Author's response to reviews

Title: Mutation screening of melatonin-related genes in patients with autism spectrum disorders

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Author's response to reviews: see over
Dear Dr Norton,

Thank you for your e-mail dated February 10, 2010 regarding our manuscript “Mutation screening of melatonin-related genes in patients with autism spectrum disorders” (MS:7814088963291315), intended for publication in *BMC Genomics*.

We are grateful for the very valuable comments that we received from the three reviewers, and have now revised the manuscript in accordance with these.

Below please find our comments to all items raised by the reviewers.

Sincerely yours,
Jonas Melke

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**REVIEWER 1**

Comments:
There is increasing interest in the circadian rhythm genes as potential susceptibility genes for autism. This manuscript describes several new rare mutations in melatonin-related genes that may be related to autism. The authors add to their previous study on genetic variation in ASMT by confirming the reported rare splice mutation in ASMT (IVS5+2T>C) in another cohort of patients as well as by describing several other rare mutations in melatonin-related genes. Although 4 of the new mutations are observed in only 1 out of 109 cases, the authors recognize this limitation by stating that their “study sample is too small to conclude that mutations in melatonin related genes are enriched in patients with ASD”. What is more interesting is their finding that 2 variants in the ASMT gene are in the upstream regulator regions of the gene. As level of ASMT transcript has been demonstrated by this same group to be associated with lower melatonin levels, it would be of particular interest to determine if the new mutations regulate ASMT expression. Likewise, rare mutations in the melatonin receptor genes may have some relevance if transcription factor binding sites or expression level are affected.
RESPONSE: Although no revisions were required from this reviewer, we are grateful for these very valuable comments. The remarks regarding the mutations in regulatory regions have been taken into consideration in the new version of the manuscript (as suggested also by reviewer 3, see below)

REVIEWER 2

A) General remarks, which can be considered Discretionary Revisions:

1. It is a pity that the authors did not have data on melatonin or melatonin metabolites in blood or urine or data on sleep. This would have made the data even more interesting.

RESPONSE: We fully agree with the reviewer on this remark and it will certainly be our focus in forthcoming research using other patient cohorts. However, for the patient population used in the present study, we have tried to measure melatonin in blood but the results are not reliable enough for scientific use since blood samples were collected at different times of the day and since too many of the patients were unavailable for recollection of fresh blood samples.

2. Although of interest the conservation analysis does not seem to add anything to the results. The authors do not refer to it in the discussion. Unless they choose to discuss it in the discussion, I think it can be omitted from the paper.

RESPONSE: The GPR50 sequence is added in the figure and the result of the conservation analysis is now also referred to in the discussion, as suggested by reviewer 3 (see below)

B) Minor Essential Revisions and typo’s.

Abstract

General comment: The abstract is too condensed en the conclusion of the abstract too bold to be able to appreciate the content of the paper.

RESPONSE: The abstract in the revised version of the manuscript is now more detailed and the conclusion sentence is rewritten as suggested in point #6 and in minor point #1 by reviewer 3 (see below).

3. In the methods the control group should be mentioned.

RESPONSE: The control group is now mentioned (page 2, line 13-15) and renamed as suggested by reviewer 3 (see below)

4. In the results in the first sentence the control group should be mentioned also.

RESPONSE: The control group is now mentioned (page 2, line 18) also in the results section.

5. In the present wording the 2nd en 3rd sentence of the abstract causes confusion. Only after reading the paper, I could easily follow this part.

RESPONSE: For better readability and clarity, the wording of these sentences is now changed
6. The sentence in the conclusion: “Our findings provide further support for the notion that the splice site mutation, IVS5+2T>C, in ASMT may infer an increased risk for ASD.” is too bold, this is not what has been studied here, and should be rewritten more like the conclusion of the paper.

RESPONSE: The conclusion sentence is rewritten in the revised version of the manuscript (page 2, line 1-8).

7. Typo: 5th sentence – ‘...mutations...has been...’ – should be – ‘...mutations...have been...’

RESPONSE: This sentence is rewritten in the revised version of the manuscript.

Introduction
8. Page 3; 3rd paragraph; 5th sentence: “Moreover, several monogenic causes of autism are well known, such as fragile X syndrome, Rett syndrome and tuberous sclerosis, and recently rare mutations and copy number variations have been found to be causative or contributory factors for autism spectrum disorders [6-9].” – Since the term autism is generally used to indicate idiopathic autism, the authors should reword this sentence to something like: “Moreover, several monogenic disorders, such as fragile X syndrome, Rett syndrome and tuberous sclerosis, are well known causes of autism like behavior patterns, and recently, rare mutations and copy number variations have been found to be causative or contributory factors for autism spectrum disorders [6-9].”

RESPONSE: This sentence is rewritten in the new version of the manuscript (page 4, line 1-5)

9. Page 4; 8th sentence: I would say that the data on the effect of melatonin on sleep disturbances in ASD are not yet that strong, that one should use ‘greatly improved’ here. Just ‘improved’ would be enough at this point.

RESPONSE: The word “greatly” is omitted and “improve” is used in the revised version of the manuscript (page 4, line 14).

10. Typo’s: Page 6; 2nd sentence – ‘raging’ – should be ‘ranging’; 3rd sentence – ‘pateints’ – should be ‘patients’

RESPONSE: Corrected in the new version of the manuscript (page 6, line 12).

Methods
11. The ‘patient recruitment and clinical assessment’ paragraph needs a table with patient characteristics. The percentages of patients in the different diagnostic groups of the second group are odd; i.e. 10% of 44 subjects is 4.4 subjects – 4 subjects = 9.1%; 5 = 11.4%. A table with actual numbers and percentages will be more clear.

RESPONSE: We have now written the actual number instead of percentages of patients (page 6, line 2-12). However, we do not believe that another table is necessary for the presentation of the clinical characteristics this relatively short paper. If the editors would like us to rewrite these numbers into a table, we will of course do so.
12. Although no statistical analyses were done, a paragraph with the ‘analytic plan’ and the considerations for the choices made is needed. Some is there in the ‘recruitment’ part, some in the ‘DNA analysis’, some in the ‘Results’.

RESPONSE: We fully agree with the reviewer on this point and we have now added a paragraph in the methods-section where the analytical plan is presented. (page 8, line 1-12)

Results
13. Page 7; 1e paragraph of Results; 4th sentence: “Of the three missense variants identified, S493N in GPR50 and V124I and K243R in MTNR1B, only the V124I variant MTNR1B was absent in controls (Table 1).” The wording is unclear. It would help to first report that of the found rare variant three were missense mutations and after that elaborate the of those three two were found in patient as well as controls.

RESPONSE: As reviewer 3 discovered (see answer to point #1, below), the two non-synonymous SNPs S493N in GPR50 and K243R in MTNR1B have now been reported to dbSNP and are considered as “previously known” in the revised version of the manuscript. Thus, this sentence is now rewritten, as well as the final sentence of the results section where the common polymorphisms are presented (last paragraph of the results section, page 9, line 19 to page 10, line 3).

Discussion and conclusion
14. Typo: Page 10; 2nd paragraph; 1st sentence: ‘...support the notion...’ – should be ‘supports the notion’

RESPONSE: Corrected in the revised version of the manuscript (page 11, line 15).

REVIEWER 3

A) Major Compulsory Revisions (5 points)

1) The text does not clearly express which SNPs are novel variants found by this study and which have already been reported as SNPs in the publicly accessible databases. As the variants c1478 G>A (rs62620754) in GPR50 and c728 A>G in MNTR1B (rs61747139) are variations already in the dbSNP database, these SNPs should be reported as such. Even though the population diversity of these database SNPs is currently undefined, calling these SNPs rare variants is confusing and also conflicts with the results in Table 1. Regarding the statement that begins the Results section:- “Six rare variants and two previously unreported polymorphisms in the investigated genes were found during the screen (Table 1).” This could be misleading. This statement should be corrected and table 1 amended (add rs codes for example) to differentiate novel SNPs from known SNPs.

RESPONSE: We apologize for this mistake and of course we fully agree with the reviewer that the polymorphisms rs62620754 and rs61747139 should be treated as “previously known” in the manuscript. This was an unfortunate consequence of the time that has passed since the submission of our first draft of the manuscript (to another journal) when these polymorphisms were not reported in dbSNP. The two polymorphisms are now omitted from the table and the results text where the novel variants are presented (page 8, line 13). In the revised version of our manuscript, the two polymorphisms (rs62620754 and rs61747139) are presented in the context of common polymorphisms (last paragraph of the results section, page 9, line 19 to
2) Focusing the results on the novel findings would put more emphasis on the bioinformatics analysis, which should be enhanced. For example, three alternative transcripts for ASMT are known. These are not considered in the report. Where are the novel 5’ SNPs in relation to the ASMT alternative transcripts? It appears that the position of the novel SNP c-38C>T and that of the ASMT-expression-altering SNP rs5989681 is identical (one nucleotide 5’) relative to the start of the ASMT-001 and ASMT-002 transcript respectively, this is interesting. Indicate the extent of the 5’UTRs of the alternative ASMT transcripts in figure 2 and use rs SNP codes and perhaps genome co ordinates to help in the presentation and discussion of this data in the text and figures.

RESPONSE: We fully agree with the reviewer and we are very grateful for the thorough examination of our bioinformatics analysis. In the revised version of our manuscript, we have included the transcription start of ASMT001 and ASMT003, as well as the splice site between exon 1 (non-coding) and exon 2 of ASMT002 (identical to the transcription start of ASMT003) in figure 2. Moreover, a potential effect of identified rare variants on alternative splicing is now discussed when the results regarding 5’-variants are referred to in the discussion (page 10, line 15). However, we believe that the reviewer have misinterpreted our figure when the positions of SNP rs5989681 and the novel SNP c-38C>T are said to be located at similar distance from the transcription start of ASMT001 and 003, respectively. The c-38C>T identified in our study is located in the 5’UTR (untranslated but expressed in all three transcripts), whereas the rs5989681 is located in an unexpressed region (for all transcripts). We have used the nomenclature of Human Genome Variation Society (www.hgvs.org), where it is recommended to use the translation start as reference. Hence, variants in the 5’UTR have negative numbers and the c in the name means that the coding DNA is used as reference sequence (see http://www.hgvs.org/mutnomen/recs-DNA.html#sub for examples). We believe that the figure is clearer now and that the indication of the different transcription starts will help the initiated reader in interpreting our results.

3) For the analysis of the novel variant c370G>A in MNTR1B where peptide sequence conservation is depicted, the valine residue also appears to be conserved in GPR50. This is not considered in the manuscript. This should be considered and discussed as this may alter the impact of this finding either way. The alignments should be shaded and discussed in the context of protein families/clans and functional domains.

RESPONSE: The GPR50 sequence is added in figure 2 and referred to in the result text (page 8, line 19). The result of the conservation analysis is in the revised version of the manuscript also referred to in the discussion (Page 11, line 1).

4) Re: Line 3/ page 6/, sentence starting “Pateints from both populations…” Did all the patients from each population get both DSM-IV and ADI-R assessment? If not give the % for each assessment in each group. If all the patients received both assessments insert “All” at the beginning of the sentence.

RESPONSE: All patients were assessed with both instruments and the sentence is now rewritten as suggested (page 6, line 13).

5) Regarding the use of terms, we consider the unaffected sample to be more of a comparison group than a control group. Indeed, the authors state (page 6 line 10) “The control group in
this study served only to investigate if mutations identified in patients were present also in the general population”. This point however is evident from the SNP and human genome sequence databases. The comparison group merely gives us an indication that the specific population surveyed is in keeping with the human genome database information regarding common SNPs. It does not properly act as a control, as the authors rightly point out. The terms “control” and “control group” should thus be replaced with an alternative, more appropriate term in a number of places in the manuscript.

RESPONSE: We fully agree with the reviewer on this remark and in the revised version of the manuscript, we have used the terms “comparison group” and “comparison subjects” instead of “controls”. Moreover, since our mistake regarding SNPs rs62620754 and rs61747139 now is corrected (see remark #1 above), it is hopefully more evident to the reader that we are not comparing allele frequencies statistically between the patient and the comparison group.

Minor Essential Revisions (6 points)

1) Abstract/ Page 3/ Conclusions/ line 2: Moreover, our results suggest that also other melatonin… should read… Moreover, our results also suggest that other melatonin…

RESPONSE: The conclusion paragraph of the abstract is rewritten in the revised version of the manuscript, as suggested in a general remark from reviewer #2 (see above)

2) Background/ Page 4/ …. Break this into paragraphs at line 14 and 19 say.

RESPONSE: As suggested, this paragraph is in the revised version divided into three paragraphs.

3) Methods/ Page 5/ line 10/ Sachska childrens hospital…presumably should be Sachska Children’s Hospital

RESPONSE: Sachska Children’s Hospital is now used (page 6, line 8).

4) Methods/ Page 6/ line 3/ … Spelling… Pateints should be Patients.

RESPONSE: Patients is now correctly spelled (page 6, line 13)

5) The grammar of the first sentence of the second paragraph of the Discussion requires correction and in the third sentence of the Discussion we read, “Moreover the most interesting of the identified variants were identified in patients…etc.” Which variants? Please specify and say why they are the most interesting.

RESPONSE: The second paragraph of the Discussion (page 10, line 4) is now rewritten and in the revised version of the manuscript, we refer to the bioinformatics analyses in the discussion (Page 10, line 20 to page 11, line 7). We are not using the phrase “the most interesting of the identified variants….” in the revised version of the manuscript, since each identified variant now is discussed in more detail (as suggested above).

6) In addition to ref. 21 line 8 page 10, cite Hu VW et al., (2009) “Gene expression profiling differentiates autism case-controls and phenotypic variants of autism spectrum disorders:
evidence for circadian rhythm dysfunction in severe autism”.

RESPONSE: The article by Hu VW et al., (2009) is cited as reference number 21 in the revised version of the manuscript.

**Discretionary Revisions**

Consider using additional bioinformatics tools to investigate the novel SNPs more thoroughly rather than the promoter regions in general. For example, an alternative scan tool might pick up a site that is altered by the c-39 G,C >A,A variation in MNTR1B. It is a pity that the work is unsupported by any association tests on the novel SNPs or the effect of the novel promoter region SNPs on gene expression but overall this manuscript offers some new data that could inform future expression studies and population genetic analysis of melatonin related genes in autism.

RESPONSE: We fully agree with the reviewer that association tests and functional studies would have strengthened our results, however, with the relatively small patient cohort we had available for the present study, the main goal was to identify novel (and previously reported) mutations in patients with ASD. However, we hope that we will be able to present new data regarding these variants when larger patient groups are available to us. In addition, that the absence of functional studies is an important limitation of our study is now mentioned in discussion (page 11, line 8).