Author's response to reviews

Title: Association of NGAL mRNA Expression with Tumor Progression and MMP-9 in Human Rectal Cancer

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Author's response to reviews: see over
Dear Editor,

Please find enclosed a revised version of our manuscript entitled “Association of NGAL mRNA Expression with Tumor Progression and MMP-9 in Human Rectal Cancer” (MS: 1203461080229281). We sincerely appreciate the in-depth critiques and constructive comments. As you will see in the following pages, we have tried our best to answer the essential questions raised by the reviewers and modified the manuscript as they suggested. We hope this improved version will meet approval for publication in “BMC Cancer”.

We appreciate your kindly consideration of our manuscript, and we look forward to receiving comments from the reviewers again.

Correspondence and phone calls about the paper should be directed to Xiao-hua Zhang at the following address, phone and fax number, and e-mail address:

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Best wishes,

Sincerely yours,

Xiao-Hua Zhang on behalf of all co-authors
Responses to reviewer 1 (Andreas Friedl):

Major Compulsory Revisions

1. Some of the conclusions are overreaching. For example, the authors state in the abstract and the main manuscript that “NGAL plays an important role in the progression of human rectal cancer”, which is not supported by their correlative data.

Response:

We truly appreciate the in-depth critiques and constructive comments. As suggested, we have revised the corresponding section in the abstract and the main manuscript as follows:

“NGAL mRNA expression was up-regulated in human rectal cancer. NGAL mRNA expression was correlated significantly with tumor progression and MMP-9 in rectal cancer. NGAL could be used for rectal cancer characterization.”

2. While the manuscript is overall comprehensible, many passages lack clarity and are tedious to read. Major editing is necessary, possibly by a native English speaker.

Response:

As suggested, the manuscript has been edited and polished by a native English speaker, who is our college’s English teacher from Australia. We are deeply sorry for our limited English writing ability. Any question, please let us know. The letter is as follows:

Dear Jack,

I have glanced through your manuscript and have carefully read through each sentence line by line. The manuscript has been well written and the English used which includes lots of medical terms have been well explained according to my knowledge.

The topic is very broad and you were able to cover the main important research points and draw very good conclusion and discussion on the experimental data gotten from the research.
There were some few minor spelling mistakes that have been checked and your sentence construction is very appealing and understandable. The work was clearly written.

Hope you succeed in this your manuscript.

Garry Wenzhou Medical College

3. As the molecule’s name implies, NGAL is expressed at high levels by neutrophils. The authors should confirm by either NGAL immunohistochemistry or even standard H&E histology that neutrophils are not present in large numbers in the NGAL positive carcinomas.

Response:

We admire you for your penetrating critiques. To be confessed, NGAL is also expressed by neutrophils. Bauer and colleagues have demonstrated by immunohistochemistry that “NGAL was detected primarily in the carcinoma cell cytoplasm but occasionally NGAL positive secretions were seen in the duct lumens. Some tumors contained NGAL positive neutrophils, whereas no staining was found in other stromal cell components” (Bauer M, et al. Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. Breast Cancer Res Treat 2008, 108(3):389-397). In the experimental design time, we also have discussed this issue. In order to reduce the interference by neutrophils, we selected the samples of rectal cancer and adjacent normal tissues from inpatients undergoing surgical operation strictly, to keep away from the section of obvious inflammation and necrosis. The adjacent normal tissues are distant from the corresponding rectal cancer tissues more than 5 cm. And then, immunohistochemistry Staining was routinely continued to perform by Prof. Guo-Rong Cheng from the Department of Pathology of the First Affiliated Hospital of Wenzhou Medical College. Some cases are excluded due to the obvious inflammation and necrosis response. However, neutrophils still can be found in small numbers in the NGAL positive carcinomas occasionally. It is to be regretted that the potential interfere by neutrophils involved can not be eliminated absolutely.
Expression of NGAL protein in human primary rectal cancer tissues by immunohistochemistry

Fig.1: The weak and negative NGAL staining in human primary rectal cancer specimen.

Fig.2: the left of the region : the weak and negative NGAL staining in adjacent normal tissues of rectal cancer (but it lies at the edge of the corresponding rectal cancer tissue, distant from the corresponding rectal cancer tissue far less than 5 cm ); the right of the region : the positive NGAL staining in human primary rectal cancer tissues.

Fig.3.4: The positive NGAL staining in human primary rectal cancer specimen.

Minor Essential Revisions

1. Table 2 is not sufficiently explained. For example, “sample means” presumably indicate mean CT values for the carcinomas, but this is not stated. The statement below the table “Randomizations 1,000 of 1,000 done” is unclear.

Response:
We have revised the remarks of Table 2 as suggested:

Randomisations 1,000 of 1,000 done.
Sample means: Mean CT values for the rectal cancer tissues.
Control means: Mean CT values for the adjacent normal tissues.
Expression ratios-nn: Expression ratios not normalised.
\(p\) values-nn: \(p\) values not normalised.

### Randomisation Data Output:

<table>
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<th>Genes</th>
<th>ref.</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>PCR Efficiencies</td>
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<td>Control Means</td>
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<td>29.776</td>
<td>28.555</td>
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<tr>
<td>Sample Means</td>
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<td>26.889</td>
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<td></td>
</tr>
<tr>
<td>Expression Ratios</td>
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<td>3.408</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(p)-Values</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Expression Ratios-nn</td>
<td>0.931</td>
<td>5.322</td>
<td>3.173</td>
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</tr>
<tr>
<td>(p)-Values-nn</td>
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<td>0.001</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Randomisations</td>
<td>1000</td>
<td></td>
<td></td>
<td>1000</td>
<td>done</td>
</tr>
</tbody>
</table>

To interpret “Randomisations 1,000 of 1,000 done”:

REST (Relative Expression Software Tool) is a standalone software tool to estimate up and down regulation for gene expression studies. The software addresses issues surrounding the measurement of uncertainty in expression ratios by using randomization and bootstrapping techniques. Prior to REST, relative quantitation in qPCR was a technique which allowed the estimation of gene expression. While useful, it did not provide statistical information suitable for comparing groups of sample versus control in a robust manner. They may calculate an average expression value indicating whether a particular subject in one group appeared to release more or less target mRNA than another subject, but without any statistical test to determine accuracy. Because the normalization and efficiency calculations involve ratios and multiple sources of error, it becomes very complex to perform traditional statistical analysis as ratio distributions do not have a standard deviation. REST overcomes these problems by using statistical randomization tests, while taking into account issues of reaction efficiency and reference gene normalisation. REST use the following randomization scenario: “if any perceived variation between samples and controls is due only to chance, then we could randomly swap values between the 2 groups and not see any greater difference than what we see between the initial groups.” In our study, the hypothesis test performs 1,000 random reallocations of samples and controls between the groups, and counts the number of times the relative expression on the randomly assigned group is greater than the sample data. For more details, please refer to the document below:

Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for

2. The authors assert that NGAL is a proven biomarker for the early diagnosis of malignancy. No such studies exist to the knowledge of this reviewer.

Response:

The suggestion that NGAL may be a valuable biomarker for early diagnosis of malignancy based on the following study:


In addition, Stoesz et al. have also suggested that “The observation that NGAL is secreted from carcinomas into normal ducts and the fact that NGAL levels are significantly higher in small tumors suggest a possible utility for NGAL expression in early diagnosis. It is likely that NGAL may be detectable in the breast fluid (Petrakis, 1986) of a subset of women with early breast cancers and may, therefore, serve as a potential early-detection marker for cancer presence”. (Stoesz SP, Friedl A, et al: Heterogeneous expression of the lipocalin NGAL in primary breast cancers. Int J Cancer, 1998, 79:565-572). This raises the possibility that NGAL measurement could also be explored as a possible utility for the early diagnosis in rectal cancer. However, till now, there are no available data on rectal cancer about this. Further studies are required to demonstrate the potential utility before NGAL could be served as a valuable biomarker for early diagnosis of rectal cancer.

3. The proposed utility of NGAL as marker is not clear. Do the authors suggest an eventual use of NGAL as diagnostic or prognostic marker?

Response:

To date, the related research about NGAL on malignancy is preliminary and
insufficient, little is known about the role of NGAL in the invasion and progression of human rectal cancer. The potential utility of NGAL as marker is indeed not clear. However, just like Bauer’s report, (Bauer M, et al. Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. *Breast Cancer Res Treat* 2008, 108(3):389-397) Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. In the future, the role of NGAL involved in the progression of malignancy will be further clarified by more and more study. We believe, with the in-depth study, NGAL may be a promising diagnostic or prognostic marker in some certain type of malignancy, but perhaps not for all type of malignancy.

**Discretionary Revisions**

*The discussion section could be shortened by omitting the discourse on the merits of qRT-PCR*

**Response:**

We fully agree with omitting the discourse on the merits of qRT-PCR on the discussion section. As suggested, we have deleted the corresponding section.
Responses to reviewer 2 (Per Eriksson):

1. The PCR analyses have been nicely performed. However, since GAPDH has been shown to be influenced by different stimuli, several housekeeping genes should have been used.

Response:

We greatly appreciate the in-depth critiques and comments concerning improvements to our paper. Quantitative real-time PCR is the method of choice for rapid and reliable quantification of mRNA transcription. Although real-time RT-PCR is widely used to quantitate biologically relevant changes in mRNA levels, there remain a number of problems associated with its use. These include the inherent variability of RNA, variability of extraction protocols that may copurify inhibitors, and different reverse transcription and PCR efficiencies. Consequently, it is important that an accurate method of normalisation is chosen to control for this error. Unfortunately, normalisation remains one of real-time RT-PCR most difficult problems. In our research, we follow several proposed strategies to normalising real-time RT-PCR data, including attempting to match sample size, ensuring good quality RNA is extracted and similar quantity used for the reverse transcription reaction and finally a reference gene also be measured. It is crucial to choose the appropriate housekeeping genes because the expression of these reference genes may vary among different tissues and may change under certain circumstances. The most commonly used reference genes include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA. GAPDH is a superior reference gene for rectal cancer among these candidates. (Radonic A et al. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun. 2004, 313(4):856-862.). In our study, We only detected rectal cancer tissues, not included other type of tissues such as stomach. The optimal conditions for the amplification of target genes were consistent respectively. GAPDH was employed simultaneously as a standard internal control. The
amplified PCR products separated on agarose gels stained with ethidium bromide, showing that they were of the expected size (NGAL 133 bp; MMP-9 167 bp, and GAPDH 141 bp). The expression of GAPDH mRNA was relatively stable. In addition, REST (Relative Expression Software Tool) is a superior software tool to estimate up and down regulation for gene expression studies. The software may calculate an average expression value indicating whether a particular subject in one group appeared to release more or less target mRNA than another subject by using simple statistical randomization tests, while taking into account issues of reaction efficiency and reference gene normalisation. REST software fully support single reference gene analysis. In the experimental design time, we also reviewed some relevant documents. In these researches, single housekeeping genes had been used simultaneously as a standard internal control (the references are listed below). There is no denying that more and more researchers advocate the use of multiple reference genes rather than relying on a single one. This is a indeed robust method for providing accurate normalisation. However, it is not always possible to measure multiple reference genes due to limited sample availability and cost. Furthermore, even if multiple genes are chosen the resolution of the particular assay remains dependent on the variability of the chosen reference genes (Huggett J et al. Real-time RT-PCR normalisation: strategies and considerations. Genes Immun. 2005, 6(4):279-284).

Undoubtedly, we fully agree with the appropriate choice of combination of more than one reference gene to improve qRT-PCR accuracy. As suggested, if several housekeeping genes could be used as internal control, the experimental design will be better. We sincerely appreciate your constructive comments again.


<table>
<thead>
<tr>
<th>N</th>
<th>T</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
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</tr>
<tr>
<td>GAPDH</td>
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</tbody>
</table>

**T**: rectal cancer tissue; **N**: adjacent normal tissue

Expression of NGAL mRNA in human rectal cancer by RT-PCR

2. *NGAL has been suggested to influence MMP9 activity by complex binding and thereby prolonging the activity of MMP9. Just measuring the mRNA expression of NGAL does not support the conclusion that NGAL plays an important role in the progression of human rectal cancer as stated in the abstract. To be more informative, the study would benefit from measuring NGAL/MMP9 complexes as well as MMP9 activity.*

**Response:**

Our study demonstrated that NGAL mRNA up-regulation correlated significantly with depth of invasion, lymph node metastasis, venous involvement, advanced pTNM stage and positively associated with MMP-9. This suggests that NGAL mRNA expression was correlated with tumor progression and MMP-9 in human rectal cancer. However, the conclusion that NGAL plays an important role in the progression of human rectal cancer is to some extent overreaching. As suggested, we have revised the corresponding section in the abstract and the main manuscript as follows:

“NGAL mRNA expression was up-regulated in human rectal cancer. NGAL mRNA expression was correlated significantly with tumor progression and MMP-9 in rectal cancer. NGAL could be used for rectal cancer characterization.”

As suggested, to be more informative, our study would benefit from measuring NGAL/MMP9 complexes as well as MMP-9 activity by gelatin zymography, Western blot analysis and so on. In the future, we will further the study to elucidate the exact regulatory mechanisms of NGAL and MMP-9 implicated in the progression of human
rectal cancer.