Author’s response to reviews

Title: High peritumoral lymphatics vessels density is a potential prognostic marker in endometrial carcinoma: a clinical immunohistochemical method study

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Author's response to reviews: see over
Dear sir:

Thank you for your reading my letter. I am very grateful for your advice and three referees’ comments about our article (High peritumoral lymphatics vessels density is a potential prognostic marker in endometrial carcinoma. MS: 9990418172878294). This could help us to make progress in the research and manuscript. At first, the statements on ethical approval, conflicting interests and authors’ contribution have been included in the manuscript and we have asked a native English speaking colleague to help us copyedit the paper. Second, according to the serious concerns of three referees regarding my article, the manuscript have been revised on the basis of reworking ,adding and deleting some of original experiment.

According to Artur Czekierdowski’s comments, the revision can be seen as follows:

1 The question posed by the Authors is in my opinion insufficiently defined. The role of lymphangiogenesis has been briefly highlighted by summarizing only some important findings that confirm the role of lymphatic vessels in local endometrial cancer spread and nodal metastases. In particular, the Authors do not state that a complex and efficient pro- and anti angiogenic balance in the endometrial and subendometrial lymphatic system exist. They briefly discuss recent data on the role of LYVE-1 and CD-44 and do not provide clear evidence for their hypothesis that LVD counting could potentially serve as an independent marker of endometrial cancer survival. Moreover, it is not clear why the Authors have used CD44 molecule to compare with LYVE-1 marker-the fact that it shares 41% homology with LYVE-1 seems somewhat insufficient explanation. CD44 is rather regarded as a mesenchymal stem cell (MSC) marker along with e.g. ITGB1 (CD29), NT5E (CD73), THY1 (CD90), ENG (CD105), PDGFRB (CD140B)- for reference see e.g. Garet et al. “Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. Biol Reprod. 2009 Jun;80(6):1136-45. Epub 2009 Feb 18”). There is only scant information on the possible role of this molecule in the Discussion section.

Revision: In fact, we have showed that P-LVD is an independent term that were predictive of overall and progression-free survival analysis in our results.

Many articles about CD44 has been looked up including “Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium”, and the research of CD44 has been added in the introduction and discussion. So it would help to explain why the we have used CD44 molecule to compare with LYVE-1 marker.

2 The paper in my opinion lacks sufficient information on currently known molecular events leading to uncontrolled spread of endometrial cancer. Interaction between endometrial tumor cells and their surrounding stroma is very important in tumor progression and metastasis. This is accomplished through a number of transmembrane receptors that interact with stromal extracellular matrix molecules. It has been documented that one of these receptors, CD44, binds to extracellular matrix component hyaluronic acid (HA). With progression from hyperplasia and with increasing atypia to adenocarcinoma, levels of stromal HA, glandular and stromal CD44s all increase (Afify AM et al, Ann Diagn Pathol. 2005;9:312-8). Also, it would be advisable to note which isoform was detected as CD44 isoforms do express variant exons usually designated as CD44v. A CD44 isoform containing the last 3 exon products of the variable region (CD44V8-10, also known as epithelial CD44 or CD44E), is preferentially expressed on epithelial cells. The Authors do not mention anything on all the above topics. More information on other lymphatic markers such as VEGF-C or VEGF-D, PROX-1, podoplanin and lymphatic endothelium VEGF...
receptor-3 (VEGFR-3) either in the Introduction or in the Discussion sections would be advisable.

**Revision:** The importance of Interaction between endometrial tumor cells and their surrounding stroma has been seen in the discussion, for instance, “HA is more abundant in the surrounding stroma adjacent to the tumor, interaction between tumor cells and HA in stroma immediately adjacent to it may play a role in tumorigenesis.”

The research of VEGF-C/VEGF-D/VEGFR-3 axis has been introduced in the discussion, which could help to explain lymphangiogenesis.

3 In contrast to the Results section, the discussion and conclusions are not well balanced. The Authors conclude that tumor peripheral LVD measurement may be a potential prognostic marker in endometrial carcinoma, moreover, they claim that peritumoral lymphatics may be a target to inhibit metastasis. The first conclusion can be inferred from the data, however, the second one is neither supported by the presented research nor in the citations used by the Authors. Moreover, there are too few citations concerning endometrial cancer and important research done before and published to date. Only two Author’s citations were published in 2008 and none of them concerns endometrial cancer and lymphangiogenesis. A well balanced review on lymphangiogenesis can be found in a paper by Achen MG, Stacker SA. Molecular control of lymphatic metastasis. Ann N Y Acad Sci. 2008;1131:225-34. On the other hand, various lymphatic vessel markers in the endometrium and endometrial cancer were well described in a paper by Stefansson et al. “Vascular Proliferation Is Important for Clinical Progress of Endometrial Cancer” published in Cancer Res 2006; 66: (6). March 15, 2006.

**Revision:** “That peritumoral lymphatics may be a target to inhibit metastasis” could not be concluded from the result directly, but the peritumoral lymphatics may play an important role in lymphatic vessel metastasis. That peritumoral lymphatics may be a target to inhibit metastasis might be supported by adding citations of “the importance of lymphangiogenesis in metastasis depending on the position of the primary tumor relative to the lymphatic network” and “P-LVD may be a determinant of metastatic spread as studies have showed in animal models that intratumoral lymphatics are nonfunctional, but peritumoral lymphatics are capable of draining fluid and cells from a tumor so they may be more important for the metastatic process”. In addition, and the result “high P-LVD were associated with lymph nodes metastasis and poor survival” may also help to deduce this.

More citations concerning endometrial cancer and important research done before and published to date has been quoted in the manuscript.

4 The Methods generally are appropriate and well described but require some minor corrections. For instance, the name of the Authors Department written in English seems odd- “1st affiliated hospital..” should rather be “1st Affiliated Hospital…of the Medical University” and not “University Medicine”. Another example is the statement- “was from R&D” no specific country source is given, it should be changed to e.g. “was purchased from R&D Systems, MN, USA”, followed by “under discussion microscope” which should be changed to “dissection microscope”. In the Discussion section there are many misspelling mistakes e.g. “Intratumol” should be changed to “intratumoral”.

The title and abstract generally agree with what was found. English language used in the whole paper needs numerous corrections. In many instances the language/translation is totally incorrect and requires several important changes before the paper is accepted. There are numerous
writing/spelling/ sentence structure mistakes. For instance, in the Background section the Authors claim that “EC …in the western world[1,2], whose five year survival..” or “the currently diagnostic technology”. Examples in the Results sections include: “Of the endometrioid carcinomas, 99 patients demonstrated endometrioid adenocarcinoma…” apparently it should be changed for example to: “of all endometrial cancer cases, 99 patients had endometrioid adenocarcinoma …” furthermore “other 9 patients died of the disease except tumor” should be changed to “died of other than endometrial cancer causes”. Generally I would suggest not to start the sentence with number in arabic., e.g in the same section a sentence which starts with “36 patients (32,1%) received…” should be changed either to “Thirty-six patients…” or “The number of patients who received…”

Revision: The English language mistakes above have been corrected and we asked a native English speaking colleague to help us copyedit the paper.

According to Mr Mario Leitao Jr comments, the revision can be seen as follows:

1 The authors use a four-point scale for determining CD44 expression based on percentage of staining cells. Is this a validated and previously published system? If so, please provide reference. The authors make a point of using the 4-point scale but then never use it in their analysis and merely use 50% expression as a cutoff. Additionally, often the intensity of staining as well as percentage of stained cells is used when determining IHC expression data.

Revision: The determination of CD44 expression has been reworked. A number of neoplastic cells labelling positively for CD44 antigen were counted in 10 high power fields (magnification factor of 400)for each tissue section and were scored semi-quantitatively as <10%, between 10% and 70%, or >70% cells positive. (Reference: Saddik M, Lai R.CD44s a surrogate marker for distinguishing intraductal papilloma from papillary carcinoma of the breast. J Clin Pathol 1999,52:862–864.)

2 There are major statistical concerns: first, the authors report that the LVD was the MEDIAN of the vessel counts obtained in three fields buy then report means +/-SD and use ANOVA to analysye. ANOVA is appropriate for means but were means or medians used? The authors then dichotomize the group in 2 groups for Kaplan-Meier estimations using the median values for LVD. The authors need to make the use of either mean or median uniform. Additionally, when reporting a median, the range is to follow, as opposed to when reporting a mean which is followed by +/-SD. There Multivariate analysis is needed for Table 1 as there are multiple findings significant on their univariate analysis.

Revision: I am sorry that it is my mistake in spelling.The LVD was the mean of the vessel counts obtained in three fields,not the median. So one way ANOVA test is appropriate for means. Multivariate analysis has been done for Table 1.

3 Lymph-vascular space invasion (LVSI) is an important factor in many carcinomas and is now felt by most to also be important in endometrial carcinoma. The authors do not mention he rate of LVSI as this may be more important than just P-LVD. This needs to be assessed and included in the analysis and accounted for in a multivariate model also. Is LVD itself important or really is it whether these lymphatics have been invaded and involved with carcinoma cells? This must be addressed.

Revision: LVSI has been assessed and included in the analysis and accounted for in a multivariate model. That LVD itself was important has been addressed in discussion.
It is difficult to understand why stage is independently associated with outcome in their multivariate model but not nodal status. Nodal status is a defining criteria in stage and defines stage IIIC. Nodal status has been shown to be one of the strongest predictors of outcome in large prospective trials conducted by the GOG. This finding makes one question the accuracy of the findings overall. 

Revision: Nodal status is only a defining criteria for stage IIIC. Stage III included not only IIIC but also IIA and IIB. That the stage is independently associated with outcome in multivariate model was the result of the analysis of all stageI, II,IIIC, IIA and IIB. That nodal involvement was not an independent prognostic factor in a multivariate survival analysis suggested that nodal metastasis may be affected by lymphangiogenesis and other factors, such as P-LVD and so on. So the research about this should be further investigated in the future.

5 Table 2 must be redone. It is generally not advisable to cut and paste tables from SPSS as they are not intuitive and it is very likely that most readers will have no idea how to interpret such tables. They should redo the table and report in the form of Hazard ratios (HR) with 95% CI.

Revision: Table 2 has been redone.

According to Mr Leslie Randall’s comments, the revision can be seen as follows:
1 Title: This is not a “case-control” study.

Revision: “A case-control study” has been substituted for “a clinical immunohistochemical method study” in the title.

2 Methods: The purpose of ER/PR staining is unclear.

Revision: The research of ER/PR has been deleted because it has little association with our study.

3 Results: 1) The table reporting the the multivariate analysis is the most important in the manuscript, and should not be delegated to the supplementary files. 2) The table reporting the multivariate analysis also a raw output from the statistical package with extra information (Wald, df) that are not explained in the footnotes.

Revision: The table has been in the manuscript and the table 2 reporting the multivariate analysis has been redone.

In addition, I am sorry that other questions such as “For example, the authors can include additional staining with VEGF-C or VEGF-D, PROX-1, podoplanin to show that this caused an aggressive growth or of any other attribute of endometrial cancer” could not be studied this time because of the limitation of our experiment condition. We would try our best to do the research in the future and go on the study of lymphangiogenesis in mechanism.

Yours sincerely,

Ying Gao