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Peer Review File

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Reviewer A

This is a very interesting report on new variants of NARS2-associated disease. A few comments to the authors:

1. On what grounds did the patient receive the diagnosis of COXPD24? There is no mention of mitochondrial biochemical investigations or respiratory chain enzyme activities. If based on patient's phenotype, elaborate why.

   Reply:

   Thank you for your kind advice.

   We made this diagnosis based on the patient's clinical phenotype and genetic test results.

   1) According to OMIM database (https://www.omim.org/), biallelic mutations in *NARS2* are associated with COXPD24 (MIM: 616239) and autosomal recessive deafness-94 (MIM: 618434).
   2) COXPD24 is characterized by early-onset seizures, global developmental delay, impaired intellectual development, myopathy, hypotonia, and hearing impairment. The patient, carrying two compound heterozygous variants in *NARS2*, mainly presented early onset generalized epilepsy, myoclonic seizures, and severe bilateral hearing impairment, which was consistent with the characteristics of COXPD24. The patient could not lift his head up stably at 3 months old, indicating early developmental delay. Because the patient died of a seizure at 6 months of age, we were not able to further assess his development.
   3) Mitochondrial biochemical investigations and respiratory chain enzyme activities analysis require tissue sample from patient. However, tissue sampling is invasive, and the parents of the patient refused the examination. It has been reported that some patients diagnosed with COXPD24 have not been tested for mitochondrial enzyme activity. When these experiments cannot be carried out due to lack of samples or experimental conditions, genetic testing of *NARS2* gene will provide strong evidence for disease diagnosis.

   Changes in the text:

   We added some relevant explanations in the text. (see Page 10, line 212)

2. The increase on myocardial enzymes is a significant finding. What did the cardiological evaluation show?

Reply:

Thank you for your kind advice.

The patient died of a seizure at 6 months of age and no further cardiological evaluation was performed before death.

Changes in the text:

We added some relevant explanations in the text. (see Page 5, line 96; Page 10, line 216)

3. Did the patient have Alpers or Leigh phenotype? It would be interesting to report on the findings of the brain MRI if performed.

Reply:

Thank you for your kind advice.

The patient did not develop symptoms similar to cortical blindness and renal insufficiency in Alpers syndrome, nor did he have brain MRI abnormalities in Leigh syndrome. But his refractory epilepsy and hypotonia were similar to Alpers and Leigh phenotype.

The patient's brain MRI was normal at 3 months of age. No more brain MRI were performed before his death. Possibly more symptoms were masked by his early death.

Changes in the text:

We added some relevant explanations in the text. (see Page 10, line 218)

4. It would also be of interest to shortly describe the outcome, i.e. how old is the patient at last follow-up and the psychomotor development and comorbidities.

Reply:

Thank you for your kind advice.

The patient died of a seizure at 6 months of age, so we were not able to further assess his psychomotor development. To our knowledge, the patient did not develop new complications until his death.

Changes in the text:

We added some relevant explanations in the text. (see Page 5, line 96; Page 10, line 216)

Reviewer B

The manuscript brings important contributions to the current literature about NARS2 gene-related conditions. Some points need to be evaluated by the authors:

1. A detailed review of language aspects by a native English speaker may increase the manuscript quality and is suggested to the authors.

Reply:

Thank you for your kind advice.

English editing has been conducted.

Changes in the text:

We have modified our text as advised. (see Page 3, line 34-36; Page 3, line 45-46; Page 5, line 82; Page 8, line 171-172; Page 12, line 258-259; Page 12, line 261)

2. The word "mutation" could be changed throughout the manuscript to variant.

Reply:

Thank you for your kind advice.

The word "mutation" have been changed throughout the manuscript to variant.

Changes in the text:

We have modified our text as advised. (see Page 3, line 28; Page 3, line 40; Page 4, line 60; Page 4, line 66; Page 9, line 191; Page 11, line 239; Page 11, line 245; Page 12, line 269; Page 16, line 340; Page 16, line 341)

3. The authors mention cardiac involvement in their described case mostly due to increased serum CK-MB and troponin I levels. However, it is not clear if other methods of imaging (structural) evaluation have been evaluated, such as cardiac MR imaging and echocardiography.

Reply:

Thank you for your kind advice.

The patient died of a seizure at 6 months of age and no cardiological imaging evaluation was performed before death.

Changes in the text:

We added some relevant explanations in the text. (see Page 5, line 96; Page 10, line 215-218)

4. Have the authors performed muscle biopsy study in the patient? Previous history study recently published (Sofou et al., 2021 - Eur J Paediatr Neurol. 2021;31:31-7) disclosed a complex phenotype with early-onset encephalopathy, sensorineural deafness, and CNS phenotypes, including Leigh and Alpers-Huttenlocher syndrome, most commonly with severe and commonly lethal presentation. As this patient phenotype brings a new presentation with cardiomyopathy and both compound heterozygous variants were VUS, it would be interesting to analyze pathologic study.

Reply:

Thank you for your kind advice.

Muscle biopsy can provide a lot of useful information. However, muscle biopsy study had not been performed, because it is an invasive test and the parents refused.

Changes in the text:

We added some relevant explanations in the text. (see Page 5, line 93)

Reviewer C

In the abstract it is mentioned that the heart was affected but in the main text nothing is reported about heart disease

Reply:

Thank you for your kind advice.

The increased serum CK-MB and troponin I levels of the patient indicating that the heart was affected (Page 5, line 86-88).

However, the patient died of a seizure at 6 months of age and no further cardiological evaluation was performed before death.

Changes in the text:

We added some relevant explanations in the text. (see Page 5, line 96; Page 10, line 215-218)

Reviewer D

This study is about genetic diagnosis of a NARS2 patient by whole exome sequencing. Although they report only one patient, the patient carried novel variants inherited from the parents, and the authors carefully assessed their functional relevances. This study fits to the journal, but with following updates:

1. Overall quality measurements of whole exome sequencing are missing. What are the coverage depth, error rates etc? Are there additional candidates that might explain the patient symptoms?

Reply:

Thank you for your kind advice.

The coverage of target region reached 99.8 %. The average sequencing depth was 204×, 98.8 % of target region reached over 20×.

There were no additional candidates that might explain the patient's symptoms.

Changes in the text:

We added some relevant explanations in the text. (see Page 8, line 168-169)

2. Does this patient show milder phenotype than previously reported NARS2 patients with truncation mutations? If yes, it would be desirable to discuss in Discussion.

Reply:

Thank you for your kind advice.

1) Previously reported NARS2 patients with truncation mutations involved two pedigrees.
   a. Compound heterozygous of **c.969T>A/p.Tyr323***; and c.1142A>G/ p.Asn381Ser (https://doi.org/10.1371/journal.pgen.1005097)
   Two siblings presented Leigh-like phenotypes including auditory neuropathy, visual impairment, myoclonic seizures, pharyngeal hypotonia and feeding difficulty with abnormal EGG and brain MRI. The two patients died at the age of 6 months and 15 months, respectively.
   b. Compound heterozygous **of c.83_84del/p.Leu28Glnfs*17**; and c.1339A > G/p.Met447Val (https://doi.org/10.1007/s10048-021-00659-0)
   The patient suffered infantile−onset severe epilepsy leading to fatal refractory status epilepticus. The disease presented at 3.5 months and the patient died at the age of 14 months.
2) In this article, our patient presented early onset generalized epilepsy, myoclonic seizures, hypotonia, severe bilateral hearing impairment and died of a seizure at 6 months of age. Laboratory tests suggested liver function and cardiac abnormalities. Possibly more symptoms were masked by his early death.
3) Seaver *et al* reported two patients in a pedigree carrying *NARS2* c.167A>G/ p.Gln56Arg; c.631T>A/p.Phe211Ile, presenting seizure, auditory neuropathy, status epilepticus and died at the age of 5.5 months and 9 months, respectively. (https://doi.org/10.1016/ j.pediatrneurol.2018.07.014)
4) Based on the above facts, we think that the clinical phenotype of the patient in this case is not milder than that of patients with truncation mutations.

Changes in the text:

We added some relevant explanations in the text. (see Page 5, line 96)