Author's response to reviews

Title: Genetic polymorphisms involved in dopaminergic neurotransmission and risk for Parkinson's disease in a Japanese population

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Author's response to reviews: see over
Response to Referee #1

Comment 1.
P6: it is unlikely that our hospital controls shared a genetic predisposition with PD. What do the authors mean? Why is it necessarily so?

▼Response to comment 1.
As suggested, we have deleted the sentence.

Comment 2. The mean male proportion was 38%, which is much lower than in most other PD populations. Was this a selected PD population?

▼Response to comment 2.
A male preponderance of PD has been reported in European countries and the USA. However, the prevalence of PD was 61.3/100,000 men and 91.0/100,000 women, showing that women were significantly more affected by PD than men (P < 0.001) (Kimura H, et al., Female preponderance of Parkinson's disease in Japan. Neuroepidemiology. 2002; 21(6):292-6). The male proportion of 38% is comparable to that of other Japanese population (40%). Although the incidence of PD may be similar between men and women in Japan, the prevalence of PD becomes more likely in women simply because women tend to live longer than men do. We think that our PD population was not biased.

Comment 3-1.
There were very few smokers. To my knowledge the rate of smokers in Japan is much higher, was this due to the high rate of females?

▼Response to comment 3-1.
According to National Health and Nutrition Survey in Japan, the prevalences of current smokers among males in their 50s, males in their 60s, males aged 70 or over, females in their 50s, females in their 60s and females aged 70 or over were 42.3%, 32.8%, 18.6%, 9.3%, 7.3% and 3.7%, respectively (please refer to the below table). The prevalence of current smokers among each age category remained steady for several years. As the male proportion was 38%, as suggested, the prevalence of current smokers among males and females combined results in a low prevalence due to the higher rate of females.
Table. Prevalence of current smokers in Japan

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>20s</th>
<th>30s</th>
<th>40s</th>
<th>50s</th>
<th>60s</th>
<th>70+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>37.9</td>
<td>47.5</td>
<td>55.6</td>
<td>49.1</td>
<td>42.3</td>
<td>32.8</td>
<td>18.6</td>
</tr>
<tr>
<td>Female</td>
<td>11.0</td>
<td>16.7</td>
<td>17.2</td>
<td>17.9</td>
<td>9.3</td>
<td>7.3</td>
<td>3.7</td>
</tr>
</tbody>
</table>

National Health and Nutrition Survey, 2007

**Comment 3-2.**
How did they calculate the p<0.0001 difference between smokers and non-smokers?
Which groups were grouped?

**Response to comment 3-2.**
The two-way chi square is a convenient technique for determining the significance of the difference between the frequencies of occurrence in two or more categories with two or more groups. We may see if there is any difference in the number of current smokers, former smokers, or non-smokers in regards to case-control status.

<2×3 table>

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smokers</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>Former smokers</td>
<td>57</td>
<td>97</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>174</td>
<td>222</td>
</tr>
</tbody>
</table>

j: smoking status (three categories)  i: case-control status (two categories)

\[ \chi^2 = \sum_{i=1}^{3} \sum_{j=1}^{2} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} \]

Degree of freedom (d.f.) = (i-1) * (j-1) = (2-1) * (3-1) = 2

The P-value is the probability that a chi-square statistic having two d.f. is more extreme than 21.37.
The P-value for the \( \chi^2 \) of 21.37 (d.f. = 2) is 0.00002289. ===> P<0.0001
**Comment 4.** In Table 2 the number of controls in the DRD2 column is less than 300, why?

**Response to comment 4.**
As suggested, that is completely our mistake. We have inadvertently written the number of subjects with the TT genotype in the space for the number of subjects with the CC genotype.

<table>
<thead>
<tr>
<th>DRD2 rs1800497</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT (ancestral)</td>
<td>29 (12.2)</td>
<td>52 (14.1)</td>
</tr>
<tr>
<td>TC</td>
<td>117 (49.2)</td>
<td>192 (52.0)</td>
</tr>
<tr>
<td>CC</td>
<td>92 (38.7)</td>
<td>125 (33.9)</td>
</tr>
</tbody>
</table>

**Comment 5.** In Table 3 the number of controls in the MAOB and DRD2 columns are difficult interpret, the authors should comment on that.

**Response to comment 5.**
The referee #1 is completely right. We have corrected our mistakes including mistakes regarding DRD4.

<table>
<thead>
<tr>
<th>MAOB rs1799836</th>
<th>GG (G) + AG</th>
<th>40/70</th>
<th>6/26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA (A)</td>
<td>134/152</td>
<td>58/121</td>
</tr>
</tbody>
</table>

| DRD2 rs1800497 | C + TT     | 108/153 | 38/91 |
|               | CC         | 66/69   | 26/56 |

| DRD4 rs1800955** | CC         | 25/37   | 10/33 |
|                  | TT + TC    | 149/184 | 54/114 |

We would like to thank earnestly the referee #1 for his helpful comments.
Response to Referee #2

**Comment 1.** A long history of case-control association analyses highlight the likelihood that modest associations, such as the ones revealed here, are merely chance findings. Many reviews could be cited that list the reasons for this. The two SNPs with reported trends to association in this manuscript (rs4680 and rs1799836) have been previously studied in many different populations (including Asian populations) and meta-analyses provide no evidence for association (one can refer to the PDGene website for this information [Lill CM, Roehr JT, McQueen MB, Bagade S, Kavoura F, Schjeide BMM, Allen NC, Tanzi R, Khoury MJ, Ioannidis JPA, Bertram L. The PDGene Database. Alzheimer Research Forum. Available at: http://www.pdgene.org/]. Of course it is possible that there are reasons why the distribution of the COMT and MAOB alleles are different in this specific case-control sample, but it is probably not specifically related to PD-risk.

**Response to comment 1.**
As suggested, the rs4680 and rs1799836 polymorphisms have been repeatedly examined in many different populations including Asian populations. As promising SNPs in one study often failed to replicate in other studies. Replication of findings is very important before any causal inference can be drawn.

We have added the some sentences to the section of discussion (lines 370-372 in the revised manuscript).

**Comment 2.** It is difficult to assess the evidence for gene x environment interactions when the environmental variable is crudely measured (such as an ever/never self report) as it is usually important to probe for dose effects and other evidence against chance findings when trying to interpret the results of such an analysis. In this case it is unclear what to make of a p=0.06 for an interaction between smoking and rs4680 genotype status.

**Response to comment 2.**
As discussed in this manuscript, discrepancies between self-reported smoking habits and biochemical verification are minimal among the general population. Furthermore, most studies have consistently reported that ever-smoking was associated with an decreased risk of PD compared with non-smoking in various settings (different
populations, different study designs) (Kiyohara C & Kusuhara S. submitted). As compared with smoking amount (i.e., Brinkman index), smoking status is crude but accurate. To calculate Brinkman index (the sum of the number of cigarettes smoked per day multiplied by years of smoking), detail information using a validated questionnaire should be required because smoking behavior changes with life-stage (dramatically in women), however. Furthermore, as smoking amount is a continuous variable, we must determine a cut-off point(s) to assess a gene-environment interaction. As researchers do not know a medically significant cut-off point(s), researchers must determine the cut-off point(s) based on the distribution in controls. We think that smoking status (an ever/never) is not inferior to a statistically determined cut-off point(s).

As the P value of 0.06 is statistically meaningful, a modest interaction between smoking and rs4680 may not be caused by chance.

Comment 3. There are many different genes involved in dopamine metabolism and transmission and each gene has many commonly-occurring polymorphisms. While it is possible to make valid arguments as to why these specific SNPs could influence risk, it seems scientifically unsatisfying to test these without being comprehensive in the approach. The scientific community has moved away from this approach in recent times. As to whether dysfunction of dopaminergic neurotransmission in the CNS impacts on the risk for PD, I think the jury remains out. Some researchers have their doubts (see J.E. Ahlskog “Beating a dead horse: dopamine and Parkinson disease”. Neurology, 2007, 69 (17), p1701-1711 for a discussion on this topic).

Response to comment 3.
As suggested, there are many different polymorphisms involved in dopamine metabolism and transmission. In this study, candidate genes were selected on the basis of their dopamine receptor and metabolism. Among them, we focused on commonly reported functional (or suspected functional) polymorphisms because commonly studied polymorphisms would provide useful information.

Although a comprehensive approach such as GWAS [for example, the study reported in Lancet in 2011 (377 (9766):641-9. Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies) may be the most comprehensive study to date of the role that common genetic factors play in PD] may be powerful, there are several limitations such as lack of
information on gene function, insensitivity to rare SNP variants, requirement for large sample sizes, biases due to selections of cases and controls and so on. Unlike hypothesis-free GWAS, candidate gene studies depend on prior knowledge or guesses regarding which genes might be important (hypothesis-driven). As described in our manuscript, studying gene-environment interactions in relation to PD risk may be valuable because positive findings would clearly implicate disease-causing exposures, clarify PD etiology, and elucidate environmental modifications for disease prevention. From the standpoint of preventive medicine, as an understanding of gene-environment interactions may help identify the high-risk group for developing PD, the results of candidate gene studies with gene-environment interaction provide us helpful strategy (high risk strategy) for the prevention of PD occurrence. From the etiologic standpoint, comprehensive studies may be more valuable to elucidate molecular pathogenesis.

As suggested, as to whether dysfunction of dopaminergic neurotransmission in the CNS impacts on the risk for PD, no final decision has been made yet. As the accumulation of putative "at-risk" alleles was significantly associated with an increased risk of PD, we think that we must continue to investigate the association between dopamine-related genes (polymorphisms) using candidate gene approach for some time in the future.

Some more specific question and comments that the authors may wish to consider:
Comment (a). What was the age-at-onset for the PD cases in the study? How many had a family history of PD and did any have PARK2 disease?

▼Response to comment (a).
The age-at-onset for the PD cases in the study was added to the revised manuscript (lines 134-135 in the revised manuscript).

The prevalences of first degree family history of PD among PD patients and controls have been added to Table 1 and lines 192-193 in the revised manuscript. As first degree family history of PD was newly included in the multivariate logistic regression model, the adjusted ORs slightly changed.

As there were no patients with PARK2 disease (juvenile PD), we have added one sentence (line 135 in the revised manuscript).

Comment (b). Is it possible that the increased smoking exposure reported in the
hospital-based controls relates to these individuals having smoking-related illnesses rather than a reduced risk of PD due to smoking (thus the apparent protective effect of smoking in this sample may be inflated.

▼Response to comment (b).
As suggested, the generally held view is that smokers are more prevalent among hospital patients than in the general population because many conditions that lead to hospitalization are caused by or associated with smoking; this increased prevalence may bias results of case-control studies of smoking-related diseases (Gordis L: Case-control and cross-sectional studies. In: Epidemiology. 3rd ed. pp. 159-176, Elsevier Saunders Philadelphia, 2004). Our meta-analysis on smoking and PD (Kiyohara C & Kusuhara S, submitted) showed that the summary ORs for ever-smokers were 0.57 (95% CI= 0.51 - 0.63) according to data from population-based studies and 0.50 (95% CI = 0.43 - 0.56) according to data from hospital-based studies. Former smoking was significantly associated with a decreased risk of PD in hospital-based case-control studies (summary OR = 0.62, 95% CI = 0.46 - 0.77) but marginally associated with a decreased risk in population-based case-control studies (summary OR = 0.85, 95% CI = 0.68 - 1.01). For current smokers, the summary ORs among population-based and hospital-based controls were 0.30 (95% CI = 0.22 - 0.39) and 0.40 (0.18 - 0.62), respectively. Although smoking had a somewhat greater impact on PD risk in hospital-based case-control studies than in population-based case-control studies, many hospital-based case-control studies including our study have an inflated opinion of the protective effect of smoking.

Comment (c). The presentation of the gene-environment interaction data in Table 3 is rather confusing (particularly regarding the choice of the reference group (which seems to be ever smokers with a different choice of specific genotype for each SNP locus).

▼Response to comment (c).
A different choice of specific genotype for each SNP locus depends on how "at-risk" allele of the SNP behaves (dominant or recessive effect). Although Table 3 may be confusing, there are no alternatives. To make Table 3 more understandable, we have made a little modification.

Comment (d). The idea to investigate the load of “at-risk” alleles is an interesting one, but I feel that having such a small reference group (with only 2 cases and 6 controls who do not possess any “at-risk” alleles) somewhat invalidates this. Perhaps there
would be better ways to examine this data.

▼Response to comment (d).
We also think that the accumulation of "at-risk" alleles is a useful strategy for assessing the risk of PD. As suggested, a small reference group (with only 2 cases and 6 controls) was only used to assess per-"at-risk" OR. As the reference category is generally the absence of exposure (risk factor), a reference group should be a group with a lower number of "at-risk" alleles based on our hypothesis. In addition to another analysis (reference group; 10 cases and 22 controls) included in original version, we have newly added an alternative result (reference group; 35 cases and 68 controls) to Table 3. Our results indicated that the accumulation of "at-risk" alleles was significantly associated with an increased risk of PD.

We have added the some sentences (lines 169-170, 247-250 and 345) in the revised manuscript.

We would like to thank earnestly the referee #2 for his helpful comments.