Author's response to reviews

Title: MMP28 (epilysin) as a novel promoter of invasion and metastasis in gastric cancer

Authors:

Pan Jian (panjian2008@163.com)
Wang Jian (wj196312@vip.163.com)
Zhu Xueming (zhuxueming@yahoo.com.cn)
Ni Jian (Ni_jian2008@yahoo.cn)
Tao Yanfang (taoyanfang1982@163.com)
Zhou Zhuan (zzhouzhuan@yahoo.com)

Version: 3 Date: 25 April 2011

Author's response to reviews: see over
Dear Judy,
Thank you for your review of our manuscript (Ref No.9102197064654903). We appreciate the concerns and suggestions provided by the reviewers, and have revised our manuscript accordingly. Our point-by-point responses are provided below, and text that has been added or modified from the original text is shown in the revised manuscript in red font. We know that your journal has high publication standards, so we have already had the language of this paper corrected by a professional language editing service that specializes in scientific manuscripts.
Upon review of our revised manuscript, we hope that you will find it acceptable for publication in *BMC* cancer and we look forward to your response.

Sincerely,

Ni Jian
Department of Cell and Molecular Biology, Cancer Institute (Hospital), Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China, 100021
Email addresses: ni_jian2008@163.com
Tel: (86)0512-62956209
Fax: (86)0512-62956209
Reviewer number: 1

Comments

There are three areas that require more information.

(1) The authors need to describe the micro array - for example, was this a targeted array?

The microarray used in this paper was a 22K Human Genome Array, a product of *Human Genome Oligo Set Version 2.1* ([http://www.Operon.com](http://www.Operon.com)). The gene array was analyzed by Bioassay Laboratory of CapitalBio Corporation ([http://www.capitalbio.com](http://www.capitalbio.com)). The data from the gene array is provided in Supplementary Data S1, and these details have been provided in the materials section of the manuscript.

(2) The data shown in Fig 3B suggest an increased growth rate for the MMP28-expressing tumors. This is a different phenotype to the enhanced invasive ability that is the focus of the paper. There is no discussion regarding this effect. The authors should at very least discuss this phenotype. Performing some staining for a proliferation marker such as Ki67 within the engraft tumor specimens could provide confirmation that MMP28 is associated with enhanced growth.

As the expression of MMP28 seemed to increase the volume and weight of tumor, we analyzed the proliferation rate of MMP-28 overexpressing clones C9 and C10, and no significant differences were observed compared control cells (data not shown). We also analyzed Ki67 expression in xenograft tumors (data presented in Fig 3D). All groups including N87, N87-Ve, N87-C9 and N87-C10, had similar expression of Ki67. As invasion related genes increased the tumor volume in the absence of significant effects on proliferation, we hypothesize MMP28 may influence the expression of other genes related to tumor growth or vascular formation, and we have added a statement to this effect into the results section.

(3) Although enhanced metastasis is described in the text, there is no data to support this suggestion. Authors should include a graph or table indicating
number and/or size of metastatic nodules to support their claim.

Following the suggestion of the referees, we have revised figure 3, and have provided a graph of lung metastasis and a table indicating the number of the metastatic nodules, figure 3D and figure 3E.

Minor Essential Revisions
(1) In the Background section 2nd paragraph, line 15, a sentence begins "This type of role for MMP28....". It is unclear what "This" refers to, and the authors should clarify.

“This type of role for MMP28” means MMPs not only act as metalloproteinases, but can also regulate cell behavior. This is discussed by Sternlicht and Werb [13]. We have edited the text to resolve this query, and the text is now unambiguous.

(2) Data in Figure 3A and description of matrigel chemoinvasion assay in methods state use of t-test. This is not suitable when greater than 2 groups are being compared, and the authors should use ANOVA with a post-hoc test for analysis.

Following the suggestion of the referees, we revised figure 3A, and the chemoinvasion assay has been analyzed using the One-way ANOVA test and the results have been revised in figure 3A. Using One-way analysis of variance all $p$ values were $< 0.0001$. The results are shown below:

<table>
<thead>
<tr>
<th></th>
<th>Pa</th>
<th>Ve</th>
<th>SPHK1-C17</th>
<th>-C24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>11.00</td>
<td>11.80</td>
<td>60.80</td>
<td>68.00</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>2.345</td>
<td>3.564</td>
<td>13.61</td>
<td>8.888</td>
</tr>
<tr>
<td>Std. Error</td>
<td>1.049</td>
<td>1.594</td>
<td>6.086</td>
<td>3.975</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>8.088</td>
<td>7.375</td>
<td>43.90</td>
<td>56.96</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>13.91</td>
<td>16.22</td>
<td>77.70</td>
<td>79.04</td>
</tr>
</tbody>
</table>

Minor Issues not for Publication
Fig 1A - axis label on graph has misspelling of 'invasive'.
Following the suggestion of the referees, we have revised figure 1A.

In the methods for semi quantitative RT-PCR, it is stated that expected sizes of PCR products are given but they are missing.
The size of PCR product of MMP28 is 258 bp and U6 control was 89bp. The methods of RT-PCR section has been revised to “Oligonucleotide primers were designed and synthesized based on the published cDNA sequence of human $MMP-28$ (GenBank accession no. AF219624) with an expected product size of 258 bp. The sense primer encompasses nt 1366-1383, whereas the antisense primer corresponds to nt 1606-1623 of the human MMP28 sequence”.

In the methods for matrigel chemoinvasion, need to superscript number for cell count

Following the suggestion of the referees, we have revised the formatting of these numbers in the method of the matrigel chemoinvasion assay.

In Results, 2nd paragraph - delete "a" from first line after title, i.e. "....expression of MMP28 in [a] gastric carcinoma tissue....."

Following the suggestion of the referees, we have revised the results.

In Results, 2nd paragraph, 5th line after title, change high to higher, i.e. "...also showed high ER MMP28 expression..."

Following the suggestion of the referees, we have revised the results.
Reviewer number Two

Comments

1. Q-RT-PCR should be done for mRNA expression analysis.

Following the suggestion of the referees, qRT-PCR has been performed to quantify MMP28 (Fig 1b), and we have revised the methods and results section and the qRT-PCR data analysis is attached in supplementary data S2.

2. Full image is needed for Western blotting of MMP28.

Following the suggestion of the referees, figure 1C presents the full MMP28 western blotting image.

3. Detailed description is needed for localization of MMP28. It seems to be localized to nucleus, which is strange among MMP family. Fluorescent staining of cancer cells transfected for MMP28 can confirm it.

Following the suggestion of the referees, we have revised figure 2A as MMP28 was mostly localized to the cytoplasm and extra cellular stroma. The picture presented before was not typical. Fluorescent staining of N87-C9 cells was used to confirm the cytoplasmic expression pattern and images of fluorescently stained N87-C9 cells have been provided in supplementary data S3.

4. Scoring method is not as usual. German score is recommended.

The scoring method applied in our study is a routine method to semi-quantitatively score immunohistochemical staining intensity, and has been used before the manuscript “Secreted LOXL2 is a novel therapeutic target that promotes gastric cancer metastasis via the Src/FAK pathway. Carcinogenesis vol.30 no.10 pp.1660–1669, 2009”. We are not aware of the “German score” method recommended by the referees, and we were unable to find any reference to “German score” using a Google search. If possible,
we would appreciate if referee could supply more information about the “German score” method. As far as we can determine, there is no regular scoring system for every immunohistochemical marker.

5. In survival curve, how many cases for each group? Prognosis is too bad, when it include considerable cases with stage I/II patients (5-year survival rate of 70 to 99%). The author should explain such bad clinical outcome. Moreover, multivariate prognostic analysis is needed.

In the survival curves MMP28 Neg/Weak group had 83 cases; MMP28 mod/strong group had 69 cases. There are only 11 cases with stage I/II patients in the survival curve analysis. Most patients included in the survival curve had stage III/IV gastric cancer, which has a poor survival rate. We have revised figure 2C, and added the number of patients in each group. Following the suggestion of the referees, we have performed multivariate prognostic analysis, and the results are presented in table 2.

6. RNA interference is the most optimal assay to elucidate MMP-28 functional role in cancer invasion and metastasis.

When we started to investigate MMP28, we searched the published literature on MMP28. As there were some papers detailing the cloning of the MMP28 gene, but few papers on RNA interference of MMP28, we decided to firstly clone the MMP28 gene and produce MMP28 stable expression cells. Currently we are trying to identify target sequences which could be used for MMP28 RNA interference and intend to study the MMP28 function and molecular mechanism using this mechanism in the future.