Author's response to reviews

Title: Few additional genetic mutations accumulate during metastatic progression in high-grade serous ovarian cancer

Authors:

Jung-Yun Lee (yodrum682@gmail.com)
Jung-Ki Yoon (neododari@gmail.com)
Boyun Kim (romanticet@snu.ac.kr)
Soochi Kim (skim245@snu.ac.kr)
Min A Kim (everest@snu.ac.kr)
Hyeonseob Lim (limhs0915@gmail.com)
Duhee Bang (duheebang@gmail.com)
Yong-Sang Song (yssong@snu.ac.kr)

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

We are very grateful to you and to the reviewers for the valuable comments on our manuscript. We have taken all the comments into account and submit a revised version of our paper.

As per the reviewers' suggestions:
- More detailed descriptions about patient's information and the definitions of terms were added.
- Additional discussion on our results including drugs targeted for somatic mutations and the significance of enriched pathway.
- Some definite expressions were modified considering the size of cohort in this study.
- Figures were combined and we clarified the legends of figures and tables to avoid ambiguity.

A full point-by-point response to reviewers' comments is included below. We thank the reviewers again for their helpful suggestions to improve our manuscript, and we thank you for your time and editorial considerations. We look forward to hearing from you.

Sincerely,

Duhee Bang
Department of Chemistry, Yonsei University, Room 437, Science Building, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Korea
E-mail: duheebang@yonsei.ac.kr, Phone: 82-2-2123-2633

Yong-Sang Song
Department of Obstetrics and Gynecology, Seoul National University, College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, Korea
E-mail: yssong@snu.ac.kr, Phone: 82-2-2072-2822, Fax: 82-2-762-3599
Reviewer Comments: Point-by-point response:

Reviewer #1

Lee et al., performed exome sequencing and copy number analysis on eleven primary and metastatic deposits collected at primary surgery from one ovarian cancer patient. Phylogenetic trees were generated using somatic and copy number data, and somatic mutations were validated by deep sequencing. The authors identified two primary site clusters (P1 and P2) and a metastatic cluster (M), although these did not directly match spatial position of collected samples. A high level of intratumoral heterogeneity was observed, however few additional somatic or copy number changes were identified in metastatic compared to primary samples. The authors suggest this indicates that clones from primary sites have metastatic potential, however this cannot be concluded from genomic analysis alone. The translational significance of the findings are unclear.

We thank Reviewer 1 for the comments and helpful suggestions, and have addressed these below.

Major Compulsory Revisions
No major revisions

Minor Essential Revisions
1) The samples should be more clearly defined beyond what is shown in Figure 1. For example, were samples taken from the ovary multiple discrete deposits or part of a single tumor mass?

The patient presented in the study had tumors in both ovaries as shown in Figure 1. During cytoreductive surgery, seven samples were collected from both ovaries. A large cystic mass (10×9×8 cm in size) with multiple solid lesions (2×2 cm) was found infiltrating the right ovary. The left ovarian tumor mass was composed mostly of solid components and was measured at 6×5×4 cm in size. Samples were taken randomly from the solid portions of both ovaries with a certain distance retained between each sample. All tissues consisted of >70% high-grade serous adenocarcinoma cells based on pathologic review. Our pathologist (Min A Kim) performed the entire procedure. As requested, we have revised the Methods section to include additional clinical and surgical information for greater clarity (line 110).

“A 71-year-old female was diagnosed with stage IIIC ovarian cancer at the time of sample collection. She had no family history for breast or ovarian cancer. She underwent BRCA1/2 germline mutation testing (Integrated BRACAnalysis®) and no mutation was found. Preoperative CA-125 level was 336 U/mL. She underwent
cytoreductive surgery followed by platinum-based chemotherapy. During cytoreductive surgery, a right ovarian cystic mass of 10×9×8 cm in size was found. Multiple solid lesions were found inside the right ovarian cystic mass. A left ovarian tumor measuring 6×5×4 cm and consisting mostly of solid tissue was also found. Seven samples were taken randomly from the solid portions of both ovaries with a certain distance retained between each sample. All tissues consisted of >70% high-grade (FIGO grade 3) serous adenocarcinoma cells based on pathological review. Adjacent normal tissues from the left fimbriae and blood were also collected to serve as normal controls. Eleven tumor samples were collected from the ovaries, right fimbriae, bladder peritoneum, and omental lesions during surgery under the supervision of our pathologist (Min A Kim) (Figure 1A).

This patient had no evidence of recurrence at the time of publication and 12 months had passed since the completion of first-line treatment. This was a platinum-sensitive case (>6 months after first-line treatment completion). This study was approved by the Institutional Review Board (IRB) at Seoul National University Hospital (Registration number: C-1305-546-487) and performed in compliance with the Helsinki Declaration."

2) For clarity, Figure 1 and 2 'normal' could also be labeled as 'left fimbriae' (or blood?). There is inconsistency between arrayed samples shown in Figure 3 and Supplementary Table 1 (i.e. bladder peritoneum indicated as arrayed but not in the figure).

The word 'Normal' appears in Figure 1A, 1B, and 1C to represent the 'left fimbriae (LF)'. We edited Figure 1 to improve clarity.

We performed SNP chip analysis on a bladder peritoneum sample. However, because this sample failed to pass our quality control, we excluded the SNP data from the bladder peritoneum from further analysis. Therefore, Supplementary Table 1 was corrected. We apologize for the error and thank Reviewer 1 for pointing it out.

3) Cut-offs (copy number/log2 ratio) used to define amplified and deleted segments should be defined.

We used SCNA calling cut-off values set to 0.2 and -0.2 (log2 scale) for the amplified and deleted segments. The sentence below was added to the Material and Methods section (line 200).

“Initially, the cut-off values (log2 ratio) for amplified and deleted segments were set to 0.2 and -0.2, respectively”
4) Deep sequencing of 122 amplicons was performed, this number should be corrected in the results section (line 209).

5) Figure labels are inconsistent with the text and should be corrected.

We apologize for the inconsistency in the figure labels and text. We corrected these errors such that the number of validated loci is correct and all figure labels are consistent throughout the manuscript.

6) Please clarify criteria for cluster-specific and sample-specific mutations. There appears to be 'cluster-specific' mutations present across individual samples in multiple clusters? How many samples within a cluster must be mutated for genes to be indicated as present in a cluster (Figure 4/Figure 1D)?

We agree that the definitions may confuse readers. First, we revised line 189 to the following:

"If any somatic mutation was found in at least three samples in 'cluster P1' or at least two samples in 'cluster P2' and 'cluster M', we concluded that the mutation truly existed in that respective cluster."

We illustrated the existence of mutations on the cluster in the 'Clusters' columns of Figure 1D. Using these 'Clusters' columns, we re-classified the types of mutations. If the mutation existed in only one of the three clusters (cluster P1, P2, or M), it was considered 'cluster-specific.' If the mutation existed in two clusters, it was classified as 'shared'. Finally, if the mutation was present in all three clusters, it was labeled 'common'.

For example, the green boxes at the bottom of Figure 1D indicate that the mutations detected RO3, LO2, or LO3. These mutations were not cluster-specific because they appeared only once, making them 'sample-specific'. If they appeared in one more sample in Cluster P2 (e.g., in any two of the RO3, LO2, and LO3 samples), they would be categorized as cluster-specific mutations, and the P2 column of 'Clusters' in Figure 1D would be colored green.

As pointed out by the reviewer, the mutation can be cluster-specific even if the mutation was detected in one sample from the other clusters because a mutation in one sample cannot represent the cluster.

We classified the mutations step-by-step as described above to avoid misclassification or misinterpretation of the time (early or late) mutations obtained from false negatives and false positives of somatic mutations.
7) The final figure (Figure 6) could more clearly summarize the main findings. Perhaps sample names should be listed on the far right side and coded with cluster labels? The term 'non-ovarian metastasis' is not clear. Cluster P2 should be labeled as common/ancestral clones as is done with cluster P1 and M.

Per the reviewer's suggestion, we added the sample names to Figure 3, changed 'ancestral clones' to 'common clone of cluster P1, P2, M,' and revised 'non-ovarian metastasis' to 'metastatic lesions'.

Discretionary Revisions

1) Did the authors look at druggable somatically mutated genes in addition to those within regions of copy number change?

Per the reviewer's suggestion, we checked for anti-cancer drug interactions with the somatically mutated genes using DGIdb. We only found one drug, sophoretin, which interacted with the \textit{PRKCQ} gene; however, this mutation was only detected in Cluster P2. This analysis has been added to the Results section (line 238):

"Also, 25 nonsynonymous mutations were considered as candidate driver mutations (Supplementary Note 1). TP53 Y220C and SPICE E152K in the Common group are the only mutations listed in the COSMIC database (Table 1). We could not identify any anti-neoplastic therapeutic agents that interact with candidate driver mutations except \textit{PRKCQ} C281S, which was found to interact with sophoretin [23]. However, this mutation was only detected in cluster P2."

2) The significance of pathway analysis is unclear and not referred to in the discussion section. Perhaps this section should be developed further or excluded?

We agree that we did not discuss details regarding the pathway analysis. Therefore, we added the paragraph below about the genotype-phenotype correlation to the Discussion section (line 313):

"Our analysis demonstrated that all metastatic samples from this patient were related to cluster P1, not P2, suggesting that the metastatic ability of ancestry clones was more accelerated in cluster P1. Based on this connection, we found that different cancer-related pathways were altered in the early divergent clones (cluster P1 and M vs. cluster P2). JAK/STAT signaling pathway genes including \textit{JAK2}, known to be related to tumor migration through the epithelial-mesenchymal transition (EMT) [24], were only amplified in clusters P1 and M, supporting the hypothesis that clones in these clusters might be under migration pressure. In contrast, genes involved in cell
adhesion pathways were only amplified in cluster P2, indicating that the clones in cluster P2 might be under an opposite pressure to clusters P1 and M."

We thank the reviewer for this comment, and are glad to bring additional information to readers.
Reviewer #2

Reviewer’s report:
This manuscript describes a study on mutational profiles and copy number alterations of whole-exome sequenced samples, along with SNP6 array data analysis, from primary tumors and the associated metastatic lesions in a single patient. Together with the somatic mutations and somatic copy number alterations, the authors further explored the clonal evolution patterns of tumors between primary and metastatic sites by constructing phylogenetic trees. The paper is relevant to understand the clonal evolutionary processes in disease progression and metastasis. The data collection and bioinformatics workflow is fairly well established. However, the a few major concerns I have with this manuscript are summarized below.

We really appreciate the devoted comments.

Major Compulsory Revisions
1. The number of study case in this manuscript is limited. High-grade serous ovarian cancers are heterogeneous diseases. Different patients may have different patterns in mutational profiles, genomic instability and clonal diversity. The study as described in this manuscript only focused on one high-grade serous stage IIIC ovarian patient, 71-year-old. The size of the cohort, i.e. one case, is not an adequate representative of HGS ovarian cancer patients. The authors claimed that "Our study has clinical implications" (Line 317), yet more than one high-grade serous ovarian cancer case should be studied in order to support the "clinical implications" and to verify the conclusions in Line 74-76.

We agree with the reviewer’s comments. Although we tried to select a representative case for high-grade serous ovarian cancer, we acknowledge that our results may not be broadly representative of HGS ovarian cancer patients. We have revised the manuscript to improve clarity and discuss our results as objectively as possible (described in detail below). Also, per the reviewer’s comments, “clinical implications” was revised in the Discussion section.

With the increasing clinical use of bioinformatics, which produces very large quantities of data for large numbers of patients, methods to use this data to categorize patients into prognostic and treatment groups has become increasingly important. Our study is significant since the patterns of intratumor heterogeneity between primary and metastatic clones may be the key to classifying patients into treatment groups effectively.

We changed the followings.
Title: Few additional genetic mutations accumulate during metastatic progression in high-grade serous ovarian cancer.

→ (line 1) Title: Tumor evolution and intratumor heterogeneity of an epithelial ovarian cancer investigated using next-generation sequencing

Abstract:
Conclusion: Although a high level of intratumor heterogeneity was evident in high-grade serous ovarian cancer, our results suggest that transcoelomic metastasis arises with little accumulation of somatic mutations and copy number alterations.

→ (line 74) Although a high level of intratumor heterogeneity was evident in high-grade serous ovarian cancer, our results suggest that transcoelomic metastasis arises with little accumulation of somatic mutations and copy number alterations in this patient.

Discussion
These findings suggest that peritoneal seeding arises with little accumulation of somatic mutations and copy number alterations.

→ (line 307) These findings suggest that peritoneal seeding arises with little accumulation of somatic mutations and copy number alterations in this patient.

Our study has clinical implications. This study suggests that clones in peritoneal implants may not be more resistant than primary tumors. As transcoelomic metastasis arises with little accumulation of genetic alterations compared with primary tumors, debulking surgery might be useful in HGSC to achieve optimal cytoreduction and adjuvant chemotherapy. Moreover, this study indicates that some clones in the primary tumor already have metastatic potential. This might be associated with the fact that about 30% of patients are ...

→ (line 339) Our study may help to further our understanding of tumor progression during HGSC. The data suggest that clones in peritoneal implants may not be more resistant than primary tumors in some patients. With the increasing clinical use of bioinformatics, developing methods that utilize the large amount of data to categorize patients into prognostic and treatment groups has become increasingly important [32]. This study suggests that patterns of intratumor heterogeneity between primary and metastatic clones might be the key for identifying the most appropriate treatment strategies for patients. In cases with metastatic patterns similar to the patient in this study (e.g., transcoelomic metastasis arising with little genetic alteration accumulation compared with primary tumors), debulking surgery might be useful to achieve optimal cytoreduction through adjuvant chemotherapy. If we identify those groups where seeding metastasis may require driver mutations beyond those required for primary tumorigenesis, debulking surgery might not be useful. In these instances, we should focus instead on the targeted therapy associated with driver mutations in metastatic lesions.
2. Lack of novelty. Previous studies have revealed that HGSC are genomically diverse across spatially separated samples collecting from individuals. HGSC are frequently characterized by genomic instability in terms of variation across population in copy number profiles, rearrangement profiles and loss of heterozygosity events. The study concluded that "HGSC has diverse intratumour heterogeneity in terms of somatic mutation and copy number variation profiles" which is a fairly well established knowledge in the previous studies of HGS ovarian cancers. The authors pointed out that "but transcoelomic metastasis arises with little accumulation of genetic alterations", yet based on a single patient data, this is not an adequate summary of the HGS ovarian cancer.

Thank you very much for your comments. Per the reviewer’s comments, NGS technology has led to progress in the evaluation of intratumor heterogeneity in various cancers.

Since more than 75% of epithelial ovarian cancer patients are diagnosed with an advanced stage, this type of cancer is the best model for researching tumor evolution and intratumor heterogeneity. However, few have evaluated intratumor heterogeneity of ovarian cancer with NGS technology. Bashashati, et al. collected multiple samples from six patients to demonstrate intratumor heterogeneity [5]. However, they focused mainly on primary tumors with less than six samples from each patient. Although two metastatic samples from a patient were included, these metastatic samples were derived from lymph nodes and the researchers did not report the findings from these lymphatic metastasized samples. Considering most high-grade serous ovarian cancer metastasizes through the abdominal cavity directly, transcoelomic metastatic samples should be analyzed. Moreover, little is known about the mutational profiles of primary tumors and the associated metastatic lesions within an individual patient. Therefore, we tried to focus on the clonal evolution of metastatic tumors compared to primary sites in a single patient with extensive tumor samples. Eleven spatially separated samples from the primary tumor, associated metastatic sites, and two normal samples were obtained from a Stage IIIC ovarian cancer patient.

We would like to publish and share our results with the scientific community and clinicians. We not only confirmed well-established findings of intratumor heterogeneity, but also investigated distinct features of this patient, such as non-spatial cluster distribution and the metastatic ability of primary tumor clones, which has yet to be confirmed by NGS methods in ovarian cancer. We believe that these...
features may help further our understanding of tumor progression during high-grade ovarian cancer and may provide important information for those who would like to study a larger cohort. We commented on the objective and significance of this study in the Introduction. (line 94)

“However, it is not certain whether the genomic alterations found in single tumor biopsy samples from primary tumors are maintained in metastatic lesions. Furthermore, intratumor heterogeneity has been proposed as the main cause of treatment failure and drug resistance in ovarian cancer and other primary cancers [4]. Recently, NGS technology has led to progress in the evaluation of intratumor heterogeneity in various cancers [5-8]. In the field of HGSC, intratumor heterogeneity has been evaluated within primary tumors and associated metastatic sites, and the divergence of genetic variants was observed [5]. Despite evident intratumor heterogeneity within individual patients, little is known about how metastatic tumors further evolve compared to primary sites. The aim of this study was to explore the mutational profiles of primary tumors and associated metastatic lesions, and to identify the evolutionary relationship between primary and metastatic clones with NGS technology.”

We agree with the reviewer’s comments. Although we tried to select a representative case for high-grade serous ovarian cancer, we acknowledge that our results may not be broadly representative of HGS ovarian cancer patients. We have revised the manuscript to improve clarity and discuss our results as objectively as possible. (See response #1 for Reviewer 2).

3. The authors pointed out "This study has important implications for the future design of personalized therapeutic solutions and investigation of drug-resistance mechanisms in HGSC” (Line 325-326). However, the rest of the section (Line 326 – 337) does not clearly support this claim and no further discussion is related to the statement. The authors should discuss the biological implication and relevance of their results in more depth and cite relevant literatures to support their findings.

We agree that this sentence is confusing and have revised it (line 351) as follows:

“This study may provide important information for those who would like to evaluate tumor evolution in a larger cohort. For future studies evaluating clonal evolution in epithelial ovarian cancer, the following should be considered. First, ..."

Minor Essential Revisions
1. In the description of patient information (Line 109 - 117), it is not clear whether the patient underwent BRCA1/2 germline mutation testing, although
the authors mentioned in the results (Line 212) no pathologic BRCA1 and
BRCA2 germline mutation was found in this patient. No information related to
the patient's CA125 level is provided. It is not clear whether the patient
relapsed or not – the platinum response information of this patient is not
provided. Does the patient have personal or family history suggestive of
hereditary breast or ovarian cancer syndrome? More details are needed here.

Per the reviewer’s recommendation, we added information about the patient to the
Material and Methods section (line 110). The revisions were described in response
#1 to Reviewer 1.

2. Line 179 – Line 181 describe the segregation of samples into clusters P1, P2
and M according to somatic mutations. Does the statement "... at least three of
four in 'cluster P1' ..." refer to "three out of four samples in cluster P1"? The
same question applies to "... at least two of three in cluster P2 ...". A concise
description is needed here.

We agree that the statement may confuse readers; therefore, we have revised the
sentence, described in response #6 for Reviewer 1.

3. Line 190 – 191 "The segments were classified as cluster P1, cluster P2, and
cluster M as well, but more stringent criteria were used". This is confusing –
does it refer to a classification of segments or a classification of samples
based on altered segments? Moreover, Line 191-Line193 describe "the
segment was altered...", but it is not clear how "... more stringent criteria were
used ..." was applied, along with the classification, to generate the clusters. A
more detailed concise description is needed.

Somatic mutations were defined as a cluster if they were observed in 'at least three
of the four' samples in cluster P1 and 'two of the three' in clusters P2 and M.
Whereas copy number altered segments were classified as a cluster if the segments
in 'all' samples in each cluster were altered. Therefore, we mentioned ‘more stringent’
criteria. However, we agree that 'more stringent' is ambiguous and unnecessary, and
thus removed the phrase “but more stringent criteria were used” (line 200).

4. Line 216 "... formed cluster M (Figure IB)...", Should "Figure IB" be "Figure
1B"?

We thank the reviewer for identifying the error and have corrected “Figure IB” to
“Figure 1B”.
5. Line 311, "our results suggest that some clones in the primary tumor already have metastatic potential...". The authors do not clarify that this statement is only supported by the data from an individual patient data. Also, it is not clear why "... transcoelomic metastasis is a simple process" and what results support this statement.

We agree that the sentence is too definitive and that the meaning is ambiguous. Thus, we revised the Discussion section as follows (line 331):

"Our results provide a clue that some clones in the primary tumor can have metastatic potential, and that transcoelomic metastasis might be a simple spreading process using existing metastatic ability rather than supporting the previous tumor evolution models (linear [27], parallel [28], or mixed [29])."

6. Line 333 – 337 "Third, genomic alterations other than ..." it is not clear how this section follows the previous context. This section needs to be rephrased.

We think that the statement is over-simplified. 'Somatic mutations' or 'somatic copy number alterations' that could cause tumor progression in ovarian cancer was not detected in this patient. Therefore, we want to suggest that other types of genetic variants such as rearrangement or microRNA must also be explored to identify the driver variant. To improve clarity, we have revised this sentence in the Discussion section (line 359) as follows:

"Third, genomic alterations other than somatic mutations and copy number changes should be considered to identify the unveiled driver variant causing tumor progression."

7. Figure legends and Figure numbers do not match. Figures are numbered as Figure 1, Figure 2 ... Figure 6. However, only three figure legends are provided (Line 388 - 403). It seems to be a miss-labeling for Figure 1 (A) – Figure 1(D)

We apologize for this mislabeling and have corrected the figure numbers and legends.

8. Supplementary Table S1 needs a descriptive legend, i.e. what does 'o' and 'x' stands for? The same question applies to the Supplementary Table S3 (columns F-H)

In Supplementary Table S1, 'o' indicates that we performed the experiment (WES, SNP chip, mPCR analysis), while 'x' means that we could not perform the
experiment or could not use the data obtained due to low quality.

In Supplementary Table S3, 'o' represents the existence of a mutation in each cluster while 'x' means that the mutation did not reside in these clusters.

We have added an explanation to each legend (line 450, line 458) as follows:
Supplementary Table S1: "o: analysis was performed, x: analysis was not performed."
Supplementary Table S3: "o: mutation found in respective cluster, x: no mutation found in those clusters."

9. Supplementary Table S8 has the last three columns color filled with blue or red for Del and Amp, yet no corresponding text is found in the legend.

Supplementary Table 8 contains the most frequently observed SCNAs (somatic copy number alterations) found in TCGA High-grade Serous Ovarian Cancer. The annotations with red (amplification) and blue (deletion) indicate the SCNAs identified in this study. We now include the following explanation in Supplementary Table 8 (line 492):

"The values in each column represent the percentage of genomic regions that overlapped with the SCNA in this study. We considered the genomic regions to be amplified or deleted if at least 70% of the regions overlapped with SCNA segments, and then color-coded the annotated regions red to signify amplification or blue for deletion."