Author's response to reviews

Title: Functional analysis of splicing mutations in exon 7 of NF1 gene

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Author's response to reviews: see over
Thank you for the opportunity to resubmit a revised version of the manuscript. We have modified the text addressing all the Editor’s and Reviewers comments.

In details,

**Editor’s Comments:**

- Ethics: a statement concerning the patient’s ethical approval for the study has been added in the Methods section of the manuscript.
- “Competing interests” section has been included between the Conclusions and Authors’ contributions.
- Language: the English style and grammar have been corrected.

**Reviewer’s Comments:**

Reviewer #1:

1) *Page1: We agree with the revision and the title has been changed according to it.
2) *Page1 (also page 10): the paragraphs “exon 7 was spliced in three alternative isoforms, including a transcript lacking NF1 exon 7” have been modified according with the revision.
3) *Page2: ”wild type” spelling has been used throughout the manuscript.
4) *Page2: ESE and ESS abbreviations have been spelled out.
5) *Page3: the typing error has been corrected.
6) *Page4: ”NF1 gene” spelling has been used throughout the manuscript.
7) *Page4: the paragraph that begins “Finally, several studies reported low expression of splice $NF1$ variants…” has been modified.
8) *Page4: the paragraph that begins “In this study we have investigated the functional consequences…” has been modified.
9) *Page4: the paragraph that begins “These included three previously…” has been modified.
10) *Page5: the typing error has been corrected.
11) *Page5: the section “Both sequence changes were found…” has been modified describing better the variant’ segregation in NF-01 family.
12) since the mRNA of the patients carrying the N335N variant wasn’t available, we couldn’t investigate exon 7 skip in vivo. We have added a section about the relationship between the c.476delC and the N335N in the discussion.

13) “SR proteins” abbreviation has been spelled out.

14) the paragraph that begins “Exon 7 of the NF1 gene and some flanking intronic sequences…” has been modified.

15) the typing error has been corrected.

16) the words order has been corrected.

17) the amount of template used in the PCR experiment has been added.

18) the words order has been corrected.

19) the typing error has been corrected.

20) the typing error has been corrected.

21) the word “hypothesis” has been replaced by “predictions”.

22) the words order has been corrected.

23) the typing error has been corrected.

24) the word “was” has been replaced by “included”.

25) the discussion has been revised in order to make it clearer.

26) “in silico” and “in vitro” have been typed in italics throughout the manuscript.

27) “in” has been replaced by “under”.

28) the reference [18] has been included.

29) “recognise” spelling has been used throughout the manuscript.

30) the paragraph “…shifts the equilibrium between …” has been modified.

31) the N335N variant was analyzed by ExonScan algorithm in order to make the creation of an ESE or the destruction of an ESS clearer. A section discussing this result has been added in the Discussion.

32) “doesn’t” and “didn’t” have been replaced by “does not” and “did not”.

33) we suggest just an hypothesis for NF1E7 role. Unfortunately we didn’t find in literature any background to support it.

34) the typing error has been corrected.

35) the paragraph “…proband’s daughter and nephew, both displaying the full-blown NF1 phenotype…” has been modified.

36) albeit a patient carrying Q315Q or L316M variant has never been reported, considering the minigene assay results, we can’t exclude that the Q315Q and L316M variants could alone cause neurofibromatosis type 1. So we hypothesize that it is important to investigate their role at the mRNA level, if they are found in NF1 patients.

37) the typing error has been corrected.

38) “onto” has been replaced by “in”.

39) author’s contribution paragraph has been shortened.

40) the typing error has been corrected.

41) the words order and the typing error have been corrected.

42) the text in the boxes has been enlarged.

Reviewer #2:

Major Revisions:

1) The sentences relating to R304X mutation have been modified, adding the references describing this mutation (Messiaen et al., 2000 and Pros et al., 2006). A section comparing the R304X analysis reported by Zatkova et al., 2004 and Hoffmeyer et al., 2004, and our results has been added in the Discussion.

2) The suggestion regarding the experiment has been considered in the revision. We have performed in duplicates RNA extraction and RT-PCR, and the SD6-SA2 PCR using 30 amplification cycles. The results’ section has been revised according to this quantification analysis.
3) A section suggesting a possible explanation for the presence of the 403 bp transcript has been added in the Discussion.
4) The Conclusions section has been revised.

Minor Revisions:
5) Reference 17 has been replaced by Ars et al., Nucleic Acids Res. 2000 Mar15;28(6):1307-12
6) Colapietro et al., 2003 and Zatkova et al., 2004 references have been added in the paragraphs dealing with the Q315Q, L316M and R304X mutations.
7) p.12: “nephew” has been replaced by “grandson”
8) p.12: “N333N” has been replaced by “N335N”
9) Figure1: variants have been included in the haplotype that has been marked by a box
10) Figure3a: lane numbers have been replaced by the amino acid change

Reviewer #3:

Major Revisions:
1) In order to make clearer that Q315Q and L316M mutations are in cis in the patient, we have designated it as “Q315Q/L316M” and we have indicated that our aim was to study their effect together and independently. We have also indicated that we have analyzed four nucleotide variants identified in three NF1 patients.
2) The typing error has been corrected.
3) A section describing skipping of other NF1 exons due to mutations and related references has been added to the Background. We think that our in vitro minigene assay can suggest a possible role for a nucleotide variant. In particular for the Q315Q, L316M and R304X mutations, our results were confirmed by in vivo analysis [Colapietro et al., 2003, Wimmer et al., 2000]. At the same time we think that, analysing different human tissues, the isoform’s amount could be different from our in vitro results, since alternative splicing is a tissue-specific mechanism.
4) Thomson et al., 2002 reference has been added in the Discussion.

Minor Revisions:
1) The section about the NF-01 family has been modified in order to make clearer the segregation of the N335N and c.476delC mutations. The discussion has been revised in order to indicate a possible role for the NF1AE7 and 430 bp transcripts. We have also explained the presence of the c.476delC variant as a result of a undetectable mosaicism, or a result of its de novo onset during spermatogenesis. The haplotypes on the pedigree have been boxed including the mutations.
2) The paragraph “We almost fully confirmed…” has been modified.
3) A section discussing the presence of the 403 bp transcript has been added in the Discussion.

Thank you for considering this revised manuscript for publication in BMC Medical Genetics.

Yours sincerely,

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