Author's response to reviews

Title: A PARK2 Polymorphism Associated with Delayed Neuropsychological Sequelae after Carbon Monoxide Poisoning

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Author's response to reviews:

Dear Prof. Cenzon,

Thank you so much for your letter dated August 9, 2013, and the referees’ comments about our manuscript entitled “A PARK2 Polymorphism (rs1784594) Associated with Delayed Neuropsychological Sequelae after Carbon Monoxide Poisoning” (Manuscript ID: 1153005392956574). Following your suggestions, guidelines and comments, we have revised the manuscript again. The revised manuscript and a response to the referees and listing the changes are updated through the Author Center on the BMC Medical Genetics online submission website. Should you have any question, please contact me without hesitate.

Thank you so much for your consideration!

Sincerely yours,

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Response to the comments of referees:
Manuscript ID: 1153005392956574
A PARK2 Polymorphism (rs1784594) Associated with Delayed Neuropsychological Sequelae after Carbon Monoxide Poisoning

First, we would like to express our sincere appreciation to Prof. Cenzon for his guidance, and also to the anonymous referee for his/her valuable comments and constructive criticism which greatly improved our manuscript. Below you will find the clarification on the remarks of the referee. The original comments of the referee are shown in italic. In manuscript, the revised comments were showed highlight.

Answers to Reviewer Ana Bakija-Konsuo:
Response to comment: Thank you so much for your approval comments.

Answers to Reviewer Conceicao Bettencourt:

Major Compulsory Revisions:

1) In the DNA extraction and genotyping section of Methods, an excessive and unnecessary description of certain details is given (e.g. DNA purity and concentration measurements). However, the genotyping method is not clearly described. Did the authors use the Infinium HD® 660W-Quad Assay or PCR followed by RFLP? Was the Infinium HD® 660W-Quad Assay used just in a few samples to help deciding which SNPs should be studied? Were all samples of each group pooled together or genotyped individually? The authors state “To assess transcript integrity, PCR products were digested…”, but all the analyses were performed using DNA not RNA samples. Was the digestion with Cail performed to see the amplicons integrity or to determine the genotypes for each SNP? This entire section should be improved and made clearer.

Response: We have revised the method section according to this comment. The first manuscript is unshaped with nonstandard description and some mistakes, even using of the restriction enzyme. In a previous study, we conducted pooling-based genome-wide association study in two independent samples of CO poisoning patients with or without DNS using the Infinium human 660W-Quad array. PARK2 was one of the promising genes (unpublished data). In the present study, another cohort was used to determine if these variants influence DNS susceptibility.

2) More details about the statistical analysis should be provided. What was the model used for logistic regression of each SNP’s main effect (i.e. what independent variables were included?). Was the major allele considered as the referent? What was the mode of inheritance used in the model (dominant, recessive, additive, co-dominant)? Did the authors really test for interactions (SNP-SNP or any other)? If so, how was the model constructed? What variables were considered? What mode of inheritance was used in the model? From tables 3 and 5, and without a detailed description of statistics in the methods, it seems that the results shown are not for any interaction, contrary to what the legends
indicate. The two tables show the coefficient for an intercept, but that intercept is not the same as an interaction. The results shown in those two tables are just the results for two models of logistic regression adjusted for each of the variables shown in the tables and without any interaction term. Moreover, if DNS is the outcome (dependent variable) it cannot be said for example “Logistic analysis for the interaction between… and DNS”. Did the authors want to say “Logistic analysis for the association between… and DNS”? I believe the authors are confusing association with interaction.

Response: There are some troubles in the analysis of the first manuscript. We have revised the method section according to this comment. The statistical analysis was performed afresh using the SNPStats, a web tool. To evaluate interactions between SNP and sex, a global test for interaction was performed in the co-dominant model, in addition to a test for the interaction in the linear trend of the nested variable (Table 5).

3) As stressed by the authors in the Background section, “the incidence of DPHL is higher in patients over 40 years of age and increases progressively with age”. Therefore, the authors should take into account a possible effect of age, and should adjust their models by age.

Response: This is a valuable comment. We have adjusted the models by age when we recomputed the data. Then some results changed a little. The result of association analysis still remained (Table3). We have revised according to this comment.

4) Are there cases of individuals exceptionally presenting signs of DNS after a latent period of more than 90 days?

Response: The patients were divided into two groups according to the result of followed-up. In my past 30 years of research work, no individuals exceptionally presenting signs of DNS after a latent period of more than 90 days. The latent period usually was 3 to 60 days. Most of papers report 2 to 40 days. (Some reference below)


5) The two PARK2 SNPs analyzed by the authors are intronic SNPs. Which functional effect do the authors expect? Are these SNPs in linkage disequilibrium with coding SNPs?
Response: Thanks for this comment. We also noticed it. These SNPs are not in linkage disequilibrium with coding SNPs. It isn’t all positive SNPs own clear function, even in Genome-wide association (GWA) studies. When we evaluated the biological functions using FASTSNP online service, rs1784594 get a medium-high risk score related to splicing regulation.

When the causative gene variation has a high penetrance nearby a genetic marker, it can lead to this marker display significant differences between cases and controls even if there is only a weak LD between them. Sometimes the marker has a long physical distance to the gene, but it still belongs to the same LD block, the marker still shows the function of this gene.

Some SNPs fall in intron. They may locus in other function sequence. For example, miRNA, regulatory sequence and the region related to chromosome structure. The data from the Encyclopedia of DNA Elements (ENCODE) project enabled us to assign biochemical functions to 80% of the genome, compared to 5% in the past (Dunham et al., 2012). It is possible those SNPs are involved in unknown function of non-coding DNA. In the whole genome level, some loci have the statistical linkage in case control study. In fact, they are not contiguous, even not in the same chromosome.


6) Besides the association found between the rs1784594 and DNS, it seems that gender is also associated with DNS. Therefore, this result should be highlighted in the manuscript, since it also contributes for the identification of a vulnerable patient group (females) to permanent disability following CO toxicity. Can the authors speculate on why females are more susceptible to DNS?

Response: To determine the gender effect, genotype and allele frequency in both sexes were assessed. The female DNS cases exhibited a significant difference in allele frequencies of rs1784594 compared to female controls. But there is no significant interaction between SNP and sex. We often say the cassation present gender difference. The result present in the first manuscript is incorrect because the analytical method. No further evidence show that gender is also associated with DNS.

DNS was a disease resulting from interactions between environmental factors (CO) and an individual’s biological background. The aim of this study was to explore genetic susceptibility about the normal human’s different consequences after poisoning. The difference caused by gender effect is hard to ignore, especially in endocrine, development, autoimmunity and stress reaction. Sex-specific association was often reported in other psychosis. The female-specific association may be attributed to the interaction between estrogen and genes related to brain development.

7) In table 2, I assume that the numbers in parenthesis are the genotype frequencies. However, they are not correct (the sum surpasses 100%). For example, for the first SNP the frequency of the CC genotype in cases should be 17.2% and it is 49.2%. The authors should carefully correct this table.
Response: Thanks! We are really sorry for my carelessness, and it is a mistake in this data. We have revised it according to this comment.

Minor Essential Revisions:
1) The gene symbol (PARK2) should be in italics.
Response: We have revised it according to this comment.
2) In the Background, the authors state “In a previous study, we conducted genome-wide SNP genotyping of CO poisoning patients with or without DNS using the Infinium human 660W-Quad array [13]”. However, reference number 13 does not belong to the authors nor mentions genome-wide SNP genotyping of CO poisoning patients with or without DNS. The correct reference should be provided. What were the most relevant results from the authors’ previous study? Does the Infinium human 660W-Quad array include the SNPs analyzed in the present study or other PARK2 SNPs?
Response: The correct reference was provided. Five SNPs met association signal in pooling-based genome-wide association study. The biological functions and risks associated with individual SNP sites were evaluated using FASTSNP online service (Yuan et al., 2006), and the GeneCards data base (http://www.genecards.org/). Finally, the PARK2 gene polymorphisms rs1784594 and rs1893895 were chosen. We have revised it according to this comment. The most relevant result from previous study is the Neurexin 3 gene.
3) Infinium HD® 660W-Quad Assay is an Illumina platform, not QIANGEN’s as stated in the methods.
Response: We have revised it according to this comment.
4) The authors say in the Statistics section of Methods: “The allele frequencies between cases and controls were first tested for Hardy-Weinberg equilibrium”. Did the authors wanted to say “The genotype frequencies in cases and controls were first tested for Hardy-Weinberg equilibrium”? 
Response: We have revised it according to this comment.
5) In the Results, it is “the 1784594 TT genotype frequency was significantly lower verses CC”, and it should be “the 1784594 TT genotype frequency was significantly lower versus CC”. Also, the authors say “Finally, rs1784594 was still associated with DNS when rs1894895 genotype and hypertension were included in the logistic analysis (Table 5)”, but table 5 shows gender instead of the rs1894895 genotype. Thus, it should be “…rs1784594 was still associated with DNS when gender and hypertension were included…”.
Response: We have revised it according to this comment.

Answers to Reviewer Nigel M Williams:

Major Revisions.
the authors state that they initially performed an analysis using pooled DNA samples with an 660W0quad array. If this is the case then the results should be presented. It is not clear how the authors selected the 2 SNPs for analysis. If it was from the genome-wide pooled analysis then the results should be genome
wide corrected. This ambiguity has to be addressed before the data can be interpreted.

Response: Pooling base GWA is difficult to reach the standard GWA due to its disadvantage. Pooling DNA in the first stage of a GWA study substantially reduces costs, and has been shown to be an efficient method to select candidate susceptibility loci for follow-up by individual. The DNA pooling focuses on initial screening of promising SNPs. It more like candidate gene studies in individual stage. As with most pooling base GWA, p-values of the individually not always come close to the genome-wide significance level. It will more perfect in that case. Even so, the result still is reliable and interesting.

Despite significant replication values of some promising SNPs found in the GWA in pooled DNA, many papers reported the lack of replication for some of the loci identified in the pooling based analysis. Some loci lost the statistical differences in individually stage. For example, two of five selected regions were found to be significant at individual level in both pooling samples and replication set in the study about atopy (Castro-Giner et al., 2009). Shifman et al. reported that nine SNPs of 194 selected SNPs had p-values below 0.005 in individually genotyped stage based on pooling result (Shifman et al., 2008). Even the loci got replication, some the pooled p-values and the p-values for the individually had a disparity (Castro-Giner et al., 2009; Liu et al., 2011; Janicki et al., 2011; Krumbiegel et al., 2011).


Minor corrections.

1. In table 1, the numbers of male/females typed in controls are incorrect.

Response: Thanks! We are really sorry for my carelessness, and it is a mistake in this data. We have revised it according to this comment.

2. In table 2, the percentage of genotypes are wrong in all situations.
Response: Thanks! We are really sorry for my carelessness, and it is a mistake in this data. We have revised it according to this comment.