Author’s response to reviews

Title: Case report: Recurrent pituitary adenoma has increased load of somatic variants

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Version: 1 Date: 26 Sep 2019

Author’s response to reviews:

Dear editors of the BMC Endocrine Disorders,

We are submitting a revised research paper manuscript entitled “Case report: Recurrent pituitary adenoma has increased load of somatic variants” following with the answers and correction based on the received reviewers’ comments on the first submission BEND-D-18-00301, and second submission BEND-D-19-00093. We are now submitting the revised manuscript with the detailed explanations to the questions raised by the reviewers (at the end of the cover letter). We enclose the revised manuscript and a detailed list of the changes made. We have also performed English editing of the manuscript. We submit this article as case report as this article contains “findings that shed new light on the possible pathogenesis of a disease . . .” as included in “Case report” criteria of BMC Endocrine Disorders. This is an observational case report that is intended to demonstrate potential mechanisms of
pituitary adenoma relapse mechanisms and included no intentional medical interventions apart from physicians appointed treatment.
The author(s) hereby confirms that neither the manuscript nor any part of it has been published or is being considered for publication elsewhere. All authors have approved the manuscript in the form it is submitted. The work will not be submitted for publication elsewhere until the editorial board of the BMC Endocrine Disorders has decided whether to publish the article. We confirm that the patient’s clinical care and the reporting of the case are consistent with the principles of Good Clinical Practice and related regulatory requirements. The patient voluntarily accepted the reporting of her clinical case, and this will not affect her future care. We are also willing to upload our raw sequencing data for review purposes upon the editor’s request and instructions for this process.
We believe that this article contains novel findings that have not been reported before and hope that the revised version will be found suitable for publishing in the BMC Endocrine Disorders.

Sincerely yours,
Janis Klovins
26.09.2019

Answers to the specific comments raised by reviewers
George Kontogeorgos, M.D., PhD (Reviewer 1).
We thank the Reviewer 1 for the comments and we have edited issues highlighted by the reviewer to make data presentation in the manuscript more accurate and avoid any misinterpretations that were not recognized in the first manuscript draft version. We consider that issues raised by Reviewer 1 have now helped to improve the data presentation and interpretation in the manuscript.

Response to the comments of Reviewer 1.
Comment 1.
The authors should follow the current terminology to define pituitary adenomas, as it was adopted by the recent WHO classification of 2017. Accordingly, somatotropinomas and corticotropinomas are now called somatotroph adenomas and corticotroph adenomas respectively.
Answer: Thank you for the comment we have thoroughly studied and edited the text of the manuscript to implement the use of the WHO classification of 2017 where appropriate.
For example, we edited the text in the Background section: "... 60.9% for somatotroph adenomas, 72.7% for corticotroph adenomas and 61.7% for lactotroph adenomas."

Comment 2.
Background Page 7, Line 108 .... "somatic mutations in PA in a primary and recurrent tumour". This is not true. See: Zahedi et al. Clin Endocrinol. 55:549,2001 Distinct clonal composition of primary and metastatic adrenocorticotropic hormone-producing pituitary carcinoma.
Answer: Thank you for the remark, and in indeed the study by the Zahedi and colleagues follows the potential clonal evolution of a recurrent pituitary tumour. We changed the text in the manuscript accordingly to highlight the different methodological approach that we have used in our study, as we have used exome sequencing while Zahedi et al. have followed the pattern of X chromosome inactivation and several microsatellite loci.
We have corrected the text in the Background section to avoid any misinterpretation: “The presented study is the first to report an exome scale profile of somatic variants in PA in a primary and recurrent tumour and indicates the contributing factors for PA clonal evolution and tumour relapse.”.

Comment 3.
Page 8, Lines 134, 135: ..."for luteinizing hormone beta polypeptide (LH), glycoprotein hormones alpha polypeptide (CGA)," .... Replace polypeptide by subunit.

Answer: Thank you for the remark we have edited the text in the manuscript Case presentation section: "Immunohistochemical analysis of the tumour tissue showed negative immunostaining for p53 protein expression, PRL, growth hormone (GH), ACTH, thyroid-stimulating hormone (TSH), follicle-stimulating hormone beta-subunit (FSH), somatostatin receptor 5 (SSTR5) and T-box transcription factor TBX19 (T-Pit); mild immunostaining for POU domain class 1 transcription factor 1 (PIT1); moderate immunostaining for steroidogenic factor 1 (SF1); and strong positive immunostaining for luteinizing hormone beta subunit (LH), glycoprotein hormones alpha subunit (CGA), somatostatin receptor 2 (SSTR2) and aryl hydrocarbon receptor-interacting protein (AIP).".

Comment 4.
Page 9, Lines 136, 137 and 152, 153: "Transitional-type immunostaining of CK8 was observed in more parts of the section" This phrase does not mean anything. The authors have to describe whether the distribution of CK8 was dot-like or diffuse.
Answer: Thank you for the comment, indeed we recognize that immunohistochemical characterization needs to be described more thoroughly.
Now, we have the data presented in the manuscript reviewed by to experienced pathologists, and we have edited the text for CK8 in the Case presentation section: “The distribution of CK8 was diffuse throughout the tumour”.

Comment 5.
Page 16, Line 303, 304: ..."rapid regrowth of PA indicates by the expansion of the tumour mutational load".. This is speculation not proved by the low Ki-67 index reported in the 1st and second operation (1.5% and 1%). The tumour regrowth is more likely attributed to the adenoma mass left behind in the 1st operation.
Answer: We agree that the Ki-67 index for both tumours is low, and we also indicate that the removal of the first tumour was partial in the Abstract section: “... who underwent craniotomy and partial resection in August 2010” and in the Case presentation section: “On August 2010, the patient underwent surgery via craniotomy, and partial resection of the suprasellar part of the pituitary adenoma was performed.”

To avoid any misinterpretation of the data, we changed the phrasing of the conclusion to clearly indicate the potential influence of the tumour leftover tissue on the regrowth but include also the main result that in our case study we detected an increase of the variant load in relapse tumour. We edited the text in the Discussion: “In conclusion, we show that in this relapse case, the regrowth of PA is accompanied by the increase of the tumour novel variant load, which could be caused by clonal expansion of the tumour leftover tissue.”.

Comment 6.
Figure 3 is suboptimal. The tumour is mostly chromophobic, not basophilic. Only the nuclei are basophilic. The "acidophilic elements" indicated by arrows in Figure legend 1, probably represent red blood cells.
Answer: Thank you for the remark, now we have had the haematoxylin and eosin staining reviewed by two professional pathologists, and we have edited the text in Figure 2 description: “Both adenomas are composed of small-to-medium size chromophobic and in some places poorly basophilic, monomorphic, rounded cells with round nuclei, disperse nuclear chromatin and in some places with a well-developed nucleolus.”
Response to the comments of Reviewer 2.

Comment 1.
The goal of the article is clear to the reader. It's novel to use exome sequencing of tumour's somatic DNA to prove the relationship of the somatic mutations in PA and recurrent tumour. Try to state more causality of mutational load and recurrence.

Answer: Thank you for this comment. We have edited the text in the manuscript to highlight the potential causality of recurrence and mutational load.

In the Abstract: “In this study, we investigated the genetic differences in genomic DNA of primary and rapidly recurrent tumours in the same patient to explain the causality mechanisms of PA recurrence.”

In the Background section: “The presented study is the first to report an exome scale profile of somatic variants in PA in a primary and recurrent tumour and indicates the contributing factors for PA clonal evolution and tumour relapse.”.

In the Discussion: “In conclusion, we show that in this relapse case, the regrowth of PA is accompanied by the increase of the tumour novel variant load, which could be caused by clonal expansion of the tumour leftover tissue.”.

Comment 2.

According to the study, the selected patient underwent twice operation with partial removal and lacked in radiation therapy. The recurrent tumour probably express lineage-specific immuno-staining. The question is whether these recurrent tumours represent a single lineage lesion or divergent differentiated cells of other lineages. It's better to seek more typical patients with multiple relapses to demonstrate your point.

Answer: We agree with the reviewer that it would be better to confirm our findings on more tumour recurrent cases, unfortunately, we had only available biological materials from a single patient. Regarding, lineage specificity of the tumour in both primary and recurrent tumour we found negative T-Pit, mild PIT1 and moderate SF1 immuno-staining, we could extrapolate that tumour has not undergone significant lineage alterations. However, in literature there are reported cases where clinically silent PA start to express hormones, therefore, demonstrating clinical functioning. We include sentence in the discussion highlighting this concept proposed by Reviewer 2 and we stress that further studies are needed to trace the lineage-specific origin of relapse PAs.

In the Discussion section: "Additionally, we did not observe any cell lineage change or significant alteration of hormone production in the presented patient’s case. There are reports that in rare cases shift from clinically silent PA to hormonally active tumours is observed (30-32), however, these do not include exact tracing according to transcription factors defined by the WHO classification of 2017. To
further assess potential shifts in cell lineage of operated recurrent PA cases comprehensive research of significant number of relapse PAs including T-pit, SF1, PIT-1 immunostaining is needed

Malak Abedalthagafi M.D., PhD (Reviewer 3):
We thank the Reviewer 3 for his comments. We now recognize that more explanation of the sequencing results should be included to better describe the landscape, potential origin and effects of the discovered genetic alterations. We have addressed all the issues raised by the Reviewer 3 and edited the text to encompass the complexity of the origin and impact of the discovered genetic variants.

Response to the comments of Reviewer 3.

Comment 1.
Please list the mutations and their allelic frequencies - are these possibly germline events?

Answer: Thank you for the comment, we have added allelic frequencies to Table 1. The somatic variants represented in Table 1 was discovered only in the somatic tumour DNA and not present in germline DNA of the patient-derived from white blood cells, therefore, we consider these variants as somatic.

We edited the text in the Exome sequencing results section to emphasize this: “Ten variants were present in the first and second tumour but not in the germline DNA derived from patients WBC.”.

Comment 2.
Were these mutations previously reported in COSMIC?

Answer: Three of the discovered variants have been reported in the COSMIC database, none of these has been previously reported related to pituitary adenoma.

We have included information COSMIC reference numbers in Table 1 and also added text to the Exome sequencing results section to present this information: “Three variants have been previously reported in COSMIC database PCDHGA1 (COSV65295567) in three cases - one prostate, one endometrium and one large intestine tumours. CLMN (COSV54187254) has been reported in one cervical cancer sample and ZNF320 (COSV67089305) in one thyroid carcinoma sample.”.

Comment 3.
Is there any functional data to suggest that they are gain-of-function mutations? Prediction algorithms are insufficient criteria for classifying a variant as a driver mutation and multiple lines of evidence should be presented if the authors are claiming pathogenicity.

Answer: Thank you for rising the point about the accuracy of the prediction algorithms. The main goal in our study was to estimate the increased load of somatic mutations in the reoccurring tumour and experimental testing for identified variants goes beyond the scope of this study. Currently, there are no functional data regarding these variants in the literature. We understand that this is not strong enough evidence for driver or even modifier variant claim and therefore, we are not claiming anywhere in the manuscript that any of the detected variants are pituitary adenoma driver variants. We, however, believe that reporting the pathogenicity scores obtained from two different prediction algorithms provides additional information to the readers.
Comment 4.
2. Did the authors found significant recurrent copy number changes or aneuploidy?

Answer: Thank you for this suggestion, we conducted CNV analysis, but no significant and recurrent copy number variations were found in the exome sequencing data. Unfortunately, CNV detection benchmark array genotyping data were not available for tumour samples.

We included information in the Exome sequencing results section: “CNVs detection using CoNIFER v0.2.2 (25) yielded negative results.”.

And to describe performed analysis also text to Supplementary 1, Materials and Methods: “CNVs were called using CoNIFER v0.2.2 in all three samples simultaneously from alignment (BAM) files and setting SVD to 1”.

Comment 5.
Prompted by the HRAS mutation identified. I think the authors should examine immunohistochemical activation of its canonical intracellular signalling cascade, the mitogen-activated protein kinase (MAPK) pathway.

Answer: Unfortunately, due to the inaccessibility of pathology material for additional investigations we were not able to perform immunohistochemical analysis of the tumour samples. Instead, we investigated transcriptome data from pituitary adenoma patients that we have obtained in another project and in majority of MAPK pathway genes (RAC1, MAP3K1, MAP3K9, MAP2K4, MAP2K6, MAPK14, PLA2G4A, MKNK1, MAPKAPK5, HSPB1, MAPKAPK2, STAT1, ELK1, MAX, MYC, DDIT3, MEF2D, ATF2KA5, HNGN1, CREB1) expression is not correlated with the expression of HRAS. The exception is MAPKAPK5 whose expression is negatively correlated with the expression of HRAS (see Figures below). These data suggest that it would be impossible (inconclusive) to draw conclusions about impact of single HRAS SNP on MAPK pathway in a single patient. Thank you for the suggestion and we think that we could investigate variations in tumour suppressor genes and impact on their pathways in upcoming studies using larges sample size.