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Prof. Stavit Allon Shalev,
Special Issue Guest Editor,
Genes

Re: genes-1650796 "*Case Review*: *Whole exome sequencing analyses identifies carriers of a known likely pathogenic intronic BRCA1 variant in ovarian cancer cases clinically negative for pathogenic BRCA1 and BRCA2 variants"*

Dear Guest Editor,

We have submitted a revised version of the **original research article** entitled "***Case Review*: *Whole exome sequencing analyses identifies carriers of a known likely pathogenic intronic BRCA1 variant in ovarian cancer cases clinically negative for pathogenic BRCA1 and BRCA2 variants***" by Wejdan M. Alenezi, Caitlin T. Fierheller, Timothée Revil, Corinne Serruya, Anne-Marie Mes-Masson, William D. Foulkes, Diane Provencher, Zaki El Haffaf, Jiannis Ragoussis and Patricia N. Tonin, for consideration in the special issue on ***Genetic and Molecular Basis of Inherited Diseases*** in ***Genes*** journal.

We appreciate the prompt handling and review of our manuscript.

In this revised manuscript, we have:

1. marked up any changes in the manuscript using the "Track Changes" function;
2. addressed the reviewers' comments, most notably incorporating the analysis of the 10 ovarian cancer risk genes from all familial cases in response to reviewer #1; and
3. edited the reference annotation, which was "mixed up" in the original submission due to a formatting error on our part.

We hope that we have more than adequately addressed any concerns regarding our findings and that the revised manuscript will now be deemed suitable for publication in the special issue on ***Genetic and Molecular Basis of Inherited Diseases*** in ***Genes*** journal.

Below we have addressed each of the reviewer's comments (point by point) shown in blue.

Respectfully submitted by,



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Response to comments and suggestions made by the reviewers:

Reviewer #1

In the paper by Alenezi *et al*., the authors examine 27 familial ovarian cancer (OC) cases and 53 sporadic early-onset OC cases all reported negative for BRCA1/BRCA2 pathogenic variants by WES analysis. The authors analyze for pathogenic variants in BRCA1/BRCA2 and report the identification of a known likely pathogenic intronic BRCA1 variant (c.5470-25T>A) in 1 familial OC case (2 sisters) and one sporadic early-onset case. The variant is a recurrent variant in Norway and results in partial skipping of exon 23. The BRCA1 c.5470-25T>A cases were also examined for variants in 10 additional OC predisposing genes and harbour no pathogenic variants in these genes. Finally, LOH analysis was performed on tumor DNA from one BRCA1 c.5470-25T>A carrier and revealed loss of the wild-type allele. The authors conclude that their findings support the BRCA1 c.5470-25T>A variant as likely pathogenic.

Taken together, the manuscript is well-written, and the data is clearly presented.

Major points

- It is strange that the authors use the alternative BRCA1 transcript NM_007300.4. Please use the most used transcript NM_007294.4 and the nomenclature c.5407-25T>A.

  REPLY: We understand that NM_007294.4 is the commonly used transcript. However, as we described in the manuscript, we chose to use NM_007300.4 as it is the canonical or the longest "most updated" transcript based on NCBI biobank (https://www.ncbi.nlm.nih.gov/nuccore/NM_007300.4). Also, this is an acceptable practice according to the ACMG guidelines (PMID: 25741868) and felt our selection of reference transcript was appropriate considering the clinical implication of our work.

- Please include pedigree data from the sporadic OC BRCA1 c.5407-25T>A carrier in Figure 1.

  REPLY: This is not possible as the family history for this case (as with all the sporadic cases not selected based on their family history of cancer) is not available from the biobank resource used to obtain cases for our research.

- Since the BRCA1 finding is of limited novelty, I would suggest that the authors include findings from the 10 other OC predisposing genes in the 27 familial OC cases as well as the 53 sporadic cases in the manuscript.

  REPLY: We agree in part with the reviewer suggestions but have only included new results of the 10 OC genes from all 27 familial OC cases in the revised manuscript, as the primary goal of our study was to re-evaluate *BRCA1* and *BRCA2* - the ovarian cancer high-risk genes - that were also independently investigated in these cases in medical genetic settings.

- In the introduction (line 82-84) the authors mention that WES analysis can identify intronic sequences. Moreover, the authors mention deep intronic sequences several times in the manuscript. I would not use WES for deep intronic analysis. Please discuss in more detail.

  REPLY: We agree that identifying deep intronic variants are limited by WES analysis, and we have revised the manuscript accordingly to clarify our comments on deep intronic variants versus those closer to the splice junctions.

- In the results section (line 215-220), the authors mention that none of the prediction programmes predict that the c.5407-25T>A variants affects splicing. However, MaxEntScan predicts the generation of weak splice acceptor site. This should be mentioned in the manuscript.

  REPLY: We used Ensemble-based Variant Effect Predictor (VEP) to annotate our germline variant calling files, which is commonly used in the practice of germline variants analysis. Such annotation did not generate any scores for MaxEntScan as we have already shown in the Table S1. To draw attention to this observation, we have included a reference this table earlier in the text in the results section that describes the used of this and other in silico predictive tools in the revised manuscript.

Minor points

- Regarding the title suggest changing "clinically negative" to "previously tested negative".

  REPLY: We respectfully disagree as the main point of this study is that the clinical genetic testing used for our cases may not have captured all pathogenic variants in known *BRCA1* and *BRCA2* risk genes.

- In the abstract, the author mention that LOH analysis is performed on tumor DNA from variant carriers. It is performed on tumor DNA from one carrier. Moreover, the authors state that the c.5470-25T>A variant is predicted to create a new cryptic splice site. However, RNA analysis has shown that the variants indeed affects splicing (PMID: 32203205), so I would suggest that the authors mention this instead of predicted data.

  REPLY: We are not clear on the comment being made by this reviewer. We followed the standard protocol for evaluating germline variants in *BRCA1* and *BRCA2* by whole exome sequencing analyses which includes the use of prediction in silico tools for interpreting the biological significance of the variants. For the strongest candidate, we also applied LOH analysis based on prior knowledge that potentially pathogenic alleles are retained while the wild-type allele is lost in the tumour tissue which is consistent with the tumour suppressor phenotype of many established cancer predisposing genes, including *BRCA1* and *BRCA2*. However, we emphasized this observation the discussion section in the revised manuscript.

- The BRCA1 c.5407-25T>A variant does not activate a cryptic splice site but introduce a de novo splice acceptor site.

  REPLY: We have not used 'de novo splice acceptor site' to avoid any confusion in the context of hereditary cancer syndrome as the variant is present in the germline of both sisters meaning 'inherited' rather than occurring in the germline during the development as has been reported for some cases of with neurofibromatosis and pathogenic variants in *NF1*.

- The manuscript lacks some references (my version only has 37 references)

  REPLY: We apologize for this error. The updated reference manager had not refreshed the reference annotations automatically, but have fixed this problem in the revised manuscript.

Reviewer #2

The manuscript describes the identification of an intronic BRCA1 varina (likely to be pathogenetic) in ovarian cancer cases negative for BRCA1 and BRCA2 pathogenic mutations by applyinf their bioinformatic pipeline and filtering criteria on whole genome sequencing data from 22 cases of ovarian cancers from 22 familes, tested negative for BRCA1/2 pathogenic variants. Interestingly they identify a variant of BRCA1 (c.5470-25T>A), not identified by commercial tests. By using SpliceAI, a relatively new in silico tool based on deep learning and pre-mRNA transcrip sequencing, they predicted a deleterious effect of this variant on BRCA1 trasncription. They then looked for the precense of this variant in other OC cohort. No claer to this referee how these ovariam cancer patients were selected. Could the authors explain?

REPLY: We have clarified the selection of the OC families in the methods section in the revised manuscript.

However this variant was not new, as alreday reported by other different groups.The informatic tool they used to detect the varian is able to capture cryptic splice sites located uo to 10 kilobase pairs from the exon-intron junctions. The references are all mixzed up.

REPLY: We apologize for this error. The updated reference manager had not refreshed the reference annotations automatically, but have fixed this problem in the revised manuscript.

Even the variant they identified is not new, tha manuscript nicely rewieved the known intronic BRCA1 variants and discuss their clinical and therapeutic implications