Author's response to reviews

Title: Whole exome sequencing identifies a novel EMD mutation in a Chinese family with dilated cardiomyopathy

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Author's response to reviews: see over
Dear Editors,

Many thanks for handling our manuscript. We appreciate the reviewers’ suggestion and the following is our revision with respect to their comments.

Major Concerns of reviewer Marcella Neri:

1. In the background they reported 97 EMD mutations known to data (HGMD) but in the LOVD gene specific database the DNA variants are 223. Among these variants, there are several deletions, in particular involving exon 1 the “c. 3_31del” is recurrent and associated to a constant cardiac involvement. It is worth to discuss these informations and to speculate a possible role of 5’ protein region in the prevalent cardiac phenotype.

   Answer:

   Thanks for your advice. 223 EMD variants were checked in the Leiden Muscular Dystrophy pages of LOVD gene specific database and updated in our manuscript.

   In the Abstract part, “about 100 mutations” was revised to “223 mutations”.

   In the Background part, “Among the 97 reported EMD mutations in the Human Gene Mutation Database (HGMD)” was revised to “Among the 223 reported EMD mutations in the Leiden Open Variation Database (LOVD)”.

   In the Mutation detection and proband reexamination part of Results, “with no record in the HGMD” was revised to “with no record in the LOVD”.

   In the List of abbreviations part, “HGMD: Human Gene Mutation Database” was replaced by “LOVD: Leiden Open Variation Database”.

   In the Reference part, the second reference was revised to “2. Dunnen JD: Leiden Muscular Dystrophy pages of Leiden Open Variation Database [http://www.dmd.nl/nmdb2/home.php?select_db=EMD]”.

   Among the 223 variants, the “c. 3_31del” mutation in the 5’ of EMD gene caused the prevalent cardiac phenotype and different degree of muscular involvement. Among the seven affected males, one with impairment of unassisted walking, two with difficulty in climbing stairs or rising from a chair but retained ability to walk unassisted, three with inability to run or walk normally, one with no evident muscular disability. The “c.26_39del” mutation reported in our study also destroyed the N-terminal of emerin protein (containing LEM domain and nuclear location signal) and caused the constant cardiac involvement but mild skeletal muscle degeneration. Both of these two mutations
located in the N-terminal of emerin and caused common cardiac disorder with different degree of skeletal muscle involvement. This information supported the hypothesis that the N-terminal mutations would be responsible for the prevalent cardiac phenotype.

A nonsense mutation in \textit{EMD} exon 6, located in the C-terminal (transmembrane domain) rather than N-terminal of emerin protein, also caused cardiac disorder with absent or mild muscle involvement (Sakata K, et al. 2005). This case indicated that the cardiac phenotype was not only caused by the mutations in the N-terminal of emerin protein.

The nuclear location signal in the N-terminal of emerin is needed in transporting the protein to nuclear and the transmembrane domain in the C-terminal is indispensable in anchoring the protein on the inner nuclear membrane. Considered these two domains are important in the process of locating emerin protein on the inner nuclear membrane, mutations impeding this locating process may result in the prevalent cardiac phenotype. Further functional studies of emerin in skeletal and cardiac muscle cells are needed to illustrate the prevalent cardiac phenotype caused by \textit{EMD} mutations.

2. \textit{The muscular phenotype of one of the patients, evaluated after the identification of \textit{EMD} mutation, is only clinically described. A CK evaluation or electrofisiological (EMG) evaluation or CT scan of muscles would be necessary to define the skeletal disorder as “mild”}.  

\textbf{Answer:}

In most EDMD cases, the onset ages of muscular phenotype are teenage years. EDMD patients’ CK levels are not more than 10-times normal levels and the higher CK levels indicate more serious muscle injury. The EMG of humeroperoneal muscles shows small amplitude narrow duration motor unit potentials with early recruitment. The routine histochemical stains of these muscles show variability in muscle fiber size with small round fibers and occasional necrotic fibers.

Patients in our study never complained of skeletal muscle problems. The creatine kinase (CK) level of proband B was elevated at 442 U/L (normal = 24-190 U/L). A male patient carrying a 5-bp deletion in \textit{EMD} exon 6 was reported suffering a severe cardiac disease but a very mild muscle disorder with CK value of $10.9 \mu$ kat/l (654 U/L).

The electromyography (EMG) evaluation of proband B identified myogenic damage. The
amplitude and duration of motor unit potentials in right biceps brachii and right deltoid were reduced, but no obvious abnormality was found in both sides of gastrocnemius muscle. The conduction velocity of motor and sensory fibers was normal.

The biopsy of deltoid showed clear cross striation and normal myofilament fibers. Internally located nuclei and fiber splitting were found. Endomysial fibrosis and sarcoplasmic condensation were occasionally noted.

Comparing to the typical EDMD, these clinical evaluations may support to define the skeletal disorder as “mild”.

In the Mutation detection and proband reexamination part of Results, “The creatine kinase (CK) level of proband B was elevated at 442 U/L (normal = 24-190 U/L). The electromyography (EMG) evaluation identified myogenic damage. The amplitude and duration of motor unit potentials in right biceps brachii and right deltoid were reduced, but no obvious abnormality was found in both sides of gastrocnemius muscle. The conduction velocity of motor and sensory fibers was normal. The biopsy of deltoid showed clear cross striation and normal myofilament fibers. Internally located nuclei and fiber splitting were found. Endomysial fibrosis and sarcoplasmic condensation were occasionally noted (Fig. 3C).” was added. The figure of histological section of proband B’s deltoid biopsy and the legend “(C) Hematoxylin-eosin staining of deltoid biopsy from proband B showed clear cross striation and normal myofilament fibers. Internally located nuclei and fiber splitting were found. Endomysial fibrosis and sarcoplasmic condensation were occasionally noted.” were added in the figure 3.

3. In the Methods, exome sequencing is not specified how was constructed the library: which kit was used? Could be responsible for the sequencing failure detected?

Answer:

The Agilent SureSelect exome capture system was used in the sequencing library construction. In the exome sequencing part of Materials and Methods, “Five micrograms of DNA from each of two affected male individuals was used for the construction of exome library” was revised to “Five micrograms of DNA from each of two affected male individuals was applied for the construction of exome library using the Agilent SureSelect exome capture system”.

The whole exome sequencing generated an average of 4.5 billion bases of sequence and a mean
coverage of 65× for each affected individual. An average of 97% of the targeted bases was sufficiently covered to pass our thresholds for variant calling. The exome sequencing results met the quality criteria provided by Illumina and would not be defined as sequencing failure.

4. In the results of WES is written “no significant variants in the exons of all reported nonsyndromic DCM causing genes” and the reference is Hershberger 2011. I would add a table with the list of the genes screened and compared it with the genes reported in the [http://www.musclegenetable.fr](http://www.musclegenetable.fr) (updated to 2013).

**Answer:**

The genes on the list of hereditary cardiomyopathies in the above website (updated to 2013) were screened and no significant variants were detected. In the **Whole Exome sequencing** part of **Results**, “No significant variants in the exons of all reported nonsyndromic DCM-causing genes were detected [3]” was revised to “Variants of 73 hereditary cardiomyopathies related genes were screened [5]”. “5. Kaplan JC and Hamroun D: Gene Table of Neuromuscular Disorders [http://www.musclegenetable.fr/].” was also added in the **Reference** part and the numbers of following references were changed in order.

Major Concerns of reviewer Luisa Mestroni:

1. **Can the authors detail their WES filtering process?**

**Answer:**

In the **Whole Exome sequencing** part of **Results**, “No significant variants in the exons of all reported nonsyndromic DCM-causing genes were detected [3]. Sanger sequencing was performed to screen the variants in the linked locus. A single-nucleotide variant c.113C>T (rs189225995) in G protein-coupled receptor 50 (GPR50) was found co-segregation with the disease phenotype.” was replaced by “Variants of 73 hereditary cardiomyopathies related genes were screened [5], the filtering conditions are as following: (1) same variants in both WES data; (2) missense, nonsense, insertion and deletion variants; (3) SNPs with minor allele frequency not more than 0.005 according to the SNP database of National Center for Biotechnology Information (NCBI). 7 variants passed the filtering conditions and none of them co-segregated with the disease phenotype. Then 140 genes in the linked locus (Xq28) were also screened according to the above conditions.
9 variants passed the conditions and only \textit{GPR50} c.113C>T variant was found co-segregation with the disease phenotype.”.

2. \textit{Did the affected family member have high CK, conduction disease?}

\textbf{Answer:}

In most EDMD cases, the onset ages of muscular phenotype are teenage years. EDMD patients’ CK levels are not more than 10-times normal levels and the higher CK levels indicate more serious muscle injury. The EMG of humeroperoneal muscles shows small amplitude narrow duration motor unit potentials with early recruitment. The routine histochemical stains of these muscles show variability in muscle fiber size with small round fibers and occasional necrotic fibers.

Patients in our study never complained of skeletal muscle problems. The creatine kinase (CK) level of proband B was elevated at 442 U/L (normal = 24-190 U/L). A male patient carrying a 5-bp deletion in \textit{EMD} exon 6 was reported suffering a severe cardiac disease but a very mild muscle disorder with CK value of 10.9 $\mu$ kat/l (654 U/L).

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Comparing to the typical EDMD, these clinical evaluations may support to define the skeletal disorder as “mild”.

In the \textbf{Mutation detection and proband reexamination} part of \textbf{Results}, “The creatine kinase (CK) level of proband B was elevated at 442 U/L (normal = 24-190 U/L). The electromyography (EMG) evaluation identified myogenic damage. The amplitude and duration of motor unit potentials in right biceps brachii and right deltoid were reduced, but no obvious abnormality was found in both sides of gastrocnemius muscle. The conduction velocity of motor and sensory fibers was normal. The biopsy of deltoid showed clear cross striation and normal myofilament fibers.
Internally located nuclei and fiber splitting were found. Endomysial fibrosis and sarcoplasmic condensation were occasionally noted (Fig. 3C).” was added. The figure of histological section of proband B’s deltoid biopsy and the legend “(C) Hematoxylin-eosin staining of deltoid biopsy from proband B showed clear cross striation and normal myofilament fibers. Internally located nuclei and fiber splitting were found. Endomysial fibrosis and sarcoplasmic condensation were occasionally noted.” were added in the figure 3.

The affected family members had conduction disease. The electrocardiogram (ECG) examinations revealed sick sinus syndrome or third-degree atrioventricular block.

Thank you very much for your attention and consideration. If any parts of the manuscript need further improvement, please inform us for revision. I'm looking forward to hearing from you soon.

Sincerely yours,

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