Author's response to reviews

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

Authors:

Xiu-Feng Zhang (legandsky520@163.com)
Xiao-Hua Zhang (oncology0607@163.com)
Su-Mei Zhou (surzsm@163.com)
Ying Zhang (lmzy1386@163.com)
Ou-Chen Wang (woc099@gmail.com)
Gui-Long Guo (guoguilong@sina.com)
Shao-Qiang Lin (linshaoqiang57@163.com)

Version: 5 Date: 7 March 2009

Author's response to reviews: see over
Honorific 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). It is a great honour that our manuscript has been the second time peer reviewed. We sincerely appreciate the in-depth critiques and constructive comments again. 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 deeply appreciate your kindly consideration of our manuscript 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:

Xiao-hua Zhang
Institute: Department of Surgical Oncology, the First Affiliated Hospital of Wenzhou Medical College
Address: No.2, Fu Xue Road, 325000, WenZhou, China
E-mail: oncology0607@163.com
Fax: 86-577-88069555
Tel: 86-577-88078236

Best wishes,

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

Major Compulsory Revisions  
None.

Minor Essential Revisions

1. The authors explained the last line in Table 2 (“Randomizations”) in their response letter. They should also add a brief explanation in the figure legend. Most readers will not be familiar with the REST statistical/software tool. Similarly, the issue of NGAL in neutrophils is addressed in the response letter but not in the manuscript. A brief comment should be added.

Response:

We truly appreciate the in-depth critiques and constructive comments. It is indeed the case that most readers will not be familiar with the REST statistical/software tool. As suggested, we added a brief explanation in the corresponding figure legend (Table 2) as follows:

Randomisations: REST (Relative Expression Software Tool) is a standalone software tool to estimate up and down regulation for gene expression study in real-time PCR by using statistical randomization tests, while taking into account issues of reaction efficiency and reference gene normalisation. The randomization scenario is as follows: “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.

In addition, the issue of NGAL in neutrophils is addressed in the response letter but not in the manuscript. Therefore, we added a brief comment in the “Methods” section of the revised manuscript as follows:

“The samples of rectal cancer and adjacent normal tissues were away from the region of obvious inflammation and necrosis. The immunohistochemistry Staining was routinely performed by the Department of Pathology of the First Affiliated
Hospital of Wenzhou Medical College in order to exclude the cases with obvious inflammation and necrosis response, and to reduce the potential interference by neutrophils”.

At the same time, we would like to mention the report by Nielsen et al (Nielsen BS, Borregaard N, Bundgaard JR, et al. Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut 1996, 38(3): 414-420). In their research, in situ hybridisation was performed on 11 of 14 tumours investigated by immunohistochemistry. A strong in situ hybridisation signal was seen in 10 of 11 cases. In contrast with the immunohistochemical localisation, no in situ hybridisation signal was seen in any neutrophils. Scattered neutrophils present in normal colon were strongly positive by immunohistochemistry, but negative by in situ hybridisation. “As expected, neutrophils present in both normal and inflamed colon contain NGAL as shown by a strong immunohistochemical staining of these cells. No mRNA for NGAL was present in neutrophils, showing that mature neutrophils in the colonic mucosa do not actively synthesise NGAL despite the potential presence of inflammatory mediators like interferon γ and TNF α, which have been shown to induce the synthesis of a variety of proteins in mature neutrophils. This implies that NGAL, present in neutrophils in colonic mucosa, is synthesised in immature neutrophil precursors in the bone marrow, where NGAL is packed in specific granules”. For this reason, the potential interference by neutrophils in real-time RT-PCR analyses perhaps was limited. However, further studies are needed to fully exclude the potential interference by neutrophils in real-time RT-PCR analyses. For more related report, maybe could further refer to the following documents:


Discretionary Revisions

None
Responses to reviewer 2 (Per Eriksson):

1. This is a revised version of a paper demonstrating an association of NGAL mRNA levels with tumor progression in human rectal cancer. A few modifications of the manuscript have been performed compared to the previous version. The comments about the house-keeping genes are ok. However, the main criticism on the manuscript, i.e. that it is difficult to interpret the relevance of just measuring NGAL mRNA levels, still remains. MMP9 and NGAL are co-regulated (both are activated by c-Jun N-terminal kinases). Why is NGAL a better predictor of the disease than MMP9? In my mind MMP9/NGAL complex or MMP9 activity should be measured. Just measuring NGAL mRNA will not add substantially to our knowledge about the disease mechanisms.

Response:

We greatly appreciate the in-depth critiques and comments concerning improvements to our paper. Till now, little is known about the role of NGAL in human rectal cancer. Its association with clinicopathologic characteristics and expression of MMP-9 in rectal cancer has not been reported systematically. Therefore, to further determine the potential involvement of NGAL in rectal cancer, we detected mRNA expression of NGAL and MMP-9 in matched rectal samples by real time RT-PCR, and further evaluated the correlation between NGAL and MMP-9 as well as clinicopathological features in human rectal cancer. Our study demonstrated that NGAL mRNA expression was up-regulated in human rectal cancer. NGAL mRNA up-regulation correlated significantly with depth of invasion, lymph node metastasis, venous involvement and advanced pTNM stage (Table 3). However, depth of invasion, lymph node metastasis and pTNM stage of rectal cancer have powerful predictive value to prognosis. Venous invasion by malignancy has also been demonstrated to be a stage-independent adverse prognostic factor. NGAL mRNA up-regulation was correlated significantly with tumor progression in rectal cancer, suggesting a more aggressive phenotype. NGAL could be used for rectal cancer characterization. NGAL detection may also provide
information for risk assessment and identification of a subset of patients requiring more aggressive adjuvant therapy.

Furthermore, the correlation between NGAL and MMP-9 was further evaluated by calculating Spearman’s correlation coefficient. In this study, the correlation coefficient between mRNA up-regulation of NGAL and MMP-9 in rectal cancers was 0.393, $P<0.001$ (Table 4). Therefore, it is reasonable to draw the conclusion that NGAL mRNA up-regulation positively correlated with MMP-9 mRNA overexpression in human rectal cancer. There is no denying that this statistical correlation perhaps indicates a kind of indirect association. As your suggestion, to be more informative, this study would benefit from measuring NGAL/MMP9 complexes as well as MMP-9 activity by gelatin zymography, Western blot analysis et al. However, in present study we mainly aimed at exploring the potential involvement of NGAL in rectal cancer, evaluating the expression level of NGAL mRNA by real time RT-PCR, and further elucidated the correlation of NGAL mRNA expression with clinicopathologic features and MMP-9 in rectal cancer. But not laying stress on elucidating their regulated mechanisms between NGAL and MMP-9. At the same time, several lines of evidence in vitro or in vivo also have demonstrated that NGAL can form a complex with MMP-9 and improving MMP-9 expression, preventing its degradation and causing increased MMP-9 enzymatic activity, thereby favoring invasive and metastatic potential of cancer cells.


3. Li EM, Xu LY, Cai WJ, Xiong HQ, Shen ZY, Zeng Y: Functions of neutrophil gelatinase-associated lipocalin in the esophageal carcinoma cell line SHEEC.

As to “why is NGAL a better predictor of the disease than MMP-9”. We also undoubtedly came to an agreement that MMP-9 plays a key role in malignant tumor growth and angiogenesis, thereby promoting invasion and metastasis. MMP-9 appears to be one of the most important since it is overexpressed in the majority of malignancies. We also known that you have done many excellent workes about MMPs and we admire your very fruitful and important finding. There is no denying that MMP-9 is a good marker for many malignancies, including rectal cancer. However, although MMP-9 detection in malignancies has good sensitivity, but its specificity is not ideal enough. In normal tissues, MMP-9 also could often be detected. For this reason, a number of screening biomarkers have been developed. NGAL as a valuable marker for early diagnosis and monitoring relapse of malignancy has been increasing reported. Fernandez et al have demonstrated that NGAL and MMP-9 complexes were identified in nearly 86.36% of urine sample from breast cancer patients whereas this molecule is undetectable in the urine of healthy controls, suggesting that urinary NGAL may represent a novel, non-invasive biomarker for tracking disease status and the effectiveness of anticancer therapy in NGAL positive breast carcinomas. Other repts are listed below:


At the same time, we also would like to mention that NGAL involved in tumorigenesis and progression not only through acting with MMP-9. Some studies have demonstrated that NGAL can bind to retinoic acid and iron via an endocytotic pathway which bypasses the transferrin receptor. The amount of iron shuttled by NGAL was sufficient to regulate iron-responsive genes inhibition of cell proliferation. NGAL also involved in epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition. In addition, NGAL directly affects tumor cell survival and proliferation and through PI3K/Akt pathway promotes tumorigenesis and progression. However, up to now the understanding of the functions of NGAL in cancer is incomplete. The precise mechanisms of NGAL implicated in tumorigenesis and progression needs to further study.

Both of the reviewers thought that the manuscript needs some language corrections. As suggested, the manuscript has been further edited and carefully polished again. In addition, we have also renew our manuscript title and modified the following main section (listed below) and others. Whether our manuscript will be accepted or not, We also sincerely appreciate your kindly consideration. Please do not hesitate to contact us if you have any further queries.

Title: Clinical significance of NGAL mRNA Expression in Human Rectal Cancer

Background:

Emerging evidence has demonstrated that Neutrophil gelatinase-associated lipocalin (NGAL) is up-regulated in multiple malignancies, including oesophagus cancer, and plays a critical role in tumorigenesis and progression. However, till now, little is known about the role of NGAL in human rectal cancer. Its association with clinicopathologic characteristics and expression of MMP-9, one of its target genes, has not been reported systematically in rectal cancer.
Therefore, to further determine the potential involvement of NGAL in rectal cancer, we have evaluated the expression level of NGAL mRNA by real time RT-PCR, and further elucidated the correlation of NGAL mRNA expression with clinicopathologic features and MMP-9 in rectal cancer.

Conclusions:

In human rectal cancer, NGAL mRNA expression was elevated. NGAL mRNA up-regulation was correlated significantly with tumor progression and MMP-9 mRNA overexpression in rectal cancer, suggesting a more aggressive phenotype. NGAL could be used for rectal cancer characterization.

Conclusions

Till now, little is known about the potential involvement of NGAL in human rectal cancer. In present study, we detected the mRNA expression of NGAL and MMP-9 in human rectal cancer by real-time RT-PCR. Our research demonstrated that NGAL mRNA expression was elevated in human rectal cancer. NGAL mRNA up-regulation was correlated significantly with tumor progression and MMP-9 mRNA overexpression in rectal cancer, suggesting a more aggressive phenotype. NGAL detection may provide valuable information for rectal cancer characterization and identification of a subset of patients requiring more aggressive adjuvant therapy. As a secreted molecule, NGAL may serve as an attractive therapeutic target. Further studies are needed to elucidate the precise mechanisms of NGAL implicated in cancer progression and its potential utility in rectal cancer treatment.