Author's response to reviews

Title: Involvement of hyaluronidases in colorectal cancer

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Author's response to reviews: see over
Patras, April 30, 2010

To: The Editor-In-Chief
   BMC Cancer

Dear Sir,

Please find attached the revised form of our manuscript, written by H. Bouga et al., which was corrected according to the reviewers comments. In the next pages you will find the corrections/answers in all comments, which also were highlighted in the text. Since one of the reviewers did not propose any corrections, we try to balance between his opinion and the comments of the other two. The paper is now greatly improved and we believe in this form it is suitable for publication in “BMC Cancer”, as a regular research paper.

Looking forward to hearing from you soon,

Sincerely Yours

Demitrios H. Vynios
Professor of Biochemistry
Editorial comments:

Was written consent obtained for the use of tissues from patients. If so, this should be documented in your revised manuscript. If consent was not required then the reasons for this should be discussed in your cover letter.

The sentence was corrected (page 6, line 14)

Referee 1:

General comments:
Hyaluronic acid plays critical role in tumor progression. There must be a critical balance between hyaluronic acid synthesis and breakdown. The breakdown is carried out by hyaluronidase that exists in different isoforms and their activities depend on pH. The role of hyaluronidases in cancer is an undefined and complex area that needs to be carefully examined.
Vynios et al has studied hyaluronidases in colon cancer. This is an extensive and valuable study using colon cancer tissues of various A/C stages. They observed a high association of the enzyme with colon cancer. The authors though concluded that the results provided new evidence on the mechanisms i.e., involvement of hyaluronidases in the colorectal cancer progression the authors did not give any detail or propose any mechanism how the progression process is influenced by the enzyme.
Our opinion is different:
It was written in the text (page 11, last para): “These results implied that hyaluronidases in the onset of pathological conditions tend to create small fragments of hyaluronan that help tumor progression.”
In addition (page 12, first para): “The increase of the activity at very late stage of cancer may be required for the final degradation and the removal of the ECM components sensitive to the enzyme.”
Therefore, we propose to readers a mechanism through which hyaluronidases act during cancer.
Specific comments:
1. The question posed by the authors well not defined. What is the significance of different methods of extraction of hyals? For example Hyal-1 and Hyal-2 are PBS extractable whereas Hyal-3 is 4M GdnHCl-1% Triton X-100. Does this property of the isoforms give any advantage in tumor progression?
The extraction of the enzymes was performed via a sequential extraction procedure to obtain the freely existed extracellular, the entrapped within the ECM and the intracellular or membrane bound enzymes. Any differences in extractability of the various hyalases would help understanding its role and provide evidence for pharmaceutical treatment. If additional differences existed when comparing normal and cancerous specimens, a hypothesis regarding tumor progression may be provided. Similar conclusions would be obtained from immunohistochemical studies, where the type (normal or cancer) cells producing special isoforms would be additionally obtained and thus one could speculate more on tumor progression.
A sentence is introcuced (discussion, end of second para) explaining the benefits of this methodology.
2. Regarding the methods were used they were well described but not appropriate. The authors would have done the immunochemical analysis of hyals in tissues from the frozen sections.
We have decided to apply the sequential extraction procedure in the study to reach to almost the same conclusions, since the number of plates required from each patient is relatively high and specific permission from the Ethical Committee is required. We have planned a detailed immunochemical study regarding the localization not only of hyaluronidases, but additional tumor and angiogenesis markers and we believe that the Committee will approve it.

3. It appears from western blot and RT-PCR that Hyal-1 is the predominant form of hyaluronidases. The authors did not interpret the results well. The results from western blotting showed a broad distribution of PH-20 in all three extracts, whereas Hyal-1 was present almost exclusively in PBS extracts. The results from RT-PCR showed increased expression of Hyal-1 in adenomas and late stages and increased expression of PH-20 in early stages. Therefore it is not so clear whether Hyal-1 is the predominant form. The last para of Discussion is describing the fact.

4. If hyaluronan breakdown products from the hyaluronidases drive processes of tumor progression like angiogenesis, the authors should have paid attention to it. At least immunohistochemistry of tissues for CD31 should have done.

See above, comment 2

5. The discussion and conclusions part should be rewritten such that they are balanced. Even the title and abstract do not accurately convey the message of the paper.

The title was revised, and some points in the discussion were clarified.

6. The authors acknowledged other work relevant to the present research article. During the revision of the discussion additional studies were referenced.

Referee 2:

In this manuscript, Bouga et al. demonstrated that several hyaluronidases was detected in colorectal tissues from patients of colorectal cancer. The profiles of different isoforms in different stages of colorectal cancer were also observed. However, the main theme is not clear and there are some points that need to be addressed by the authors as noted below.

Major compulsory revisions
1. 35 colorectal carcinomas are a small series for a surgical department in Europe. Is this series really consecutive? Or what are the criteria for selecting these patients?
   A sentence was introduced (materials and methods, tissue source) to explain the criteria
2. The authors need to describe why the three-step extraction was used to measure the activities of different hyaluronidases?
   See above, referee 1, comment 1.
3. How were the activities of different hyaluronidases distinguished and measured in the same extract?
   The measured activity in zymograms is described as hyaluronan-degrading activity and by only the migration of the bands one can distinguish enzymes possessing the same activity. In our paper we described that, from the zymography experiments, we obtained only quantification of total hyaluronan-degrading activity.
4. According to Figure 2 (B), why hyaluronidase activity was higher in early and late stages but lower in middle stages? Can it be explained by the profiles of different isoforms?
   An explanation of the question was written in the text (page 11, last para and page 12, first para). In addition, in the last para of page 12 it was written that degradation of
hyaluronan seemed to proceed via the synergistic action of specific isoforms. All these concluded from the measured hyaluronidase activity and the profile of the different isoforms.

5. As described in the Results section, Hyal-2 and Hyal-3 mRNA expressions were not identified in any samples examined. Why were these data not consistent with the Western blot data shown in Figure 3? It is important to mention how quickly the RNA was obtained from the time of blood collection (the longer it takes to transport samples prior to RNA isolation, the gene expression may change; therefore, it is important to describe how long this time was or how samples was preserved until time of RNA analysis)

We think the reviewer has misunderstood the method applied for mRNA expression analysis. Tissue samples were used and not blood samples. Nevertheless, the problems described (preservation, transportation of samples, etc) were carefully controlled, as written in page 6, tissue source.

Of course, the notice of the reviewer that western blotting gave positive results and RT-PCR analysis gave negative results in the cases of Hyal-2 and Hyal-3 is correct, but the answer, to our opinion, does not correspond to mRNA deterioration in the samples obtained, and other reasons, such as long half-life of the proteins, mutations of the genes or alternative splicing, should be considered. A sentence was introduced in the results section (page 10, line 13) to indicate it.

Minor Essential Revisions

Linguistics review should be necessary. For example:
Page 8, row 24-25: “…..were mostly of advanced stages…..”.
Page 10, row 9: “……; significant also overexpression……”.
The text is revised and grammar and syntax errors are corrected

Referee 3:

The article by Bouga et al is an interesting, methodologically-oriented analysis comparing expression of different type of hyaluronidases using zymography and western blotting. Their expression was also examined by RT-PCR. Examine of macroscopically normal and cancerous tissues, high association of hyaluronidases in colorectal cancer was observed. This observation would be important in aspects from understanding the tumor developement and diagnostics of CRC. Therefore the question posed by the authors is actual. The applied methods are reasonable and well documented and the data are sound. But instead of end point analysis the reiewer would suggest performing at least SYBR GREEN assay, which requires 60-120 pb length of products. The perfect method for the correct quantification would be the Taq-Man assay. The discussion and conclusion are well ballanced and adequately supported by the data. Literature and references were correctly used. But it would be better using more and recent relevant references. The title and the abstract are covering the contents of the publication. The writing is acceptable and well understanding. Based on consideration of the above mentioned criteria I suggest for acceptation of this publication without any changes.

Since the other two reviewers suggested major revision of the manuscript we follow their comments, and according to this reviewer we use more and recent relevant references in the revised discussion.