Author's response to reviews

Title: Mammaglobin B expression is an independent prognostic marker for reduced risk of recurrence in epithelial ovarian cancer

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Author's response to reviews: see over
Dear Dr. Norton,

Thank you for consideration of above manuscript. Enclosed is our response to the Reviewers’ comments.

Reviewer: Angele Oei

1. It is well known that endometrioid ovarian neoplasm have better survival prognosis as compared to serous epithelial ovarian neoplasms! Isn't it logical to find correlation with improved disease free / overall survival when human mammaglobin B is strongly correlated with endometrioid ovarian cancers? This should be further declared or noted in the discussion

We agree with the reviewer that ovarian cancer histology is an important prognostic factor. Consistent with this view, patients harboring ovarian endometrioid tumors are known, in general, to have a better outcome when compared to those developing ovarian cancer with other histology. However, several studies have previously demonstrated that the prognostic value of ovarian cancer histology is mainly dependent on the stage distribution of the ovarian cancer patients. Indeed, when compared stage by stage and grade by grade the prognostic variable “histotype” loses its significance, see for example: Heintz AP, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, Ngan HY, Pecorelli S, Beller U. Carcinoma of the ovary. FIGO 6th Annual Report on the Results of Treatment in Gynecological Cancer. Int J Gynaecol Obstet. 2006 Nov;95 Suppl 1:S161-92; Malkasian GD Jr, Melton LJ 3rd, O’Brien PC, Greene MH. Prognostic significance of histologic classification and grading of epithelial malignancies of the ovary. Am J Obstet Gynecol. 1984 Jun 1;149(3):274-84.

Consistent with these findings, our statistical analysis didn’t show any significant interaction between MGB-2 expression status and the different ovarian cancer histology (i.e., endometrioid, serous, and others) in the multivariate analysis for DFS, PFS or OS. This has been further clarified in the discussion section on page 20, line 11 by the following sentence: “It is noteworthy that MGB-2, although showing higher expression in ovarian cancer with endometrioid histology when compared to other histologic types, maintained its independent prognostic value for DFS in the multivariate analysis”.

May 29, 2009

Dr. Melissa Norton, MD
Editor in Chief
BMC Cancer

Re: MS#: 1316393508252807, entitled: “Prognostic significance of human Mammaglobin B expression in epithelial ovarian cancer” by Renata A Tassi, Stefano Calza, Antonella Ravaggi, Eliana Bignotti, Franco E Odicino, Germana Tognon, Carla Donzelli, Marcella Falchetti, Elisa Rossi, Paola Todeschini, Chiara Romani, Elisabetta Bandiera, Laura Zanotti, Sergio Pecorelli and Alessandro D Santin
2. On page 7 it is stated that only 98 tumor samples contained at least 70% neoplastic epithelial cells. What happened with the other 8 ovarian tumor tissues and why were they excluded with less than 70% neoplastic epithelial cells? Please explain.

_Eight samples with less than 70% neoplastic epithelial cells were excluded from RNA extraction due to their high contamination with stromal cells (i.e., higher than 30%). On page 7, line 17 the sentence has been revised to better clarify this point._

3. On page 9 also omental metastases were included for total RNA extraction, why not the primary ovarian tissue?

_For those 9 patients, primary fresh frozen ovarian tissue (unlike their metastatic tissue) was not available in our tissue bank._

4. In the discussion it should be mentioned that by dividing the different histopathological groups, the numbers became small and results should be interpreted with caution. (max no of patients per group is 54 most groups < 20 pts)

_In agreement with the reviewer’s suggestion, a sentence stating: “Although histological subgroups were somehow limited in their sample size.....”, has been added on page 1, line 7 of the Discussion. Indeed, for the reason mentioned by the reviewer, in our survival analysis ovarian cancer patients were not stratified by histology (Table 4). To emphasize further this point, on pages 21, last line, in the Conclusion section, we have also declared that: “Further studies on a larger patients’ cohort are warranted to validate the prognostic impact of MGB-2 expression on survival”._

5. In table 4 and the result section; the presence of ascites and involvement of lymph nodes had a significant impact on DFS, OS, PFS. Isn't this logical at it is generally known that FIGO staged matters and these component; positive lymph nodes and ascites are contained in FIGO stage? If the author wishes to leave this data unchanged a comment on this issue should be stated in the discussion section.

_Although we agree with the reviewer that the F.I.G.O. stage already includes the presence of both ascites and positive lymph nodes, we believe the presence or absence of these single parameters may be important to potentially explain the different biology of ovarian tumors presenting with a similar stage and may also significantly affect their management. As requested, a comment on this issue has been introduced in the discussion (page 19, line 16): “In this regard, although FIGO stage includes lymph nodal status and positive cytology, these parameters have been analyzed separately because of their ascertained clinical prognostic relevance [3]”. _

6. With regards to the statistics I do think the ROC and univariate and multivariate analysis are the right approach. However I am still not a statistician.

_No response is necessary on this point._

7. The discussion should include a section where is discussed how to optimally measure mammaglobulin B in ovarian cancer patients (HOSE, PCR?) with regard to the results of this paper.
The discussion now includes a paragraph specifically addressing the reviewer’s concern. Indeed, on page 20, line 16 of the Discussion (second paragraph) we have now clearly stated that PCR is a more accurate technique than immunohistochemistry for MGB-2 signal quantification. Furthermore, a comment on the potential limitations in the use of this laboratory technique in the clinical setting has been also added (Discussion page 20, line 19).

8. An earlier study should be discussed (ref18). These authors were unable to find a significant difference between the mammaglobulin B expression between the different histopathology. Why did the authors of this paper did find a difference?

We believe the most reasonable explanation for this difference in results is the “higher statistical power” of our current study when compared to the previous one (i.e., the number of samples evaluated in the new study was larger (i.e., 106 samples) than the one analyzed in the previous study (i.e., 68 samples). As requested, a statement explaining this point has been introduced in the Discussion section, (page 19, line 9), in the paragraph entitled: “Association between MGB-2 expression and clinicopathological features”.

**Reviewer:** Menelaos Zafrakas

**Reviewer's report:**

Discretionary Revisions (which are recommendations for improvement but which the author can choose to ignore)

1. Pages 14-15: There is only one paragraph under the subheading “Expression patterns of Mammaglobin B protein in normal ovary and in epithelial ovarian cancer by immunohistochemistry”. This section could be divided in at least two paragraphs (e.g. the 2nd paragraph could start from “Finally…” in page 15, line 15).

The paragraph on page 14 has been split following the reviewer’s request and a second subheading had been introduced in the text on page 15, line 5: “Relationship between MGB-2 protein expression and tumor histology”.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1. Page 3-line 19, page 16-lines 3 and 22, and page 17-line 23: “mRNA level” is more accurate for most readers than “gene level”.

“Gene level” has been converted in “mRNA level” as requested throughout the manuscript.

2. Page 6-line 20: “FIGO” stands for “International Federation of Obstetrics and Gynaecology” (in French) – the word “International” is missing from the text.

The word “International” has been now included in the text.
3. Page 7-line 2: Please change “did not received” to “did not receive”.

_The sentence has been corrected._

4. Page 7-line 7: “expired” could be changed to a more formal verb (e.g. died or succumbed).

_The verb “expired” has been changed to “died of”._

5. The authors should provide all details concerning manufacturers of reagents and equipment used, i.e. city and country of origin (e.g. page 9, line 8 etc.)

_Since the same manufacturers are cited multiple times in the text, their details have been reported once._

6. The Discussion section should be divided in paragraphs.

_According to the reviewer’s suggestion, the discussion has been divided in two paragraphs, each section is preceded by a subheading._

7. Page 21-last line: Please change “design” to “designed”.

_The word has been corrected._

Major Compulsory Revisions (which the author must respond to before a decision on publication can be reached)

1. Although this is a well-written, original study with very interesting findings, the title does not convey these new findings.

_Following the reviewer’s suggestion the title of the manuscript has been changed and now reads: “Mammaglobin B is an independent prognostic marker for reduced risk of recurrence in epithelial ovarian cancer”._

2. The authors state that “this study was performed on 106 consecutive cases of epithelial ovarian cancer” (page 6). Did the authors include any of the cases included in their previous publication in Gynecol Oncol (Tassi RA, Bignotti E, Rossi E, Falchetti M, Donzelli C, Calza S, Ravaggi A, Bandiera E, Pecorelli S, Santin AD. Overexpression of mammaglobin B in epithelial ovarian carcinomas. Gynecol Oncol 2007;105: 578-585)? In this previous paper, the authors analysed Mammaglobin B expression in ovarian cancer and concluded that evaluation of the clinical utility of Mammaglobin B in future studies is warranted. This has been done now, as described in the present paper. However, the authors should clearly state whether previously published data were in part used in order to generate new results for the present study or not.

_On page 5, the last paragraph of the Background section has been revised to clarify this point and now reads: ... “in the present study we extended our molecular and immunohistochemical MGB-2 expression findings previously reported in ovarian cancer [18] and analyzed MGB-2 in a larger cohort of clinically well characterized EOC patients”._
3. Pages 9-10: The authors should provide the primer sequences for Mammaglobin B and GAPDH.

*The primer sequences for Mammaglobin B and GAPDH used in our study were obtained as “Assays on Demand” from Applied Biosystems (Applied Biosystems, Applera UK, Cheshire, UK). Although the sequences are commercially available, they are considered proprietary information by Applied Biosystems and are not provided to customers.*

4. Page 11-lines 20-23: Why did the authors divide immunohistochemical results in two groups, i.e. negative and positive (with positive including weak, moderate and strong immunoreactivity)? One could argue that negative and weak staining should be grouped together, while moderate and strong immunostaining should comprise a second group or that the four distinct categories of immunoreactivity should be used individually for statistical analysis. Would the results and conclusions be the same?

*In our study we divided immunohistochemical results for statistical analysis on the basis of presence or absence of Mammaglobin B expression in ovarian tumor cells. This is because our working hypothesis was to evaluate whether ovarian carcinoma characterized by Mammaglobin B expression (i.e., 1+ 2+ and 3+ tumors) may differ in their biologic behaviour/aggressiveness when compared to similar stage ovarian cancers found negative for Mammaglobin B expression (i.e., 0). Our statistical results support this hypothesis.*

5. Page 19-lines 12-15: The authors argue that MGB-2 “…could be an attractive diagnostic candidate and prognostic biomarker for ovarian malignancy [18], similarly to its homologous Mammaglobin A (MGB-1) for breast cancer [30]”. In the next two sentences, the authors go on discussing the possible clinical utility of MGB-1, based on previously published studies [citations 29, and 31-40].” However, in another paper [Zafrakas et al. BMC Cancer 2006; 6:88], published later than those cited by the authors, MGB-1 was not found to be as breast-specific as previously thought, and thus its utility as a diagnostic marker in breast cancer appears to have certain limitations. Moreover, in the same paper MGB-1 was found to be expressed in gynaecological malignancies, including ovarian cancer. Since the authors chose to discuss the role of MGB-1 in this extent, these findings should have been also included.

*Sensitive to the reviewer’s critique and because our current report focused exclusively on the prognostic relevance of MGB-2 expression in epithelial ovarian cancer without examining its diagnostic potential, we have revised the discussion and deleted comments and references about the diagnostic utility of its omologous MGB-1. Specifically, the following paragraph has been deleted “ MGB-1 is indeed considered as a sensitive molecular detection marker for axillary lymph node micrometastases as well as occult breast cancer cells by multimarker real-time RT-PCR assay. Accordingly, multiple citations supporting this point have also been removed from the Reference section.*

6. Page 20-lines 17-21: The authors mention “… a new intra-operative molecular assay that qualitatively detects the gene expression of two breast cancer markers (Mammaglobin A and Cytokeratin-19)…”. This development (concerning another
type of cancer, other molecular markers, and sentinel node biopsy, a technique not used in ovarian cancer) is not relevant to the findings of the present study, and it should be omitted.

Following the reviewer’s request the statement in question has been deleted from the discussion and references 44-45 have been removed from the manuscript.

7. Page 20-lines 21-22: The authors state that they “…don’t have any explanation for the biological mechanism linking MGB-2 expression with reduced risk of recurrence…” Which could be the possible biological explanations? Are there any hypotheses on this issue? What should be done in the future in order to clarify these biological mechanisms?

Following the reviewer’s recommendation, a paragraph discussing the limited information available on MGB-2 and other secretoglobin family members, including their potential function and regulation in cancer has been introduced in the Discussion section (page 20, line 23).

8. The authors should clearly discuss the limitations of this study.

We believe the main limitation of this study is represented by the small sample size for the evaluation of MGB-2 prognostic value in survival analysis. Multiple sentences emphasizing this point have been introduced in the discussion of the manuscript (see also our response to the first reviewer above). Another potential limitation of our study is the fact that the most sensitive method used for the molecular determination of MGB-2 expression level was found to be RT-PCR. Cancer marker detection by real-time PCR technique is not currently performed in routine clinical practice, whereas immunochemistry has current practical application in ovarian pathology. A statement regarding this second potential limitation is also now present in the discussion section of the paper (page 20, line 16).

Reviewer: Christer Borgfeldt
Reviewer's report:

Was lymph node dissection performed in all cases of stage I disease?

Yes, it was.

Early ovarian cancer is in general stage I disease not stage II.

In ovarian cancer stages I and II are commonly defined “early stage” disease while stages III and IV are defined as “advanced stage” disease, see for example: Heintz AP, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, Ngan HY, Pecorelli S, Beller U. Carcinoma of the ovary. FIGO 6th Annual Report on the Results of Treatment in Gynecological Cancer. Int J Gynaecol Obstet. 2006 Nov;95 Suppl 1:S161-92). In the present study we adopted an even more stringent definition including stage IIC in the “advanced” tumor subgroup since they are characterized by the presence of ascites or positive peritoneal cytology, both of which are considered indicators of a worse prognosis.
Were all immuno-histochemically stained slides stained in the same batch?

*No, they were not stained in the same batch. However, positive and negative controls were added to each experiment and the same primary and secondary reagents were used.*

The endpoints cancer relapse and cancer progression are in general considered the same endpoints since relapse of ovarian cancer is not a curable disease. Use just one of these endpoints!

*Cancer relapse and cancer progression are not the same, as defined by Ovarian Cancer Consensus Conference of 2004. Therefore, we did not use these two endpoints as synonymous, since the meaning is different: (i.e., relapse means the reappearance of cancer following a period of disease free, while progression means the progression of a disease already present).*

How often were the patients scheduled for follow up?

*Ovarian cancer patients are usually clinically evaluated every three months for the first two years after the completion of their adjuvant treatment.*

Was increased value of CA 125 used as marker of progression?

*Yes it was. We commonly use CA 125 as a marker of progression, particularly in patients found to have an elevated level of CA 125 at the time of diagnosis.*

The CA 125 Threshold = 200 U/ml for pre-menopausal patients & Threshold = 35 U/ml for post-menopausal patients. Why not the median value of CA125?

*We referred to a commonly adopted clinical criterion (see response below) instead of adopting a median value not useful for our research purposes.*

Why a CA 125 threshold 200 U/ml for pre-menopausal patients?

*In pre-menopausal women, elevated levels of CA 125 are associated with a variety of common benign conditions. We therefore adopted a CA 125 threshold of 200 U/ml following The American College of Obstetricians and Gynecologists recommendation for the evaluation of pre-menopausal patients with a pelvic mass that is suspicious for a malignant ovarian neoplasm (Committee on Gynecologic Practice, Society of Gynecologic Oncologists. ACOG Committee Opinion Number 280: The Role of the Generalist Obstetrician-Gynecologist in the Early Detection of Ovarian Cancer. Obstetrics and Gynecology, 100(6), 2002, 1413-1416).*

To evaluate the effect of MGB-2 mRNA expression on prognosis and not choosing the median value imposes going “fishing” for the best value. Use the median value!

*There is no reason, neither statistical nor biological, for assuming that the median value is the best choice, or even more strictly, the “right choice” when categorizing a continuous variable. Generally speaking “categorization” leads to some loss of*
information: the fewer levels we choose, the more information we might lose. Clearly in survival analysis we don't want to compare survival curves for continuous values. As for the median, any quantile (tertiles, quartile, etc.) is an acceptable choice as the categorization is performed without using any information related to the output variable we are interested in (the survival here). If the null hypothesis is true (no relation between survival and the marker) we don't expect a difference among groups, despite which splitting we have employed. Clearly a choice must be taken, basically depending on a trade off between simplicity (too many groups won't be easily interpreted) and sample size (we need enough events within a group) and on the other side information (too little categories might produce information loss). Similarly, from a clinical point of view, we wanted to compare groups as more extreme as possible.

In multivariate models the number of events should be at least 10 for each included parameter. In this paper the events analysing overall survival were 37 and the included parameters in the multivariate model were eight.

We agree with the reviewer on the commonly used rule of thumb of 10 events for estimated parameter. On the other hand we would like to stress that we are not actually interested in estimating and testing all the parameters, but rather only in MGB2 effect “corrected” for all the other known prognostic variables. The main issue suggesting having “enough” events is related to variance estimation and therefore confidence levels. We are aware that having relatively few events compared to factor levels might produce unstable estimates and therefore wider confidence intervals. On the other hand, our purpose was to evaluate the effect of the proposed marker, given the effect (whether significant or not) of known clinical factors. Finally, we stressed the presence of a selected model where we accounted for model fit and the number of parameters, through AIC criterion, delivering a more parsimonious model. Both were basically consistent.

In the figures the number of events should be expressed and the patients at risk showed at the marked time points (months).

*Figure 2 has been revised following the reviewer’s recommendation (new figure 2).*

It would be very interesting to have some thoughts about the explanation for the biological mechanism linking high MGB-2 expression to reduced risk of recurrence.

*According to the reviewer’s request, a new paragraph has been introduced in the Discussion regarding this point (page 20, line 23).*

We believe that the comments of the Reviewers have significantly strengthened the paper and we hope that the revised manuscript is now acceptable for publication in BMC Cancer. As requested, the revised manuscript has been copyedited to improve the style of written English.

Please send all proofs and correspondence to the following:
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Thank for your consideration

Sincerely,

Renata Alessandra Tassi PhD