Author's response to reviews

Title: Increased PADI4 expression in blood and tissues of patients with malignant tumors

Authors:

Xiaotian Chang XC (changxt@126.com)
Jinxiang Han JH (jinxiang.han@163.com)
Li Pang PL (chang-xt@163.com)
Yan Zhao YZ (zhaoyansdams@163.com)
Yi Yang YY (yiyang@163.com)
Zhonglin Shen ZS (zhonglinshen@163.com)

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Author's response to reviews: see over
Dear Joseph Dunckley:

We really appreciate reviewers and editor for their comments to our submission. Based their suggestions and instructions, we made correction and modification with our manuscript. The point-by-point response to the concerns is attached at end of this letter.

We also made formatting changes to our manuscript according to instruction of editor. Details of these changes are:

(1) Abstract was structured according to the guidelines of BMC Cancer.
(2) Key acronyms such as "PADI4" were written in full for the first instance of their use.
(3) The manuscript is resubmitted as a research article. We are so sorry for the mistake.
(4) The Manuscript Presentation Service (www.biomedes.co.uk), a professional copyediting service, polished the English writing of the resubmitted manuscript. We would be grateful if our work is accepted for publication on your journal
(5) We removed table 2, because the standard of positivity we set up (150% higher in tumors than the average of healthy controls were considered as positive) was feasible.
(6) Recent studies (cited our paper about PADI4 expression in tumors) of others showed that PADI4 antagonized regulation of p53 to tumor suppressor genes, indicating the importance of PADI4 for tumorigenesis. We added some discussion


If editors and reviewers are not satisfied with our revision, please let us know immediately and we will make further correction with our submission.

We would be very grateful if our submission is accepted by BMC cancer.

With best wishes,

Xiaotian Chang

Tel: +86-531-82919606
Facsimile: +86-531-82951586
E-mail: changxt@126.com

Point-by-point response to comments of reviewers

I. For comment of Dr. Lorne Hofseth

We really appreciate comment and suggestion of Dr. Lorne Hofseth to our submission. Based on his comment, we made correction and modification to our manuscript. The detailed is as following:

1. “State the criteria for positivity in immunohistochemistry (IHC). This needs to be
We added new interpretation and analysis to our immunohistochemistry results in the resubmission. According to the paper this reviewer assigned, we evaluated the histological section by following protocol. The percentage of positive cells was scored as 0 (0%), 1 (10%), 2 (10–50%), 3 (51–80%) and 4 (>80%). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The immunoreactive score (IRS) was obtained by multiplying the percentage of positive cells and the staining intensity. Immunohistochemical result with IRS of 0-1 was considered as negative, 1-2 weak and 6-12 positive.

2. “What are the positive and negative controls used for IHC results?”

We prepared a series of control experiments with the anti PADI4 antibody in the present study. We also tested the specificity of the antibody by (1) western blot and (2) blocking the antibody with the PADI4 oligo-peptide or recombinant protein before immunohistochemistry and western blotting. We used synovial membranes of rheumatoid arthritis as positive control, because we and others had confirmed the significant expression of PADI4 in the diseased tissues. We also used cultured tumor cell lines as positive controls, because Abcam (www.abcam.com/index.html?datasheet=38772) and Dong et al. (Mol Endocrinol 2007; 21:1617-29) had detected PADI4 expression in some tumor cell lines such as Hela cells. Because PADI4 was originally present in some CD34 cells in both normal tissues and diseased tissues, there seem no PADI4- absolutely negative tissues. But brain and muscle is normally PADI4-negative according to our study.

As the response to question 1, we quantitatively analyzed and interpreted the immunohistochemical results in our resubmission based on IRS protocol of Denkert et al’s. The detailed change is seen in the revised manuscript. In addition, we recently investigated the expression of PADI4 by immunoflorescent method and we are analyzing the result by automated IHC quantification system. The result of immunoflorescent labeling is corresponding to the result of immunohistochemistry.

The following figure is immunoflorescent detection to various types of ovary cancer.
Figure 1. Immunodetection of PADI4 in various kinds of ovarian tumors by immunohistochemistry (A) and immunofluorescent labeling (B). Tissue section: 1A-B serous adenocarcinoma, 2A-B mucinous adenocarcinoma, 3A-B immature teratoma, 4A-B dysgerminoma, 5A-B clear cell cancer, 6A-B endodermal sinus tumor, 7A-B sibnet-ring cell carcinoma metastatic, 8A-B adenocarcinoma metastatic, 9A-B squamous cell tumor, 10A-B malignant cells, 11A-B malignant thecoma, 12A-B granulosa cell tumor, 13-14A-B ovarian cyst, 15-16 A-B the same sample having both malignant tumor tissue and normal adjacent tissue (SP×400)

4. “Because points 1, 2 and 3 above were not done, this reviewer disagrees with some interpretation of IHC. Only a few examples are that the authors state (page 10) that no significant immunosignals for PADI4 were detected in various benign tumors, including leiomyoma of the stomach, myoma of the uterus, endometrial hyperplasia of the uterus…’. The first issue is to define ‘significant immunosignals’. The second issue, here, is that if one takes a close look at Figure 1, there are clearly signals in all of the above named tissues (leiomyoma of the stomach, myoma of the
As described in the response to question 1, we evaluated the histological section according to IRS protocol of Denkert et al.’s in the resubmission. Immunohistochemical result with IRS of 0-1 was considered as negative, 1-2 weak and 6-12 positive. The immunosignals of leiomyoma of the stomach, myoma of the uterus, endometrial hyperplasia of the uterus was low and ration of positive cells was lower than 5%. Thus, we suggested that there were no significant immunosignals for PADI4 in these benign tumors. In our previous study, we found that PADI4 is present in CD34 and CD34-derived cells in both normal tissues and diseased tissues. Thus, we accordingly detected some positive signals in benign tumors including leiomyoma of the stomach, myoma of the uterus, endometrial hyperplasia of the uterus. However, IRS of these tissues is 1-2, much lower than the malignant tissues, indicating the low expression of PADI4 in the tissues. In addition, western blot analysis and real time PCR also demonstrated the low levels of PADI4 in the benign tissues and normal tissues.

5. “Because of the lack of quantification, to convince the reader/reviewer, the authors should also take a low power and high power magnification of each tissue they quantified. Add the pictures to supplementary data”.

In our previous report (Molecular Carcinogenesis 2006;45:183-96), we provide a figure with low power and high power magnification to show the expression of PADI4 in various malignant tissues (see following figure). In the previous study, we used tissue array containing 204 various tumor tissues to confirm the expression of
PADI4 in various types of adenocarcinoma. In the current submission, we found that PADI4 was expressed not only in adenocarcinoma but also in other malignancies with a great numbers of samples and quantative methods.

Figure. Immunodetection of PAD4 in various tumors and their corresponding normal tissues. Panel A shows PAD4 expression in tumor tissues. Original magnification, 100×. Panel B is a partial magnification of panel A. Original magnification 400×. Panel C shows PAD4 expression in normal tissue corresponding to panel A. Original magnification, 100×. Tissue sections: 2A-2C duodenum adenocarcinoma, 3A-3C esophagus adenocarcinoma, 4A-4C prostate adenocarcinoma, 5A-5C parotid adenocarcinoma, 6A-6C stomach adenocarcinoma, 7A-7C liver cholangiocellular carcinoma and 8A-8C testis seminoma. Arrows indicate expression of PAD4.

6. “Figure 3: Standard Error bars are shown, but there are no statistics to indicate where significance is.”
We added statistics to indicate significance.

7. “Figure 4: There are no Standard Error bars shown. I would envision there are standard errors with the numbers the authors indicate”.

We added standard errors to Figure 4 in the resubmission.

8. “Figure 4: What is at the bottom of the figure?”

The words at bottom of Figure 4 describe the blood samples we tested. In the resubmission, we omitted this description, because we describe the samples in legend of the figure.

Minor essential revisions:

“(1) Benignant and/or benignancies’: this word is not usually used in a medical context. I think the authors mean ‘Benign’. Please change at appropriate places.

(2) Abstract: 6th line down: change ‘…blood of many malignant tumors as compared with chronic…’ to ‘…blood of many patients with malignant tumors as compared to patients with chronic…’.

(3) Introduction: 3rd line from the bottom: change ‘…leukemia cells.’ To ‘…leukemia cells, respectively.”

We really appreciate this reviewer for his kind suggestion. We made correction at appropriate places according to his instruction.

II. For comment of Dr. Sunil Badve

We really appreciate comment and suggestion of Dr. Sunil Badve to our
submission. Based on his comment, we made modification with our manuscript. The detail is as following:

1. “Based on the author’s data, PADI4 seems to predominantly expressed in epithelial cells. The authors compare RNA levels in normal and tumor tissues and claim that in put RNA quantity was similar so differences in PADI4 are real. Normal tissues tend to be less cellular have a significant amount of stromal, muscle etc. Normalization of the data needs to be performed with (?) Keratin to assess the relative amount of PADI4. This will significantly change a number of results described by the authors”.

For this study, we also performed real time PCR with beta actin and GADPH as references to assess the relative amount of the PADI4 transcript and got similar results. The following figure shows the relative level of the PADI4 mRNA in ovary cancer. In most of cases, beta actin and GADPH are used as references to normalize the expressions of target genes. There are many papers about the expression of keratin in tumors. So far, 20 kinds of keratin are identified. Different types of tumors and tissues expressed different types of keratin at different levels. As suggestion of this reviewer, we will get new result if we use keratin to assess the relative amount of PADI4.
**Figure.** Relative expression of PADI4 transcription determined by real-time RT-PCR. β-actin was used as reference to normalize expression of the PADI4 mRNA. Lane 1-5 showed the levels of the PADI4 mRNA of 5 different samples of ovarian cancer, lane 6-7 metastatic carcinoma, lane 8 teratoma, lane 9 and 10 adjacent normal tissues to the tumor samples, lane 11 ovarian cyst, lane 12 serous cystadenoma, lane 13 mucinous cystadenoma.

2. “The authors seem to confuse endothelium with endoderm (page 15). Adenomas of the thyroid are derived from endoderm and have nothing to do with endothelium”.

   Based on the suggestion of this reviewer, we changed the confusing description and removed the discussion about origin of adenoma. We added two new references about increased numbers of CD34 cells in adenoma tissues.

3. “Keratin is NOT a tumor marker (page 3) nor does it play an role in cell differentiation and apoptosis.”

   We agreed the comment of this reviewer. As suggestion of this reviewer, keratin should not be actually considered as tumor marker. We corrected the discussion about this issue. Many studies reported the important role of keratin during carcinogenic process. For example, (1) Accumulation of ubiquitin-conjugated cytokeratin