Author's response to reviews

Title: Decreased Hsp90 expression in the continuum of breast lobular lesions.

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Author's response to reviews: see over
To
The Editorial Board of *BMC Cancer*

Athens, 02/03/2010

Dear Editor,

I am sending for publication to your distinguished Journal the revised manuscript entitled: "Decreased Hsp90 expression in the continuum of breast lobular lesions". This is the first study to focus exclusively on the continuum of breast lobular lesions, going beyond lobular neoplasia (LN) and assessing Hsp90 expression in infiltrative lobular carcinomas (ILC). The fact that Hsp90 expression is lower on LN and ILC lesions is a finding contrary to what might have been expected, given that high Hsp90 expression is a trait of invasive ductal carcinomas.

We have made all the revisions indicated by your distinguished reviewers and have coloured them in RED throughout the manuscript. In the next page please find a point-to-point reply.

Yours sincerely,

George C. Zografos,
Professor of Surgery
University of Athens
"This paper addressed the HSP90 different expression among normal ducts and lobules, lobular neoplasia and infiltrative lobular carcinoma. The conclusions were potential interesting. The statistical methods used in the paper were enough. However, there were many studies about HSP90 expression in breast cancer. The authors only used immnohistochemical staining to verify their views and the methods were considerably simple."

"Secondly, this paper gave detail information about the patients and the results only assessed the different expression among normal ducts and lobules, lobular neoplasia and infiltrative lobular carcinoma"

-We would like to thank you for your constructive comments which have allowed substantial improvement to our manuscript. In light of this suggestion, the comment has been adopted and the variety of studies on breast cancer has been defined; nevertheless the scarcity of data specifically on lobular carcinomas (i.e., the subject of this study) has also been highlighted (Introduction, paragraph 2, last four lines). We believe that the incorporation of this proposal has added to the dialectic presentation of the study background to the scientific audience of the Journal.

"Thirdly, the authors did not thoroughly discuss the reasons that HSP90 expression in the nucleus of epithelial cells and if the counting results included the nucleus positive or not."

-This study has evaluated cytoplasmic staining for the calculation of Allred score. The significance of nuclear expression remains elusive, as some studies have not documented any nuclear Hsp90 expression in invasive ductal carcinomas (Pick et al, 2006), whereas other researchers (Diehl et al, 2009) have. In light of the above controversies, a detailed comment has been added in the Discussion; likewise the fact that the nuclear expression was scarce (i.e., <5%) and thus not included in the calculation of Allred score has been noted. (Discussion, paragraph 5).

"Finally, in the methods part, the paper did not address how many fields used in one section."

- We would like to thank you for the comment, which allowed us to provide details on the immunohistochemical examination. Ten fields x40 magnification were evaluated.

"Anyway, the paper was not fit to publish in BMC cancer.
Level of interest: An article of insufficient interest to warrant publication in a scientific/medical journal."

- We would like to thank you for the comment, although we disagree. We believe that the novel finding, i.e. reduced expression of Hsp90 in infiltrative lobular carcinoma, as well as its potential clinical implications (such as the modification of the efficacy of the Hsp90 inhibitors), adds to the value of the manuscript and may thus render it publishable.

"Quality of written English: Needs some language corrections before being published. Statistical review: Yes, and I have assessed the statistics in my report."

- English has been revised by an English speaking person.
Referee 2
Reviewer Wolfgang J Köstler

“In their manuscript, Zagouri et al expand their previous observation (Zagouri F et al BMC Cancer. 2008 Oct 28;8:312) on decreased expression of Hsp90 in non-invasive lobular neoplasias of the breast to a somewhat larger cohort. Moreover, unlike their previous paper, the current report also includes invasive lobular carcinomas. The authors demonstrate a modest, but progressive decrease of Hsp90 expression en route to malignancy. Their findings form a relevant basis for analyzing the clinical efficacy of Hsp90 inhibitors according to histological subtype.”

- We would like to thank you for the encouraging comments on our manuscript, as well as for the time spent on revising our manuscript. Following your comments, we believe that the revised version of the manuscript has been substantially improved.

“Major compulsory revisions: Due to universal ER expression in non-invasive lesions the authors did not observe any interaction between ER and Hsp90 expression in their last report. I would be most interesting to know correlation between Hsp90 expression and ERalpha and HER2 status - at least in the subset of patients with ILC.”

- We would like to thank you for this insightful comment which allowed us to perform additional analyses in our dataset. The correlations between Hsp90, ER-alpha and HER2 have been added in the subset of ILC. No significant associations were demonstrated (Results, paragraph 3). A Table containing the features of ILC has been added, so as to provide the audience with all the necessary details (Table 3).

“Minor essential revisions: In tables 1&2, LN samples should be divided into ALH and LCIS cases and comparisons made accordingly.”

- We would like to thank you for this comment. Unfortunately, separate data were not available for ALH and LCIS, as they had both been categorized as “lobular neoplasia”, according to the most recent WHO classification. As suggested, a comment has been added to the Limitations paragraph (Discussion, last paragraph, last four lines). Please also keep in mind that data on Table 2 have been divided into LN and ILC, according to reviewer #4, comment #5.

“Discretionary revisions: On page 3, consider adding a citation to your previous work.”

- As requested, the citation to our previous work has been added on page 3.

“Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.”
Referee 3
Reviewer Gulisa Turashvili
- We would like to thank the reviewer for the encouraging comments as well as for the time spent on revising our manuscript. We certainly agree with the revisions indicated, all of which we have incorporated in the final version of the manuscript.

“- Major Compulsory Revisions
Material and Methods:
1. Specify the host (mouse) for anti-Hsp90 monoclonal antibody.”
- In the revised version, the host (mouse) has been added (Methods, paragraph 1, line 1).

“2. Specify the dilution and incubation time for the primary antibody.”
- In the revised version, the dilution (1:500) and incubation time (18-24 h) has been added (Methods, paragraph 1, line 3).

“3. Specify which avidin-biotin detection system was used.”
- In the revised version, the avidin-biotin detection system (Envision, Dako) has been added (Methods, paragraph 1, line 4).

“4. Correct the duration (18h) of antigen retrieval.”
- The duration has been corrected (15 min); we would like to apologize for the typing error pertaining to incubation time.

“5. Give more information about the Allred score. Describe in detail how it was calculated. Did the authors use Allred score 0 to 8? How did they interpret these scores? Give the table describing the scoring procedure.”
- We would like to thank you for this comment which will provide the audience with more profound understanding of the score used in this study. Table 1 contains all the details for the calculation of the Allred score.

“6. Was Allred score calculated based on the cells with cytoplasmic expression only?”
- We would like to thank you for this comment which allowed us to provide details on the immunohistochemical algorithm of our study. This study has exclusively evaluated cytoplasmic staining for the calculation of Allred score; this has been included in the revised version (Discussion, paragraph 5).

“7. What was the interobserver variability? Did the authors run kappa statistic?”
- We would like to thank you for this comment which allowed us to present the background issues behind our methodology. Kappa statistic has been performed and the results are provided in the Results section (paragraph 2)

“Results:
1. What was the proportion of cells with nuclear Hsp90 expression?”
- We would like to thank you for this comment. Nuclear expression was scarce (i.e., <5%); as a result, nuclear staining has not been included in the calculation of Allred score. In any event, the significance of nuclear expression remains elusive, as some studies have not documented any nuclear Hsp90 expression in invasive ductal carcinomas (Pick et al, 2006), as opposed to other researchers (Diehl et al, 2009). In
light of the above controversies, a detailed comment has been added in the Discussion (Discussion, paragraph 5).

“2. Results section is not very extensive, so add the data on interobserver variability including kappa statistic.
-We would like to thank you for this comment which allowed us to present the background issues behind our methodology. Kappa statistic has been performed and the results are provided in the Results section (paragraph 2)

“Figures
1. Fig. 1 shows normal TDLU with strong Hsp90 immunostaining.”
- Legend on figure 1 has been appropriately corrected.

“2. Combine figures 2-4 (LN) and 6-7 (ILC) into one.”
- New, informative, combined figures highlighting the study findings have been added as requested (please see comment below).

“Discussion:
1. Normal TDLU and two of the three ILCs show strong Hsp90 expression, so it is unclear how Hsp90 expression is ‘significantly decreased’ in ILC in comparison to adjacent normal breast tissue.”
- New, informative, combined figures highlighting the study findings have been added as requested.

“2. Any speculation regarding any association of Hsp90 expression with HER2 status would require HER2 data for these ILC patients and subsequent statistical analysis. HER2, ER and PR status of cancer patients should be reported and their association with Hsp90 expression should be analysed.”
- We would like to thank you for this insightful comment which allowed us to perform additional analyses in our dataset. The correlations between Hsp90, ER-alpha and HER2 have been added in the subset of ILC. No significant associations were demonstrated (Results, paragraph 3). A Table containing the features of ILC has been added, so as to provide the audience with all the necessary details (Table 3).

“3. The comment regarding proliferation advantage is unclear. Do the authors have Ki-67 data?”
- We would like to thank you for this valuable comment, which allowed us to perform analysis of Ki-67 data which have been available for all ILC cases. Ki-67 and Hsp90 expression were not associated with each other; as a result, the hypothesis that the proliferation advantage in ILC is Hsp90-independent seems further supported.

“4. If Hsp90 expression is decreased in LN and ILC, Hsp90 targeting drugs are unlikely to be effective in ILC.”
- We would like to thank you for this remark. This issue has been clarified, as the statement “effectiveness may vary accordingly” has been replaced by “effectiveness may be accordingly limited” (Discussion, paragraph 4, last line).

“5. When discussing ‘larger sample’, do the authors mean the size of the primary tumor or small number of patients (if so, use ‘larger sample size’)?”
We would like to thank you for this remark. The term “larger sample size” has been adopted, as appropriate (Discussion, paragraph 6, line 4).

“6. Automated quantitative procedure is unlikely to be superior to the scoring performed by two qualified pathologists.”
-Given this insightful remark, the statement has been deleted in the revised manuscript.

“7. By ‘less sensitive assessment’, do the authors mean IHC procedure as such or visual scoring?”
-A parenthesis clarifying this sentence has been added, as we refer both to IHC procedure and visual scoring (Discussion, last paragraph, line 4).

“8. ‘The results need to be confirmed by other methods apart from immunohistochrometry’. What other methods do the authors refer to?”
-We would like to thank you for this clarification which may indeed provide stimulus for future studies in our setting, as well as in other settings. One example of a useful method may be Western blot; this suggestion has been clarified in the manuscript. (Discussion, last paragraph, line 8)

“9. Conclusion: ‘Hsp90 expression is lower on LN and ILC lesions’ – do the authors compare LN and ILC lesions to normal TDLUs? If so, please specify.”
-As declared in the Methods section, LN and ILC lesions have been compared to the adjacent normal breast ducts and lobules (please refer to Methods, paragraph 2, line 7). The relevant statement has been added in the conclusion.

“10. As Hsp90 expression is reduced in ILC and LN, it is unclear why ‘Hsp90 deregulation does not seem to be a major event in lobular carcinogenesis’.”
-Following this thoughtful remark, this statement has been eliminated in the revised Conclusion.

“- Minor Essential Revisions
1. Neolplasia is misspelled in Abstract.”
- This typing error has been corrected.

“2. Include abbreviation - TDLU (terminal duct lobular unit).”
-As requested the abbreviation has been adopted.

“- Discretionary Revisions: None.
Level of interest: An article whose findings are important to those with closely related research interests.
Quality of written English: Needs some language corrections before being published.
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.”
-We would like to thank you for the encouraging and enlightening comments. English has been revised by a native speaking English
Referee 4  
Reviewer: Matthew H. Herynk

“The manuscript entitled “Decreased Hsp90 expression in the continuum of breast lobular lesions” by Zagouri et al describes immunohistochemical evaluation of Hsp90 in lobular neoplasias and infiltrating lobular carcinomas. The authors find reduced Hsp90 in these lesions when compared with normal adjacent tissue. The work is intriguing and may have important clinical implications for the use of Hsp90 inhibitors in breast cancer.”

-We would like to thank you for the encouraging comments regarding the content and the clinical impact of our study. A set of changes have been made according to your comments and we believe that substantial improvement has been accordingly made to the manuscript.

Major Compulsory Revisions

“1) The authors acknowledge the small sample size and its’ limitations in producing statistical significance. A larger cohort, confirmation through additional methods (as listed in the discussion), or a few in vitro mechanistic studies would allow the authors to make definitive conclusions thereby significantly enhancing the impact of this manuscript. (See related comment #2)”

-Acknowledging the major importance of this comment and adopting its point of view, a relevant statement has been added in the Discussion, i.e. “in vitro mechanistic studies seem indispensable for definitive conclusions”. Moreover, the inability to perform such studies in the clinical setting, in which this study was performed, is noted (Discussion, paragraph 7, lines 8-11).

“2) Previously, the same group has reported reduced Hsp90 in lobular neoplasias in 44 patient specimens (BMC Cancer 2008, 8:312). This current manuscript reports 65 patients with lobular neoplasia with similar staining percentages and intensities as previously reported. Are these 65 new patient samples or the same 44 plus an additional 21? Please describe in the text.

a. If these are an additional 21 (for a 50% increase in sample size), this small increase in the sample size does not warrant publication.

b. If this is 65 new cases (for a 150% increase in sample size), the authors should consider combining both cohorts (if the staining and quantitation methods allow) to increase the power of the study.”

-We would like to thank you for this important comment which allowed us to provide further details about our study sample. Indeed, among the included cases, 44 had been analyzed in our previous publication; this has been declared in the text, as requested (Methods, paragraph 1, lines 6-7).

Please note that the increase in the total sample size is not equal to 50%, as this study has additionally included the ILC cases, reaching a sample size equal to 97. Indeed, the net increase in sample size is equal to (97-44)/44=120%.

We believe that this study merits consideration for publication, as it expands the finding upon ILC cases, which had not been included in the previous publication. The inclusion of LN cases serves mainly as a secondary finding in the present publication.

“3) The LN data does not demonstrate statistical significance as analyzed and the three pictures demonstrate a broad range of staining. Analyses of the data
comparing differential levels of staining within the group may provide additional insight.”

-Please refer to the comment below, as each lesion was evaluated as a whole. We would like to apologize for the inadvertent statement regarding any preselected areas. We declare that no preselection of areas has been performed. As a result, no differential effects within each lesion may be detected.

“4) The authors state “the area of maximum staining intensity was preselected...”.
Please add the percentages of high, medium, and low expression. It has been reported that ductal carcinoma shows increased Hsp90 expression and herein lobular carcinomas show reduced Hsp90 expression, it may be pertinent to quantitate the lowest levels of expression rather than the highest levels as these will be the areas that deviate most from normal.”

-We would like to thank you for this insightful remark. Please note that the statement concerning the preselection of maximum intensity had been inadvertently included in the manuscript. Your comment allowed us to clarify that the score was based on the lesion as a whole (10 x 40 fields) (Methods, paragraph 3, lines 3-5).

Minor Essential Revisions
5) Separate the patient characteristics by LN and ILC.
-We would like to thank you for this comment which allowed us to present further details of our dataset. The patient characteristics have been separated by LN and ILC, as indicated (Table 2).

6) Please provide a dilution factor for the antibody used.
- In the revised version, the dilution factor (1:500) has been provided (Methods, paragraph 1, line 3).

7) Are the numbers listed standard error or standard deviation?
- In the revised version, this issue has been clarified; regarding Allred score and Ki-67 percentage, all numbers were provided as mean±SD (Methods, paragraph 4, lines 2-3)

Discretionary Revisions
8) Figures
a. It would be easier to view the figures as grouped composites, i.e.normals (Figure 1), LN (Figure 2A-C), and ILC (figure 3A-C).
b. As the quantitation of normals exhibits a similar standard deviation as the LNs, and the LN pictures demonstrate different levels of staining, please provide multiple representative images for comparison
- New, informative, grouped figures highlighting the study findings have been added as requested.

“Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.”