Author’s response to reviews

Title: Whole exome sequencing of benign pulmonary metastasizing leiomyoma reveals mutation in the BMP8B gene

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Author’s response to reviews:

Rebuttal Letter (MGTC-D-17-00169)

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Dear Dr. Pasini,

We would like to thank the proficient referees whose suggestions and comments were very valuable and helpful. These suggestions were taken into account in preparing the revised manuscript and we hope that our paper now meets all the criteria to be published in the journal of “BMC Medical Genetics”.

Our responses to the reviewers’ comments along with the main changes are listed below in the order of reviewers’ comments. The line numbers are given according to the highlighted version of the manuscript.
Reviewer 1.

Ping Yin: The article by Sõritsa et al, "Whole exome sequencing of benign pulmonary metastasizing leiomyoma reveals mutation in the BMP8B gene" is a detailed case report. The study uncovered a possible deleterious somatic heterozygous de novo mutation in the BMP8B gene in pulmonary metastasis of uterine leiomyoma. The authors confirmed the uterine origin of the pulmonary lesion by X-chromosome inactivation assay. Interestingly, the mutation was not detected in uterine leiomyoma. The authors did not examine further the impact of the mutation on protein function because of limited tissue material. Although the article focused more on being descriptive and the conclusion was speculative, it contained valuable information to those with closely related research interests. The paper was well written and is suitable for publication.

We thank the referee for the comment.

Reviewer 2.

Netta Mäkinen: Here, the authors have performed whole exome sequencing on a pulmonary metastasis and peripheral blood sample from a patient diagnosed with benign metastasizing leiomyoma (BML) to examine somatic mutational landscape of pulmonary BML. They identified a missense mutation in BMP8B gene, suggesting the gene to have a facilitating role in the metastasizing of BML. Also results from X-chromosome inactivation assay proposed monoclonal origin of the pulmonary BML.

Since this manuscript is based on data from only one BML patient, a case report could be a better-suited format for the paper instead of a research article.

The manuscript is now formatted as a case report.

Major concerns:

1. Because the manuscript is based on data from only one pulmonary BML, it makes it rather difficult to draw conclusions from the data.

-Without validating the BMP8B mutation further (more samples, presence of the mutation on cDNA level, etc.), it is difficult to rule out the possibility that this mutation is just a passenger change.

We agree that our conclusions are rather speculative and therefore we also suggest that the link between mutations in the BMP8B gene and pulmonary BML needs to be corroborated by further studies involving additional patients and exploring the functional consequences of the here-described mutation. Unfortunately, as BML is a rare disease, we did not have any other samples to investigate the recurrence of this mutation. Furthermore, in a very recent paper, Wu et al. (2017) demonstrated that using a panel of 409 cancer mutations resulted in ten different recurrent mutations in three cases of synchronous uterine and pulmonary leiomyomata. Therefore, it is
possible that all these mutations, described in a paper by Wu and colleagues or in our manuscript, are just passenger changes or there are many different mutations that may lead to the development of BML.

We also tried to validate the presence of this mutation on cDNA level but unfortunately the quality of RNA extracted from the archived FFPE tissue was insufficient to be amplified.

-Could it be just a coincidence that both the pulmonary metastasis and uterine leiomyoma showed non-random X-chromosome inactivation with the same allele being inactivated?

Yes, basically it may be just a coincidence. Clonal origin of both tissues (pulmonary metastases and uterine leiomyomas) has been repeatedly demonstrated but as there are only two possibilities which of the X-chromosome alleles is inactivated, the probability to have the same allele inactivated by chance is high. We now mentioned this possibility also in the manuscript (Page 10, line 229).

2. Filtering criteria for the exome sequencing data:

-Why were the splice variants not taken into account in the analysis? (exon-intron boundaries are usually well-covered in the exome data)

Thank you for pointing out this shortcoming. The splice variants were taken into account in the analysis but unfortunately this fact was not mentioned in the description of the analysis criteria. This is now added (Page 6, line 137). There were 10 splice region-related putative variants shown in the Additional file 2 (Additional file 1 in the previous version of the manuscript).

-SIFT and Polyphen2 do not give predictions to indels, thus did the authors exclude all indels from the analysis? Could an additional in silico prediction program be used for indels?

All short indels called by GATK (Additional file 2) were manually inspected but excluded from further consideration for validation because of the very low counts of the alternative alleles. Deletion of one nucleotide in BRD7 gene has comparable coverage of both alleles but was excluded as it has also been described in dbSNP database (NCBI) as a genomic variant (rs145896392) having heterozygozity of 0.04.

-Why were the heterozygous mutations required to harbor similar depths between the alleles? How did this affect the filtering of the variants? Were some variants excluded from the analysis based on this criterion?

In my opinion, if some alternation in nucleotide sequence is functionally related to the onset of BML, it has to be already happened in the uterine leiomyoma tissue (if this is a source of the disease) or rather early in the tissue growth in ectopic location. In our case, the pulmonary metastasis was histologically estimated to consist almost entirely of smooth muscle cells (>90%). Therefore, it seemed reasonable to assume that the depth of alleles in case of heterozygous mutations should be similar. All potential candidate mutations are listed in Additional file 2 but only the one corresponding to all criteria was selected for validation. If the proportion of
surrounding pulmonary tissue was larger, also alternative alleles with smaller read counts compared to the wild type would have been considered for validation.

-Due to the criteria (4), is there a possibility that the authors are missing potential novel candidate mutations?

Basically, it is possible that we miss some potential novel candidate mutations that have occurred later in the disease development and have dissimilar allele counts. However, it would also be very difficult to validate variants present in only a small subset of cells.

Minor comments:

Introduction:

-line 69: Since chromosomal aberrations are also mutations, it would be recommended to use another term than 'mutational landscape' in this case.

We now use more specific, and more suitable in the current context, term - single nucleotide variations (SNVs) and short insertions/deletions (indels) – instead of mutational landscape.

Methods:

-line 79: addition of 'uterine' before leiomyoma

Added.

-line 84: Did the authors mean 'smooth muscle cells' instead of 'leiomyoma cells'?

We now changed leiomyoma to smooth muscle cells.

-line 89: Was the quality score of extracted DNA the same (0.8) for each FFPE sample?

The DNA quality was screened only for a pulmonary metastasis to check the suitability of FFPE tissue for WES. To avoid misunderstanding, we removed this sentence from the revised manuscript.

-line 93: In the beginning of the Sequencing paragraph, it would be preferable to describe, which samples entered exome sequencing.

DNA from blood and pulmonary metastasis entered exome sequencing. This information is now added before the description of WES (Additional file 1).

Results:
lines 178-179: How did the authors define the absence of CNVs? Did they use any specific software designed for exome data or manually compare the coverages between the metastasis and peripheral blood sample?

The coverages between the metastasis and peripheral blood samples were compared manually. This is now also mentioned in the manuscript (Page 6, line 129).

-Why was the endometrioma sample not part of the X-chromosome inactivation analysis?

Histological analysis of the endometrioma sample showed that in addition to endometriosis-specific glands and stroma it also contained patches of surrounding ovarian tissue. The HUMARA analysis was not performed as it would not be possible to estimate the extent of endometrioma-specific X-chromosome inactivation in this mixed tissue. Further, as endometrioma consists mostly of uterine glands and stroma and does not contain smooth muscle cells in remarkable proportions, X-chromosome inactivation pattern of this lesion would not help to explain the origin of pulmonary lesions.

Figures:

-Figure 2: Addition of the used magnification to the figure legend.

The used magnification is now added to the figure legend.

Ethics approval:

-The used informed consent form does not need to be approved by the ethics committee?

Written informed consent for sequencing analysis and any other DNA manipulations and for using these data in research was obtained from the patient. As the data presented are part of the clinical practice at the Tartu University Hospital and are being published retrospectively, the ethics committee was not involved.

Yours sincerely,

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