Author's response to reviews

Title: Systematic analysis of mitochondrial genes associated with hearing loss in the Japanese population: dHPLC reveals a new candidate mutation

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Author’s response to reviews: see over
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BMC Medical Genetics Editorial Board

Dear Sir or Madame,

We are enclosing the revised form of manuscript entitled “Systematic analysis of mitochondrial genes associated with hearing loss in the Japanese population: dHPLC reveals a new candidate mutation”, originally entitled "A new candidate mutation in the mitochondrial 12S rRNA, 904C >T, associated with hearing loss: systematic analysis by dHPLC" for re-consideration as a publication in BMC Medical Genetics.

We appreciate all the comments from the 3 reviewers. We added several data including audiogram of the probands, chromatogram of dHPLC, revised the entire manuscript including abstract, and shortened Methods and Discussion. We also asked an English editing service for proofreading of the manuscript.

We affirm that the manuscript has not been published previously and is not being considered concurrently by another publication. The research carried out here is in compliance with the Helsinki Declaration, and was approved by the ethics committee of National Tokyo Medical Center. All the authors have read and have abided by the statement of ethical standards for manuscripts submitted to BMC Medical Genetics.

We appreciate your review of this work.

Sincerely yours,

Tatsuo Matsunaga, M.D., Ph.D.
Chief, Laboratory of Auditory Disorders
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Reply to Reviewers' comments

To Dr. Malgorzata Rydzanicz

Discretionary Revisions
1) The general remark is that authors use the definition of mutation in huge simplification as a any change in a gene sequence. I recommend to describe the sequence variants as mutations in a case of known or suggestive pathological changes, but not for all observed changes, including non-pathogenic polymorphisms and rare sequence variants with unknown function.

   We really appreciate the advice. Yes, there were debate among ourselves about the usage of "mutation" and "variant". We decided to use the term "mutation" to describe m.1494C>T, 1555A>G, 3243A>G, 7445A>C/G/T, 7472insC, and 7510T>C, and use "variant" for all other nucleotide changes.

2) Affiliation 2 and 3, it is probably not obvious for all readers where these institutions are located, please give at least the city names.

   We added the cities where the institutions (Affiliations 1,2, and 3 are in Tokyo, Affiliation 3 is in Nagano) were located.

3) Background, page 3, the authors give the frequency of 1555 A>G mutations in range 0.6-5.3% depending on the ethnic group. However, it may be even higher in the Chinese population and Hispanic patients, please verified.

   We verified that Estvill et al. reported that in Spanish families with nonsyndromic hearing loss, 105 out of 649 families (16.2 %) had m.1555 A>G mutation (J Med Genet 2003, 40:632-636). The frequency of m.1555 A>G was estimated as 3.96% in Chinese population with hearing loss by Lu et al., (Mitochondrion 2010, 10:69-81). We corrected the sentences to "...(0.6–16%, depending on the ethnic group)..." (p.3)

4) Methods>Screening for mtDNA mutations by dHPLC, page 6 “Initially, whole mtDNA from each patient was amplified in three fragments (1351–8197, 6058–12770, and 11706–2258)...”, please add overlapping fragments.

   We replaced "three fragments" with "three overlapping fragments". (p.6)

5) Table 1. I suggest to show all sequence variants detected in the control group. Such
data gives an additional valuable information about mitochondrial genome variability in different populations.

We added data of m.752C>T, 1009C>T, and 1107T>C variants, detected only in the control group, in the Table 1.

Minor Essential Revision
1) The title of the article is not accurate and should be rewritten.

In response to the comment, we corrected the title as "Systematic analysis of mitochondrial genes in Japanese patients with hearing loss by dHPLC: A new candidate mutation associated with hearing loss".

2) Abstract should be rewritten. I have found some mistakes, including "degenerating high-performance liquid chromatography", I suppose, instead of denaturing high-performance liquid chromatography (dHPLC). The authors states "...mutational analysis of several mtDNA genes", which genes, the entire sequence of mtDNA was screening for deafness-related mutations/sequence variants? As abstract is one of the most viewed part of the paper, such information make it more informative for the readers and may be more valuable than “Subjects with pathogenic mutations of GJB2 or 1555A>G and 3243A>G in mtDNA frequently found in hearing loss patients were excluded from the study”, which may be or even should be given in the Materials and Methods section, where studied group is characterized. Moreover, the authors mentioned in the abstract about other mutations 961insC and 961delT+Cn in 12S rRNA gene; however, I have not found even a word about substitutions 1005 T>C and 7501 T>A which are wide discussed in the next parts of the article.

In response to the suggestions by all the reviewers, we edited the abstract significantly. Wording error of "denaturing high-performance liquid chromatography" and nomenclature of the genes were corrected. Description of m.1005 T>C and 7501 T>A variants were added in the abstract.

3) Methods>Subjects, page 5: “Prior to this study, the patients were confirmed not to have 1555A>G and 3243A>G mutations in mtDNA nor pathogenic mutations in GJB2 by our routine methods of RFLP-PCR or direct sequencing [13, 42, 43]”. It is not clear if the referred papers contain information about technical detail or described the results of molecular analysis, including screening for 1555 A>G, 3243 A>G and GJB2 mutations for the same group of patients. Please explain.

These articles were cited simply in order to refer to the technical information of
RFLP-PCR and direct sequencing. The patients studied here were not involved in the previous studies. We modified the text as "... by our routine methods of RFLP-PCR or direct sequencing. The methods for the "pre-screening" have been described elsewhere [13, 42, 43]." (pp.5-6)

4) The authors need to show the audiograms of all affected subjects carrying sequence variants 904 C>T, 1005 T>C and 7501 T>A considered as putatively pathogenic in this study.

We added the audiograms of all the proband patients carrying m.904C>T, 1005T>C, and 7501T>A variants, and some of the family members carrying heteroplasmic m.1005T>C variant onto Fig. 1-3. Those of other subjects were not available.

5) The authors should show the molecular data (for example: sequence chromatograms, PCR-RFLP analysis, dHPLC profiles) to improve that identified substitutions were indeed homoplasmic and/or heteroplasmic.

In response to the comment, the chromatogram of dHPLC of the heteroplasmic m.1005T>C variant in 12S rRNA was added to Figure 2D.

6) Please give the GeneBank accession number of the rCRS.

The GenBank accession number of the rCS is AC_000021. the accession number was described in Methods and Supplemental Table.

7) Discussion, page 15. The first sentence of the discussion suggests, that the authors analyzed entire sequence of mtDNA and have found sequence variants only in 2 genes; 12S rRNA and tRNASer(UCN). What about the other mitochondrial genes, no changes? Did the authors define the mitochondrial haplogroup of affected subject.

We did not find any variants in the other mitochondrial genes analyzed, that is, tRNA_{Leu(UUR)}, tRNA_{Leu}, tRNA_{His}, tRNA_{Ser(AGY)}, and tRNA_{Glu}. Since we focused on the systematic screening of the mtDNA genes previously reported to be associated with hearing loss, in this study, we did not conduct sequencing of the entire mtDNA genes in the subjects to detect novel genes associated with hearing loss. In the Discussion, some sentences were modified to " No variants in tRNA_{Leu(UUR)}, tRNA_{Leu}, tRNA_{His}, tRNA_{Ser(AGY)}, and tRNA_{Glu} were detected in the subjects studied here, suggesting that the mutations in these genes associated with hearing loss are not common in the Japanese patients." (the first paragraph of the Discussion,
p.15) and "It would be perceptive to await further investigation, such as haplogroup analysis or generating the lymphoblastoid cell lines for measurement of the endogenous respiration rates, to determine pathogenicity of the m.7501T > A variant" (p.16)

8) The manuscript text should be read by the native speaker, a number of spelling and typo errors were found throughout the paper. Some sentences are really difficult to follow.

In response to the suggestions by all the reviewers, we sent the revised manuscript to an Scientific writing editing service.
To Dr. Carmela Scuderi

Discretionary Revisions
The methods section could be shortened; in particular, in the second paragraph, the detailed description of the mutation analysis by the Mitoscreen assay kit and, in the fourth paragraph, the list of mammalian species used for the evaluation of nucleotide conservation, appear unnecessary.

In response to the comment, method of mutation analysis by Mitoscreen kit was omitted. Since the mammalian species used for the evaluation of nucleotide conservation were selected originally and therefore inconsistent with the previous paper ([51], Leveque et al., Eur J Hum Genet 2007, 15:1145-1155), the list of the animals and the accession numbers of the mtDNA was moved to Supplemental Table.

Minor Essential Revisions
1. use the correct nomenclature for the mitochondrial mutations described in the manuscript (for example, m. 904C>T)

   We corrected the nomenclatures as suggested in the entire manuscript.

2. In the abstract, use the term “denaturing” instead of “degenerating”

   We corrected the error as suggested. In addition, in response to the suggestions by all the reviewers, we edited the abstract significantly.

3. In the background section, the term “a mutation of” should be correct in “the mutation” (for example “the m.1555A>T mutation” instead of “a mutation of 1555A>T”)

   In response to the comment, the term "a mutation of" was corrected to "the m.*** mutation" in many cases. In addition, in response to other reviewer's comment, we decided to use "mutation" when the nucleotide change was confirmed to be pathological, and use "variant" when the nucleotide change was nonpathological or not proved to be pathological.

Major Compulsory Revisions
1. The English style must be revised

   In response to the suggestions by all the reviewers, we sent the revised
2. The authors have focused their manuscript on the new mutation 904C>T in 12S rRNA; however it has not been demonstrated the pathogenicity of this mutation, since the mutation segregation in other family's members has not been verified; on the contrary, the family tree suggests an autosomal recessive disorder. On the other hand, the work presented by Mutai et al. is interesting because it provides new information about the association between mtDNA mutations and hearing loss in the Japanese population. For this reason, I suggest the authors to modify the title giving emphasis to their study on the whole.

In response to the comment, we changed the title to “Systematic analysis of mitochondrial genes in Japanese patients with hearing loss by dHPLC: A new candidate mutation associated with hearing loss”.
1. In the abstract, it is stated that ‘…were subjected to mutational analysis of several mtDNA genes.’ The authors should clearly state in the abstract which genes they tested in order to help researchers access their paper when using keywords in databases (i.e. PubMed).

In response to the comment, we added the sentence “… subjected to mutational analysis of mtDNA genes (12S rRNA, tRNALeu(UUR), tRNASer(UCN), tRNALys, tRNAHis, tRNASer(AGY), and tRNAGlu)” in the “Methods” of the abstract.

2. In the abstract, the statement ‘…1555A>G and 3243A>G in mtDNA frequently found in hearing loss patients’, is somewhat confusing. Can we actually refer to these mutations as ‘frequent’ ones? The A1555G mutation is indeed the most common mtDNA mutation worldwide but mtDNA deafness mutations in general are not ‘common’. The A3243G mutation is even more rare.

3. In the background section, the authors state that ‘…Mutations in mitochondrial DNA (mtDNA) are frequently responsible for hereditary hearing loss’. Again, this statement is wrong as it is known that mitochondrial mutations account for <1% of sensorineural deafness cases worldwide.

Our understanding is that the frequency of mtDNA mutations found in patients with hereditary hearing loss varies widely among ethnic groups. In Spanish families with nonsyndromic hearing loss, 105 out of 649 families (16.2 %) had the m.1555A>G mutation (J Med Genet 2003, 40:632-636). In Japan, the frequency was 3.4% (J Med Genet 2000, 37:38-40). The frequencies may not be very high (certainly lower than that of GJB2) but are definitely much higher than that of many of the deafness genes. We corrected the sentence in the abstract as "… not having pathogenic mutations of GJB2 nor m.1555A > G and m.3243A > G were subjected to mutational analysis..." in the abstract. We also corrected the sentence in Background as "Among them, the m.1555A > G mutation in the 12S rRNA is found relatively frequently (0.6–16%...."(p.3).

4. At the end of page 3/start of page 4: “Mutations of 7445A>C/G/T [14-16], 7472insC [17], and 7510T>C [18] in the tRNASer(UCN) are also associated with aminoglycoside-induced or nonsyndromic hearing loss.” The 7472insC mutation is also associated with syndromic deafness combined with neurological dysfunction, while the
A7445G mutation has also been reported in cases with deafness and palmoplantar keratoderma.

We really appreciate the comment. The sentence was corrected as "...in tRNA^Ser(UCN) are also associated with aminoglycoside-induced, nonsyndromic, or syndromic hearing loss." (p.4)

5. At the end of page 5, the authors state that GJB2 mutations were excluded by PCR-RFLP and/or bidirectional sequencing and give references [13,42,43]. It would be helpful to mention in brief what is their strategy, i.e. they first use PCR-RFLP for frequent mutations of GJB2 in Japan (235delC) and in heterozygotes they sequence the whole coding region in order to explore the existence of a second mutation?

The sentence was corrected as "... the patients were confirmed not to have the m.1555A > G and m.3243A > G mutations or not to be diagnosed as GJB2-caused hearing loss by RFLP-PCR, or together with direct sequencing in case when the heterozygotic 235delC mutation was detected in GJB2". (pp.5-6)

6. In page 6, the authors set the age limit for prelingual deafness to be 4 years old and for postlingual to be 5 years old but they need to clarify if this is an objective classification which follows certain guidelines or it is subjective.

Our understanding is that the 0-1 year old is always called "prelingual period". In this study, the peri-lingual period (2-4 years old) was also merged into "prelingual" according to the previous paper ([1], N Engl J Med 2006, 354:2151-2164). We corrected the sentence to "...subjects were classified into prelingual hearing loss (onset before 5 years old, 20 males and 34 females) or postlingual hearing loss (onset at 5 years old or later, 31 males and 49 females)[1]". (p.6)

7. The manuscript is too wordy and could be significantly reduced in length. Especially the 'Prediction of pathogenicity of mtDNA mutations' and the 'Discussion' sections.

In response to the comment, the sentences were shortened, omitted, or transferred to the Supplemental Table. The 'Prediction of pathogenicity of mtDNA mutations' section was reduced from 2,166 to 1,438 letters. 'Discussion' was reduced from 4,807 to 4,014 letters

8. In page 16, ‘...the inheritance of hearing loss in the child is likely due to the transmission of an autosomal genetic mutation, not mtDNA, from the proband.’ I
assume the authors mean ‘from the father’?

As indicated by the reviewer, hearing loss in the child is likely due to the father, the proband. We corrected the sentence to "...transmission of an autosomal genetic mutation, not mtDNA, from the male proband,..." (p.16).

9. In page 17 and start of page 18, the authors discuss that the uncertainty of the role of the T7501A mutation which was found in three patients but not in controls will be enlightened by further studies. It would be essential to add that the studies needed are i) isolation of total mitochondrial RNA from lymphoblastoid cell lines derived from individuals with the T7501A mutation in order to examine the steady-state of the tRNA-Ser(UCN), and ii) measurement of the endogenous respiration rates of cell lines by determining the O2 consumption.

We appreciate the comments. Unfortunately, we could not obtain the consent from the patients to isolate blood cells for generating cell lines. We corrected the sentence as "It would be perceptive to await further investigation, such as haplogroup analysis or generating the lymphoblastoid cell lines for measurement of the endogenous respiration rates, to determine pathogenicity of the m.7501T > A variant....". (p.16)

10. There are a few typo errors in the text (‘otoxic’ instead of ‘ototoxic’, etc).

In response to the suggestions by all the reviewers, we sent the revised manuscript to an Scientific writing editing service.