Peer Review File

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Reviewer A

Niinimäki et al. explored the potential prognostic value of HSP90 expression in patients with pulmonary carcinoid tumors in this study. The authors found that high expression of HSP90 was associated with metastatic disease and disease-specific mortality.

I congratulate the authors for this critical study that sheds light on a potentially targetable gene expression. The manuscript is very well written and the methodology used is appropriate. I have a few comments that I hope the authors find constructive.

Comment 1: Abstract, results, page 2 line 42, the authors wrote "HSP90 protein expression was found to be 42 associated with poor patient outcome". Please specify what you mean by poor patient outcome. As mentioned later in Figure 4-A it appears that this refers to disease-specific survival. It needs to be stated clearly. Also the results of the regression analysis "and increased risk of disease-specific death (P=0.023)" needs to be expressed in HR and CI, instead of P-value.
Reply 1: Thank you for the comment. We modified the abstract accordingly.
Changes in the text: Page 2, lines 37-39, *Through immunohistochemical analysis, HSP90 protein expression was found to be associated with* ***shorter disease-specific survival*** *(P=0.009) and increased risk of disease-specific death (****HR 6.4, 95% CI 1.3-31.8****).*

Comment 2: Methods section, page 5, lines 168-171, the authors wrote "The Kaplan–Meier method and log-rank test were used to evaluate the effect of immunohistochemical HSP90 expression on patient outcome by estimating cumulative survival probabilities and drawing disease-specific survival (DSS) curves." This needs to be rephrased, Kaplan Meier / log-rank test don't assess potential effects on survival, but rather estimates survival probabilities and compare differences in survival. I recommend rephrasing to "The Kaplan-Meier method and Log-rank test were used to estimate survival probabilities and compare differences in disease-specific survival".
Reply 2: Thank you for the valuable comment. We rephrased this sentence according to the suggestion.
Changes in the text: Page 10, lines 168-170, *The Kaplan–Meier method and log-rank test were used to* ***estimate survival probabilities and compare differences in disease-specific survival (DSS)****.*

Comment 3: The authors need to elaborate on the regression analysis. It is unclear what variables were included and if they had any relevant demographic or clinical variables. Also, it is not clear if this was only univariable or if the authors did a multivariable

model. From going through the results section, it appears that the authors only used a univariable analysis.

I strongly encourage them to elaborate on this analysis in their methods and results section. Analysis should include a multivariable model to adjust for potential confounding factors. It is important to include patients' demographics and the clinical characteristics of the tumors. The authors need to specify in the methods section which variables were included, and the criteria for variables to be included from univariable to multivariable model. In the results section, the authors need to include a detailed table of the analysis that includes all the univariable and multivariable results.

Reply 3: Thank you for the important comment. A reliable multivariable analysis could not been performed because of the low number (six) of disease-specific deaths. A larger study/higher number of events is required to obtain more results: *"for every variable included in a multivariable Cox model a minimum of 10 (better 20) events should have been observed"**. However, we edited the Method section and added the variables used in the regression analysis to Results section. We also added Table 2 to Results to present the numbers of the univariable regression analysis, and the known poor prognostic marker, Ki-67, was added to the DSS curves. Furthermore, we modified the strength and limitations section in the Discussion and added a sentence on why we didn't use multivariable analysis.

** Campbell, Michael J., David. Machin, and Stephen J. Walters. Medical Statistics : a Textbook for the Health Sciences. 4th ed. Chichester: Wiley, 2007. Print. Page 195.*

Changes in the text: Page 10, lines 172-175, *A **univariable** Cox regression model was used to calculate hazard ratios (HRs) and **95%** confidence intervals (CIs) **for HSP90 status and other factors associated with** the risk of disease-specific death.*

Changes in the text: Page 13, lines 232-237, *Based on univari**able** Cox survival regression analysis, **high** HSP90 expression was associated with an increased risk of shorter survival (HR 6.4, 95% CI 1.3-31.8, P=0.023; **Table 2**). **In addition, AC subtype, metastatic disease and high Ki-67 labeling index were associated with an increased risk of disease-specific death (all P values < 0.05). Other potential risk factors, such as sex, age, or tumor size, were not associated with DSS.***

Changes in the text: Page 13, lines 227-230, *In addition, Kaplan–Meier survival analysis was performed between PC subtypes, metastasized and nonmetastasized disease**, and high and low Ki-67 labeling index** to demonstrate the effect of well-known indicators of poor prognosis on the outcome of patients with PC disease (Figure 4b-4d).*

Changes in the text: Page 16, lines 304-307, *A drawback of our study was the limited number of disease-specific deaths despite a relatively long follow-up. **Based on this low number of disease-specific deaths, a reliable multivariable analysis could not be performed, and a larger study is required to obtain more detailed results.***

Changes in the table: Table 2 "Univariable Cox regression analysis in PC tumor sample series" was added.

Changes in the Figure 4: Ki-67 proliferation index was added to the DSS curves.

Comment 4: The authors used the term "univariate" they should use the term "univariable" instead as the term "univariate" implies multiple outcomes.
Reply 4: We replaced words *univariate* with *univariable*.
Changes in the text: Page 13, line 232-234, *Based on univari**able** Cox survival regression analysis, **high** HSP90 expression was associated with an increased risk of shorter survival (HR 6.4, 95% CI 1.3-31.8, P=0.023; **Table 2**).*

Comment 5: The authors defined disease-specific death as "time elapsed between the day of the primary tumor surgery and either the end of follow-up or death, whichever came first." This is the definition of overall survival and not disease-specific survival. Disease-specific survival is defined as the time from surgery to the time of death from the disease (i.e., pulmonary carcinoid tumor). The authors also need to report the median follow-up period of patients. Survival curves should include the number of patients at risk for each group.
Reply 5: Thank you for the valuable comments. We had made a mistake here and modified the sentence accordingly. We had reported median follow-up time for patients who did not experience death but revised this to be the median follow-up time for all the patients. We also added the number of patients at risk for each group in survival curves.
Changes in the text: Page 10, lines 170-172, *The association of **HSP90** expression with disease-specific survival was determined using the time elapsed between the day of the primary tumor surgery and **the time of death from pulmonary carcinoid tumor**.*
Changes in the text: Page 12, lines 223-224, *The average follow-up time for **all** patients was **12.3 years (range 1.1–27.8 years, median 10.2 years).***
Changes in the Figure 4: The number of patients at risk for each group in survival curves were added.

Comment 6: Page 5, lines 174-176, the authors wrote "Cox regression model was used to calculate hazard ratios (HRs) and confidence intervals (CIs) to assess the effect of HSP90 protein expression on the risk of disease-specific death." Using the term "assess effect" implies causation. Regression analysis studies the association and terms implying causation should not be used. Please use "factors associated with" instead of "assess effect".
Reply 6: Thank you for the valuable comment. We modified the sentence accordingly.
Changes in the text: Page 10, lines 172-175, *A **univariable** Cox regression model was used to calculate hazard ratios (HRs) and **95%** confidence intervals (CIs) **for HSP90 status and other factors associated with** the risk of disease-specific death.*

Comment 7: It will be helpful to include the surgical procedure, and whether there was a positive margin at the time of resection or not, in Table 1 and also in the regression analysis, as those are known to affect the outcome.
Reply 7: We added the surgical procedure in the Table 1 but unfortunately we are not able to find out whether there was a positive margin or not. Seven different surgical procedures were used (which exceeds the number of disease-spesific deaths), so the

affect cannot be reliably evaluated by regression analysis. The surgical procedures for the six patients who died due to PC were 4 lobectomies, 1 bilobectomy and 1 pneumoectomy.
Changes in the text: No changes.
Changes in the table: Surgical procedures were added in Table 1.

Reviewer B

The manuscript "HSP90 expression is associated with outcome in pulmonary carcinoid tumor patients" by Jenni Niinimäki et al. investigates 128 patient biopsies to identify biomarkers for pulmonary carcinoid tumors using RNA sequencing and immunohistochemistry. The authors found that elevated HSP90 expression was associated with poor patient outcome and increased mortality. Therefore, the authors suggest that HSP90 expression could be used as a prognostic marker for pulmonary carcinoid tumors.

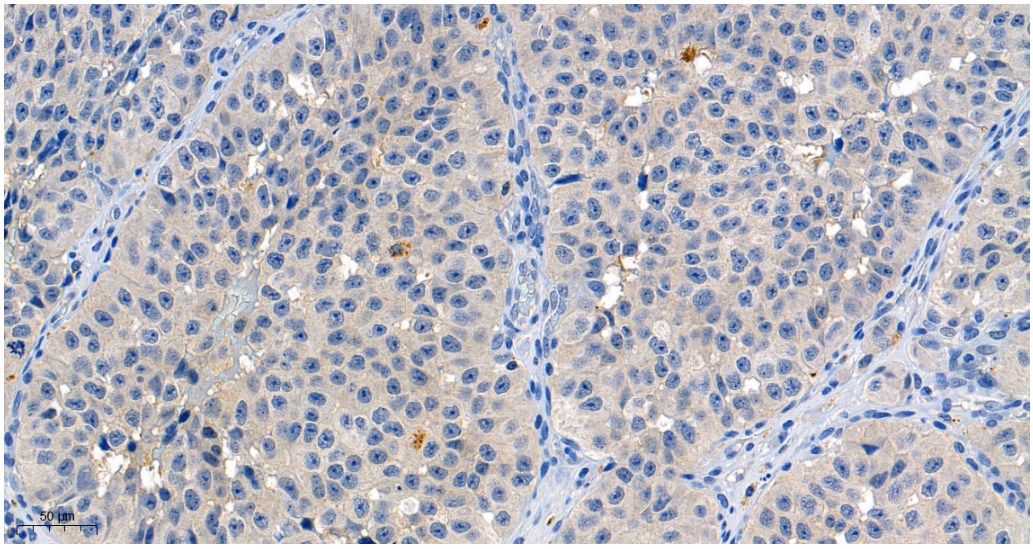
In general:
The paper is technically sound
The claims are convincing
The claims are on the whole supported by the experimental data presented
The manuscript is clearly written and the English is sufficed

The topic is interesting and the results could have clinical implications.

Some issues need to be addressed:

Comment 1: In addition to surgical removal, did the patients included in this study receive any adjuvant treatment regimen?
Reply 1. Yes, eleven of the patients with metastatic disease received also adjuvant treatment. The information was listed in Table 1, but it was presented per treatment, not per patient. We modified the Table 1 so that the information is now patient-specific.
Changes in the table: Table 1 was modified for adjuvant treatments.

Comment 2: The authors describe that the immunohistochemical expression analysis of HSP90 was not associated with Ki67 score. Was the Ki67 expression evaluated in the same biopsy? Please add additional information to Materials and methods.
Reply 2: Yes, Ki-67 PI was previously evaluated from the same TMA punches of the highest labeling region of at least 2000 cells with QuPath software. We added this information to the Results.
Changes in the text: Page 12, lines 216-218. *Moreover, HSP90 expression was not associated with tumor size, the Ki-67 proliferation index* ***(previously analyzed from the same TMA cores with the QuPath software (20))*** *or patient sex or age.*



Comment 3: Did the authors observe homogeneous expression of HSP90 in the tissue sections? Did they observe areas of lower expression or hot spots with elevated expression? If so, how were these inconsistencies scored? Did heterogenicity of HSP90 expression have impact on the prediction of the survival probabilities?
Reply 3: The tumor cells stained very homogeneously within one TMA spot and between parallel TMA spot from the same tumor. Please see a few more examples below. No hotspots or areas of lower expression were seen in the tumors.
Changes in the text: Page 12, lines 211-213. *Tumor samples were scored from 0 to 3 based on their staining intensity (Figure 2).* ***The staining patterns were highly homogeneous in the cytoplasm of the tumor cells.***

Comment 4: Figure 2: Add a size reference to the images.
Reply 4: We added the scale bars to the images.
Changes in the figure 2: Scale bars were added.

Comment 5: Figure 1: Please increase the text size.
Reply 5: We increased the text size as much as we could.
Changes in the figure 1: We increased the text size.

Reviewer C

Comment 1: In this article, the authors report that HSP90AB1 expression was elevated in metastatic pulmonary carcinoid tumors compared with non-metastatic tumors, and HSP90 protein expression was associated with poor outcome. However, elevation of HSP90 protein expression in metastatic tumors was not observed by IHC. This is inconsistent with mRNA expression results. As authors themselves pointed out, this may be due to non-specificity of antibody. As the specificity of antibody is doubted, the association of HSP90 protein expression and poor prognosis cannot be concluded.

Because this conclusion was one of the main conclusions of this article, the manuscript cannot be accepted.

Reply 1: Thank you for your comment. It is true that we could not see difference in immunohistochemical HSP90 expression between metastatic and nonmetastatic tumors. However, high HSP90 expression was associated with shorter disease-specific survival and typically patients, who die due to pulmonary carcinoid tumor, have a metastasized disease. Thus, we see these results as a strong indication to study HSP90 expression further in larger patients series.

Moreover, we intended not to question the specificity of the antibody, but rather to highlight the uncertainty of immunohistochemistry in general. In the manuscript, we present the results we obtained with this antibody clone, but perhaps the results might have slightly differed with another clone targeting other areas of HSP90 protein. Naturally, we would not present our results if we do not think they are reliable.

Changes in the text: Page 15, lines 277-279. *This may have been due to the **altered affinity of the primary antibody to the epitope as a result of** translational and posttranslational modifications.*

Reviewer D

This is a carefully designed study that explored genes and proteins related to Heat shock proteins (HSPs) in surgical specimens from patients with Pulmonary Neuroendocrine Tumors (Typical Carcinoides and Atypical Carcinoides). The writing is clear and concise allowing the reader to easily follow the text. The methodology is described in detail, highlighting the statistical analysis. The article is of interest to general readers.

Comment 1: However, my major concern is related to the evaluation of HSPs protein expression. Carefully examination of the panel in Figure 2, we noted that the staining pattern is homogeneously diffuse in the cytoplasm of tumor cells, perhaps suggesting that a score from zero to 4 may not be adequate to assess expression intensity. Hence the lack of statistical significance in HSP protein expression between metastatic and non-metastatic PNETs. Authors are suggested to use the scanned slides at a magnification of ×400 and submitted to an automatic staining vector analysis, followed by total tissue area detection, separation of tumor from non-tumor areas in each slide, and finally, automatic cellular detection. Then, a membrane algorithm to obtain the H-score of the HSP cytoplasmic staining. This algorithm consists of multiplying each staining membrane score, i.e., 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining), by the percentage of positive cell (0-100 %) at that intensity to reach a final H-score ranging between 0- 300. If the H-score was equal to or higher than the mean value of all samples, HSP protein expression was classified as positive, whereas an H-score lower than the mean value was classified as negative HSP protein expression.

Reply 1: Thank you for the valuable instructions and advice. We scored the tumor spots with intensity score 0-3 (negative, weak, moderate, strong, respectively) which we

found suitable for scoring since staining pattern was really homogenous as you noticed. We do not currently have access to automated image analysis tools which would analyze cytoplasmic protein expression. However, we will take this comment into account in our future works utilizing immunohistochemistry. We also discussed utilizing automated image analysis.

Changes in the text: Page 16, lines 297-304. *Our study can be considered representative since it comprised a large number of* ***well-characterized*** *PC tumor patients with up-to-date clinical follow-up and survival data. All tumors were re-evaluated by an expert pathologist according to the latest WHO classification.* ***With our TMA method, adequate amount of tumor tissue was stained using*** *a monoclonal antibody* ***and the staining pattern was highly homogeneous****.* ***Scoring was carefully performed by two independent, experienced researchers. Automated image analysis tools were not utilized in this series, but we acknowledge their value in analyzing series of hundreds of tumors.***