Author's response to reviews

Title: Ser80Ile mutation and a concurrent Pro25Leu variant of the VHL gene in an extended Hungarian von Hippel-Lindau family

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Author's response to reviews:

Dear Dr. Graham,

We would like to thank your comments and suggestions. According to your and the Reviewer’s comments, we revised our manuscript (MS: 1597987078162808) entitled “Ser80Ile mutation and a concurrent Pro25Leu variant of the VHL gene in an extended Hungarian von Hippel-Lindau family.”

Please find enclosed our point-by-point responses to concerns raised by the Reviewers.

We also included an Authors’ contributions section before the Acknowledgment and Reference list.

We hope that the revised manuscript will meet your acceptance.

Sincerely yours,

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Detailed Responses to Reviewer’s Comments

Reviewer 1:

1. The Pro25Leu is a well known polymorphism (see: NCBI:dbSNP127). The prevalence data 1:36000 and 1:85000 must be checked and are incorrect.

Answer: We thank the Reviewer for clarification and we included the identification numbers for this variant in Introduction and Result sections. The prevalence data were corrected.

2. The classification of VHL has meanwhile been softened and reads now not exclusively, but predominantly without pheo (type 1) predominantly without RCC (2A) and (predominantly) only with pheos (2C): See WHO Classification of Tumours; Pathology and Genetics, Tumours of Endocrine Organs 2004.

Answer: We thank the Reviewer for clarification and we corrected the classification accordingly: Page 3, first paragraph.

3. Renal cysts occur in VHL, but should be only carefully be assigned to VHL.

Answer: We agree with the Reviewer that renal cysts alone should be carefully assigned to VHL but in patient IV.13 other manifestations of VHL disease (brain, retinal) were diagnosed in addition to renal cysts.

4. Pancreas carcinoma is not a manifestation of VHL: Mostly one understand here pancreas adenocarcinoma, but in VHL there occurs islet cell pancreatic tumor; the authors must check.

Answer: We thank the Reviewer for this comment. This patient (II.4) died because of a metastatic pancreatic tumor without histological confirmation and, therefore, this family member was not considered as having VHL (Page 7 lines: 13-16).

5. Table 1 must be checked for case II5 and III1: Not RCC but Pheo? Case II4 must be removed from the table unless islet cell tumor has been confirmed.

Answer: We thank the Reviewer for this question. Both cases: II.5 and III.1 had bilateral renal cell carcinomas and the case III.1 had also unilateral pheochromocytoma. Case II.4 was removed from this table due to the lack of histological confirmation.

6. Suggestion: The symbols of figure 1 are somewhat confusing: What they use for Not tested is usually the sign for dead person, what they use for Ser80Ile is mostly the sign for disease manifestation.

Answer: We thank the Reviewer for this suggestion and we completely redrew the pedigree according to standard conventions (see new Figure 1: filled boxes
represent affected persons, Ser80Ile mutation carriers are labeled as M+, Pro25Leu polymorphism carriers are labeled as P+. Members not available for DNA testing are labeled as NT (not tested).

REVIEWER 2.

- Major Compulsory Revisions

1. The authors suggest that the Pro25Leu variant may present a protective variant for VHL disease caused by the Ser80Ile mutation. This is solely based on the observed compound heterozygosity in individual II.1 in the pedigree (Figure 1) who is clinically unaffected. However, it cannot be excluded that individual II.5, who has clinically proven VHL syndrome, is a compound heterozygote as well. This is based on the observation of the Pro25Leu variant in individual III.6 and of the Ser80Ile mutation in individuals III.7 and III.8. Moreover, no molecular basis (e.g. based on functional analysis of VHL proteins carrying the respective amino acid changes) is provided by the authors to support such a protective effect. Therefore, the suggestion(s) that Pro25Leu is a possible protective variant for VHL should be removed in its entirety from the manuscript.

Answer: We thank the Reviewer for these comments and suggestions. We agree that our conclusion that the Pro25Leu variant might have a protective effect was based exclusively on pedigree analysis. Namely, the oldest individual who is a compound heterozygote for Pro25Leu and Ser80Ile is clinically free of disease, while other family members harboring the Ser80Ile variant alone are affected with the disease. Therefore, we accepted the Reviewer proposal and we removed our proposal of a „protective role“ of this variant throughout the entire manuscript.

2. The symbols used in Figure 1 are not according to standard conventions for pedigree drawing. This may be confusing. Clinically affected persons should be shown as filled symbols and unaffected persons as open symbols. Slashed symbols are used by the authors to identify individuals not tested, however this symbol is usually used for deceased persons. Persons not tested may be labelled as “NT” e.g. To indicate whether a person carries the Ser80Ile mutation and/or the Pro25Leu variant, the authors could use an asterisk combined with a different symbol.

Answer: We thank the Reviewer for this suggestion and we completely redrew the pedigree according to standard conventions (see new Figure 1: filled boxes represent affected persons, Ser80Ile mutation carriers are labeled as M+, Pro25Leu polymorphism carriers are labeled as P+. Members not available for DNA testing are labeled as NT (not tested).

3. The resolution of Figures 2A and 2B (at least, in the PDF-version that I have downloaded and printed) is far from optimal, the text cannot be read properly, especially in 2B.

Answer: We thank the Reviewer for this comment and we split our Figure 2 into two figures: new Figure 2 and new Figure 3.
4. The authors do not use the standard HGVS nomenclature for the description of sequence variants, while it is generally recommended to do so. At least, the authors should provide a full description of the VHL changes identified in their patients, i.e. including the nucleotide changes as well as the predicted amino acid changes.

Answer: We thank the Reviewer for drawing our attention to the use of the standard HGVS nomenclature. In the revised manuscript we described the changes not only on protein level but on the nucleotide level, as well.

- Minor Essential Revisions
5. Page 2, line 7: downstream
6. Page 3, line 13: 1:36000 and 1:85000 = 1:36,000 and 1:85,000 in
7. Page 3, line 18: germline mutation or deletions = germline mutation or deletion
8. Page 4, line 9: downstream = downstream
9. Page 10, line 7: Ser802Ile = Ser80Ile
10. Page 14 (legend Figure 2), line 2. gene = protein

Answer: We thank the Reviewer for these comments and we corrected them throughout the manuscript.

11. It would be useful to run the Align GVGD software tool for both Ser80Ile and Pro25Leu. Align-GVGD is a freely available, web-based program that combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral. Align-GVGD is an extension of the original Grantham difference to multiple sequence alignments and true simultaneous multiple comparisons. Data from this program may help to confirm the pathogenicity of Ser80Ile and the neutrality of Pro25Leu.

Answer: We thank the Reviewer for this suggestion. We performed a Multiple sequence alignment analysis of protein sequences combined with computational prediction of amino acid changes using Align GVGD freely available web-based tool (Page 5: last paragraph, page 6: first paragraph).

12. Extension of the multiple alignment analysis of the vhl protein from different species by including orthologs from evolutionary more distant species, such as fugu and drosophila, can be informative. The depth of the alignment provided by the authors may be insufficient.

Answer: As suggested by the Reviewer, we extended our previous alignment analysis by including protein sequences of Gallus Gallus (ENSGALG000000013678), and evolutionary more distant species Drosophila melanogaster (CG13221_CG13221-RA), Xenopus tropicalis (ENSXET0000001448) and Takifugu rubripes (SINFRUG00000121189) (Page 5, new Figure 2).