Author's response to reviews

Title: Osteopoikilosis and multiple exostoses caused by novel mutations in LEMD3 and EXT1 genes respectively - coincidence within one family.

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Author's response to reviews: see over
Dear Editor,
We thank for reviewing our ms entitled “Osteopoikilosis and multiple exostoses caused by novel mutations in LEMD3 and EXT1 genes respectively - coincidence within one family.” ms# 6233717734744763. Please find below our answers to the reviewer’s comments.

Reviewer: Jan Hellemans

Major Compulsory Revisions

1. Description of the c.1732G>A mutation (page 9): Additional phenotypes are identified in patients with both a LEMD3 and EXT1 mutation. Please check whether parents, sibs or kids that lack a LEMD3 mutation (and osteopoikilosis) show similar phenotypes to distinguish 2 possibilities: a) the extra phenotypes result from the combination of LEMD3 and EXT1 mutations, or b) the extra phenotypes result exclusively from the EXT1 mutations. Related to this remark is the statement that “None of these pathologies was observed in the mutation unaffected family members”. It is impossible to appreciate the value of this statement because it is unclear how many relatives underwent a detailed clinical (and genotypic) analysis.

We recognize importance of this consideration. Clinical evaluation was only possible with two of the family member affected with LEMD3 mutation who showed no EXT1 mutations, and in four family members unaffected with either of the mutations. We therefore corrected statement:

“None of these pathologies was observed in the mutation unaffected family members” - to:

“None of these pathologies was observed in the examined family members who carried LEMD3 mutation only (patients III:10 and IV:8), nor in the family members who were free of mutations in both genes (patients III:5, III:8, III:14, IV:3). However, since we were unable to examine other unaffected family members, the relevance of this observation remains uncertain.”

The patients who underwent genotyping and clinical evaluation were marked in the figure 2 with horizontal bars and the appropriate description was introduced in the figure legend.

“Horizontal bars over symbols mark patients who underwent clinical examination and molecular testing “

2. Patient V:1 was screened on both blood and progenitor cells from and exostosis. Is there any indication for additional hits in the affected bone?

Both LEMD3 and EXT1 genes were sequenced in blood and bone cell derived genomic DNA from patient V:1. There were no additional/different point mutations detected in the bone cells, however exon deletions can not be excluded. EXT1 gene was also sequenced in the cDNA from bone cells, and the only detected mutation was the described in frame deletion.
We recognize that mutations might have occurred also in other genes, which we did not examine. Further, it would be likely that the mutation would not only occur in mitosis (differences between blood and bone sample) but also in meiosis. While the analyses of the real mutation rate would be extremely interesting, it goes beyond the scope of this manuscript.

3. The hypothesis that LEMD3 inactivation may lead to increased mutation susceptibility may be worked out a bit more. A number of sporadic co-occurrences of osteopoikilosis with other anomalies have been reported in literature – on the other hand I am not aware of an increased risk for cancer which would contradict the author’s hypothesis.

We expanded discussion of this hypothesis:

“In this context it is interesting to note that osteopoikilosis was previously reported to coincide with other pathological entities, including various types of cancers: synovial chondromatosis [18], synoviosarcoma [19], chondrosarcoma [20], osteosarcoma [21], giant cell tumor [22], metastatic breast carcinoma [23], as well as developmental dysplasias: dental, facial abnormalities, coarctation of the aorta, double urether, mental retardation and other reviewed by Gunal et.al. [24].”

Minor Essential Revisions

1. Use a dot as a decimal separator rather than a comma, 0,4 mM # 0.4 mM. Also 20-µl # 20 µl

Corrections were introduced

2. References to figure 2 at the end of the Clinical History section seem to be inappropriate. A similar comment applies to the reference to figure 2 and 3 at the bottom of page 9.

Corrections were introduced

3. The enumeration of patients on page 8 is not correct: IV:13 # IV:8

The enumeration was corrected

4. Figure 1A: in contrast to what the legend is stating, the figure does not contain any arrowheads

The missing arrowheads were introduced and the legend formulated more precisely:

“Hyperostotic spots are seen bilaterally in the distal parts of radius and carpal bones (arrows) as well as in the phalanges of the hands and feet and in the pelvis (arrowheads).”

5. Figure 2: Use different icons to distinguish the different phenotypes in the pedigree, e.g. half filled symbols, and describe them in the figure legend. Also
describe the meaning of the horizontal bars above individual symbols.

We introduced two colours to distinguish phenotypic presentations - The patients who underwent genotyping and clinical evaluation were marked in the figure 2 with horizontal bars and the appropriate description was introduced in the figure legend.

“Osteopoikilosis phenotype is shown in black, whereas multiple exostoses syndrome is represented with brown colour. Horizontal bars over symbols mark patients who underwent clinical examination and molecular testing.”

**Discretionary Revisions**

1. It would be better if the preferred mutation nomenclature would be used consistently throughout the manuscript, e.g. p.Arg578Thr, c.123-2T>C

   We introduced corrections and now use a uniform mutation nomenclature, e.g. p.R735X.

2. Readers that are unfamiliar with the described disorders may find it helpful if adverbs like dominant/recessive or heterozygous/homozygous would be used in the Background section when describing the phenotypes and observed mutations.

   We added adequate adverbs in the background section.

3. Table 1: Interpretation may be easier if the EXT1 mutation status would be included as well.

   Table 1 has been changed according to the suggestion of both reviewers. EXT1 status, age, and sex of the patients have been included.

**Quality of written English:**

*Needs some language corrections before being Published*

The manuscript was now copyedited by a language proficient person.

We thank Dr. Jan Hellemans for reviewing the manuscript.
Reviewer: YUN ZHANG

Questions:
In the Results of the Abstract, mutation should be presented in standard format as (c.2203C>T) and (p.R735X), which later is corrected stated in the main text.

We introduced corrections and now use a uniform mutation nomenclature, e.g. p.R735X.

In the Methods section, if the authors mentioned all the details in the PCR mix, Mg++ should also be mentioned.

Magnesium content of the PCR buffer was added:

“PCR amplification of LEMD3 gene was performed in a 20 µl final volume, contained 1U of Taq polymerase (FIRE Pol) with buffer supplemented with 1.5 mM Mg²⁺, 0.4 mM dNTP, 8 pmol of each forward and reverse primer, and 30 ng of DNA.”

“PCR amplifications of EXT1 gene were performed in a 20 µl final volume, contained 1U of Taq polymerase (Invitek) with buffer supplemented with 2 mM Mg²⁺, 0.4 mM dNTP, 8 pmol of each forward and reverse primer, and 30 ng of DNA.”

As cDNA sequence result was mentioned in the Results section, therefore cDNA sequencing should be mentioned in the Methods section.

Following information was added:

“cDNA was synthesised using random hexamer primers as described below. PCR amplification of the EXT1 cDNA fragments was done with primer pairs listed in Table 3. The amplification conditions were like for the genomic DNA sequencing. PCR products were sequenced with the DNA Sequencing Kit BigDye™ Terminator v3.0 Cycle Sequencing (Applied Biosystems) on an ABI 3730 automated sequencer.”

Table 3 was added:

<table>
<thead>
<tr>
<th>PCR name</th>
<th>F Primer sequence 5' - 3'</th>
<th>R Primer sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXT1 cDNA1</td>
<td>GCTGCTCGCCCGCCCTGGGTG</td>
<td>GTGGTGAAGCCATTCCTAC</td>
</tr>
<tr>
<td>EXT1 cDNA2</td>
<td>CTCAGCTGGCCTTTGTCTCG</td>
<td>CTCGGTGAAGTCAGGCAAG</td>
</tr>
<tr>
<td>EXT1 cDNA3</td>
<td>CTTGAGAACAATGGTAGG</td>
<td>CCTATGACGGCAGCTTAC</td>
</tr>
<tr>
<td>EXT1 cDNA4</td>
<td>GTATGATTATCGGGAAATG</td>
<td>CTGGGCACAGTGATGCCCTG</td>
</tr>
<tr>
<td>EXT1 cDNA5</td>
<td>GTCTCTCTCAGTCCAGC</td>
<td>GTCATTTTGTCTCTTATAC</td>
</tr>
<tr>
<td>EXT1 cDNA6</td>
<td>GCCATCATTCAAAGTG</td>
<td>CTCATTTTGTCTCTTATAC</td>
</tr>
</tbody>
</table>
Results part, page 9 line 17, the splice site mutation in exon as detected in DNA from the blood lymphocytes, and from cDNA. Please add which tissue cDNA was synthesised from though this has been mentioned in the Methods section.

Inforation on cDNA source was added:

“Sequencing of the exostoses derived cDNA (obtained from the cells isolated from the affected bone) showed that splice site mutation resulted in the in-frame deletion of 9 bp of the exon 5 leading to a deletion of three amino acids (pos. 429-431 - two conserved isoleucin residues and a conserved glutamic acid residue) (Figure 3A, B).”

Figure legend:

Should be legend for Figure 1 put in front of the one for Figure 2?

The numbering order was corrected

Figure 2. (A), please indicate clearly whether the sclerotic changes are in both hands, right or left feet, right or left side of the pelvis. Adding on arrows would be helpful to the readers. The same problems in (B).

We corrected figure legend and added arrowheads to indicate hyperostotic changes

“Hyperostotic spots are seen bilaterally in the distal parts of radius and carpal bones (arrows) as well as in the phalanges of hands and feet and in the pelvis (arrowheads).”

Figure 3.(B) amino acid not aminoacid (also in Page 9 text).

corrected

Table 1. Indicating the patients’ age and sex would be helpful.

Table 1 has been changed according to the suggestion of both reviewers. EXT1 status, age, and sex of the patients have been included.

We thank Dr. Yun Zhang for reviewing the manuscript.

Ethical Approval

We appreciate you had ethical approval, but could you also add a statement confirming whether had consent to include the clinical information.

A confirmatory statement has been added:
The local ethics committee approved the study and written, informed consent was obtained from all participants or their legal guardians for publication of this case report, including clinical data, pedigree and X-ray images.

We hope that reviewer questions, suggestions and comments have been answered and the ms is now acceptable for publication in BMC medical genetics.

Sincerely

Mateusz Kolanczyk