the same pathogenic pathway as the invasive carcinoma. However, the histologic diagnoses in the study cases comprise both serrated (SSA, SA) and conventional adenomas (TA, TVA) and there is no histologic classification of the invasive colon cancers; for instance MSI-H and conventional carcinomas are two endpoints of different pathological sequences with different precursor lesions.

This is an important point. However, it is not true that we provided no classification of the colorectal cancers: because of our long-standing interest in DNA repair, we routinely test colorectal tumors for mismatch repair deficiency using immunohistochemistry for five mismatch repair proteins (MSH2, MSH6, MSH3, MLH1, and PMS2) and assays of
microsatellite instability, and on the basis of these analyses, we specified in the original (and revised versions of the) manuscript as well that “All tumors were sporadic lesions with a functional DNA mismatch repair system” (Methods section). As for the serrated pathway of tumorigenesis, its relevance has always been mentioned in our Background section, where we state that in both adenomatous and serrated lesions, lesion size is equally important for tumorigenesis. Serrated precancerous lesions accounted for only 5 of the 42 lesions we examined. Since these lesions are sometimes the precursors of MLH1-deficient cancers, we also verified their mismatch repair proficiency with the methods mentioned above. We decided to include these five lesions in our analysis because they did not emerge as outliers in preliminary analyses of our datasets. In addition, their exclusion did not have any significant effect on the data reported in Tables 2 and 3. This point has been clarified in the revised Discussion (page 8, second paragraph).

Point 2
-In order to a better classification of polyps not only based on the size but in its oncogenic potential (taking into account other features such as histology) I would suggest the Guidelines for Colonoscopy Surveillance after Polypectomy published GASTROENTEROLOGY 2006;130:1872–1885. Is there any rationale for the cut-off of 20mm for dividing precursor lesions into small and large preinvasive lesions?

As specified in the original and revised versions of the Methods section, we restricted our transcriptomic analysis to lesions > 10 mm: otherwise, removal of part of the specimen for microarray analysis might have interfered with the histologic assessment of the lesion. Thus, we could not adopt the 10-mm cutoff recommended by the US Multi-Society Task Force on Colorectal Cancer for efficient postpolypectomy surveillance. We were aware of the clinical implication of lesion size, and we mentioned this in the original Background section (and in the present version). This part was also referenced –Ref 3- with a paper in Gastrointestinal Endoscopy. The growth of a tumor is a continuous process that parallels its progression to malignancy. Although the cut-off we used to define small and large precancerous lesions (20 mm) is admittedly arbitrary, it provided us with two similarly sized subgroups of lesions (19 small, 23 large), which was statistically advantageous. The choice was also dictated by our previous observations, which indicated that these two size-based subgroups were characterized by biological differences. This is now specified in the Methods section (page 8, second paragraph).

Point 3.
-The authors presented a bioinformatic analysis of the microarray results but did not validate them using other techniques such as immunohistochemistry, western blot or qPCR. It could be difficult to validate pathways but there are key molecules such as RB1 or p16 that could have been used for this purpose.
The aim of our bioinformatic analysis of transcriptomic data was to identify pathways that might be involved in colorectal tumor progression. Two previous reviewers asked that we validate our findings by repeating the analysis on a different microarray data set. We did so, and these data have been provided in the first revision of this manuscript. Bench-based validation studies of our pathway data are undeniably necessary, but it is beyond the scope of the present study. It will require new, carefully planned, long-term studies. We believe the results of the present study deserve to be published because they can serve as a foundation for future studies, in our lab or others, designed to identify biological aspects of pathways whose roles in colorectal cancer have not been fully explored. Many published findings reported in our manuscript (e.g., those regarding the cell cycle, RB and p16) have been extensively investigated by many groups in the past and with different experimental approaches. We have mentioned these in the Results & Discussion section.

Point 4. -Apart from histological diagnoses there are also differences in term of location between SPLs and LPLs. For instance, there are 5 LPLs located in the cecum whereas there are no SPLs with this location. This fact can bias the profile obtained for each group of lesions. Besides, there is no information of matching for age or gender and this could also bias the results.

As reported in Table 1, precancerous lesions from the right colon (i.e., cecum, ascending and hepatic flexure) accounted for 63% (12/19 lesions) of the small lesions and 61% (14/23) of the large lesions. The distinction between lesions in the ascending colon vs. the cecum may be of limited biological relevance since these segments are consecutive components of the proximal colon. (There is an excess of proximal lesion in our series since our gastroenterologists are increasingly detecting Ilia lesions—slightly elevated nonpolypoid lesions—with the aid of high-resolution instruments and chromo-endoscopy.) Collection of human tissues is no simple task, and limiting our sample to specific tumor subsets would have considerably increased the time needed to obtain an adequate number of specimens. For this reason, each of the stage-specific tumor sets we analyzed displayed some degree of heterogeneity related to endoscopic morphology, histology, location within the colon, etc. (Table 1). We agree with the Reviewer that this variability probably reflects differences at the molecular level as well. Nonetheless, our analysis has pinpointed a large number of fundamental pathway alterations that are likely to occur during the development and progression of most tumors in the large intestine. Additional research in larger tissue series will be needed to explore pathway regulation in more specific subsets of tumors.

In a prospective collection of samples like the one used for this study, matching by age and sex would substantially prolong recruitment considering, for example, the size criteria we applied (> 10 mm). The average age at diagnosis of subjects with larger lesions was, as expected, higher (by 2 years) than that of subjects with small lesions. The transcriptomic changes observed in large lesions are those that usually occur in older persons, so for the purposes of this study, age-matching was not absolutely necessary. As
for sex, the ideal approach would be to focus on one sex at a time (even in clinical trials), but for the reasons mentioned above, this is rarely possible. Nevertheless, in our opinion, the inclusion of transcriptomic data from normal colorectal mucosa from the same patients should mitigate any analytical problems related to our decision to omit age and sex matching.

Point 5
-The authors should clearly state which are the main findings of this work compared to the previous ones.
We have revised the Conclusions section, along with the Abstract and Background, to clarify the original aspects of our study.

Point 6
-The results and discussion part is very long and difficult to follow.
As specified in our reply to Reviewer 1 (Point 4), we have shortened the Results & Discussion sections by 3 pages.

Point 7
-“Farther” refers to distance: I guess the authors would like to say “further”
We have revised the sentence in question.

-The term preinvasive is not entirely correct since many of these lesions would remain as non-invasive if let untreated. The term adenoma would be more advisable.
The terms “preinvasive” and “precancerous” are universally used in this field to indicate benign lesions with the potential to become invasive, i.e. to become cancer. It’s true: lesions > 10 mm might remain benign for 10 or 20 years, but after 30 or more years, who knows? This is why it is compulsory to remove them at endoscopy. For these reasons, we preferred to leave these terms in the manuscript. Substitution with the term “adenomas” would be problematic since the sample also included a few nonadenomatous (i.e., serrated) lesions (see Point 1).