Author’s response to reviews

Title: Identification of differentially expressed genes using annealing control primer system in stage III serous ovarian carcinoma

Authors:

Yun-Sook Kim (drsook@schch.co.kr)
Jin Hwan Do (jinhwan.do@gmail.com)
Sumi Bae (ahnlab4@catholic.ac.kr)
Dong-Han Bae (dhbae@schch.co.kr)
Woong Shick Ahn (ahnlab1@catholic.ac.kr)

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Author’s response to reviews: see over
Dear Editor,

The revised manuscript entitled “Identification of differentially expressed genes using an annealing control primer system in stage III serous ovarian carcinoma” is attached for your kind consideration of its suitability for publication in BMC cancer as a research paper.

The manuscript has been revised according to the recommendations of the reviewers. A point by point response is given in the ‘Response to Reviewers’ section below. We hope the paper is now ready for publication.

Very sincerely yours,

Professor Woong Shick Ahn
Department of Obstetrics and Gynecology,
The Catholic University of Korea,
505 Banpodong, Seocho-ku,
Seoul, Korea, 137-040
E-mail: ahnlab1@catholic.ac.kr
Response to Reviewers

Comments from Editor

Unfortunately, some serious issues have been highlighted by the expert referees for this submission regarding validation of the findings and the comparator control groups for the analysis. However, it is clear that the methodological approach described (annealing control primer-based PCR) is state of the art and critically its application here has been to a poorly-understood, aggressive cancer type. As such, I would encourage the authors to respond to the criticisms and formulate an approach to be able to address key points raised by the reviewers in a timely manner so that the article can be re-considered.

Other comments:

Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm), and any experimental research on animals must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.

Informed consent must also be documented.

We modified a sentence in “Patient information” of the Methods section as below:

Original: After obtaining informed consent and performing surgery at The Catholic Medical University (Seoul, Korea), samples of primary epithelial ovarian cancer were snap frozen in liquid nitrogen and stored at -80°C.

Modified: After obtaining written informed consent from all patients included in the study, samples of primary epithelial ovarian cancer were snap frozen in liquid nitrogen and stored at -80°C. Analysis of tissues from patients was approved by the Institutional Review Board of The Catholic University of Korea (Seoul, Korea).
Reviewer's report #1

Title: Identification of differentially expressed genes using an annealing control primer system in stage III serous ovarian carcinoma

Version: 1 Date: 29 June 2010
Reviewer: Gary Edward Gallick

Reviewer's report:

The manuscript of Kim et al uses annealing control primer-based PCR to identify differentially expressed genes in stage 3 serous ovarian cancer tissues compared to normal tissue. This is a robust technique that eliminates many of the past problems associated with other strategies. The approach is promising and the authors’ use of the genes to examine survival analysis and survival analysis associated with chemoresistance. The results are promising. The problem this reviewer has with the work is lack of validation. Specifically major compulsory revisions should be:

(1) Several identified changes should be confirmed by Q-rt-PCR

: Thanks. We have confirmed the results of the annealing control primer (ACP)-based PCR by quantitative real-time PCR for 38 differentially expressed genes (DEGs) out of 114 DEGs. Please note “Confirmation of ACP observation by quantitative real-time PCR and cluster analysis” in the Methods section.

(2) For those changes, protein levels should be examined.

: Thanks for your keen point. As you know, our goal is to identify DEGs between normal ovary and ovary with serous cancer stage III by ACP-based PCR. The total RNA of normal ovary is commercially available, but it is very difficult to obtain normal ovary tissue from which total proteins can be isolated. That is the reason that we could not carry out comparative analysis in protein level.

Resolution of these issues would make this work a good contribution in understanding genes important to ovarian cancer progression and/ or genes that may be prognostic or predictive markers.
Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published. English was improved by a native English speaking-scientist.

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare I have no competing interests
**Reviewer's report #2**

**Title:** Identification of differentially expressed genes using an annealing control primer system in stage III serous ovarian carcinoma

**Version:** 1  **Date:** 8 July 2010

**Reviewer:** Jeremy Chien

**Reviewer's report:**

Manuscript Summary: In this manuscript, Kim et al described the identification of differentially expressed genes (DEGs) in serous carcinomas of the ovary. They used the annealing control primer (ACP) assays to compare differences in gene expression between normal ovary and late-stage serous carcinomas. A total of 114 DEGs were identified using ACP-base assays, and 38 of those were used in the quantitative PCR confirmatory study. Supervised cluster analysis and survival analysis were performed to correlate with DEG expression and clinical outcomes.

Major compulsory revision: One of the serous limitations with current study is the use of normal ovary as the control for differential expression of genes in high-grade, late-stage serous ovarian cancer. Normal ovary contains various tissue types, e.g., ovarian surface epithelium, stroma, granulosa cells, theca cells, and germ cells. Current study design is therefore unable to determine whether differential gene expression observed in serous tumor is the result of aberrant transcriptional regulation or the result of different tissue types. It is therefore recommended to use normal ovarian surface epithelium as a control. In addition, emerging evidence indicates that fallopian epithelium may also represent another point of origin for high-grade serous ovarian cancer, and as such it should be included as a control. When fallopian epithelium is used a control, it is also advisable to take into account the hormonal cycle under which the tissue was collected, given that gene expression of histologically normal fallopian epithelium in luteal phase is more similar to serous cancer than the expression between fallopian epithelium in follicular phase and serous ovarian cancer. Without knowing these exact cell type and the hormonal status of the normal ovary used in this study, it is unclear whether differential gene expression is the result of 1) transcriptional deregulation in cancer, 2) tissue-specific differences, 3) hormonal differences, or 4) a combination of all of the above. Therefore, the biological significance of genes identified in this study is not clear.

*Thanks for your keen point. First of all, the origin of serous ovarian cancer was the*
The choice of control depends on the purpose of a given study. For the comparison of normal epithelial cells and its transformed phenotype such as cancer cell, normal epithelial cells should be used as control. In addition, the immortalized ovarian surface epithelial cell line such as IOSE29 has also been as control in the gene expression analysis of serous ovarian cancer. These controls could represent characteristics of epithelial cells, but could not represent the integrated property of ovary including many types of tissue.

We believe that the ovary at serous cancer stage III might have different gene expression patterns or gene regulation networks compared to the normal ovary. The goal of our study is to identify DEGs between normal ovary and ovary with cancer. This is the main reason why we use the normal ovary as control. Another reason is that the control used is commercially available, which make it universal use.

Even it is facts that an organ consists of various types of tissue, the function of an organ is mainly govern by the organ specific gene expression. There have been many studies on organ-specific gene expression (Toxicol Appl Pharmacol. 2007;220(2):186-96, Int J Cancer. 2008;123(12):2735-40, J Appl Toxicol. 2010 Jul 9. PMID: 20623750, Liver: Int J Pharm. 2010 Jul 23. PMID: 20656001). We believe that our results might provide some clues to the understanding of the ovary system (including various types of tissue) with serous cancer stage III.

Specific critiques:

1. Figure 3 is difficult to follow. Such complicated data can be best represented by heat map diagram. It is also striking to see that some samples (such as 5, 10, and 14) have, in general, lower expression of candidate genes. Please comment on the quality of these samples.

2. Figure 4 may also be shown in heat map. Otherwise, please provide legends for each symbol used in the graphs.

: Thanks for you kind point. Figures 3 and 4 were combined into a single heat map in Figure 3 and the numerical data of this new figure was added as an additional file 1. The quality of the total RNA extracted from samples was checked by the Agilent Bioanalyzer 2100 and all of the extracted total RNAs showed 8~10 RIN, where RIN represents the RNA integrity number. Intact RNA has an RIN if 8~10 while degraded
RNA has an RIN of 1~3 (See Molecular Aspects of Medicine 27:126-139, 2006, for more detail). Therefore, the lower expression of candidate genes in some patients might be due to individual genetic variation rather than the quality of the sample.

3. Figure 5-7, although DDAH2 and TCEAL2 expression was associated with survival outcome in univariate analysis, no significant association was observed when adjusted for chemoresistant phenotype. Please comment on possible explanation for this discrepancy. In addition, it is advisable to include other clinical variables, such as optimal debulking status and performance status as these factors also influence overall survival.

: Thanks for your points.

1. DDAH2 and TCEAL2 had a similar proportion of up/down-regulation between the chemo-resistant and chemo-sensitive groups (See below table). This might lead to no significance in the overall survival by the Cox multivariate analysis involving chemoresistance and gene expression.

2. We added the following sentence in the last paragraph of Survival analysis in result section (page 7).

The RNase K showed significance in both univariate (gene expression) and multivariate (gene expression and chemo-resistance) analysis while DDAH2 and TCEAL2 showed significance only in univariate analysis. This was mainly due to the similar proportion of up- and down-regulation of DDAH2 and TCEAL2 between the chemo-resistant and chemo-sensitive groups.

3. We tried to add patient age as additional variable in the multivariate analysis, but there was no significant ($p$ value < 0.05) association with overall survival. Information on the other factors such as debulking and performance status was not available.
**Level of interest:** An article of limited interest

**Quality of written English:** Needs some language corrections before being published. 
*English was improved by a native English speaking-scientist.*

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:** I declare that I have no competing interest.