Author's response to reviews

Title: Role of the H1 haplotype of microtubule-associated protein tau (MAPT) gene in Greek patients with Parkinson's disease

Authors:

Nikolaos Refenes (nrefenes@pharm.uoa.gr)
Juliane Bolbrinker (juliane.bolbrinker@charite.de)
Georgios Tagaris (tagaris@otenet.gr)
Antonio Orlacchio (a.orlacchio@hsantalucia.it)
Nikolaos Drakoulis (drakoulis@pharm.uoa.gr)
Reinhold Kreutz (reinhold.kreutz@charite.de)

Version: 5 Date: 15 May 2009

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Version: 2  Date: 15 May 2009
Dear Editor,

We are grateful for the helpful comments of the reviewers of our paper. We have taken into account all suggestions and responded to each comment point by point as you can see from our responses to each reviewer, respectively. We have revised our manuscript accordingly.

Reviewer #1:

1. “The choice of only two "H1-SNPs" in addition to the H1/H2 division would not account for the entire diversity of the MAPT locus in their study population and coverage is therefore incomplete. A more comprehensive approach would be to identify/genotype haplotype-tagging SNPs representative of the Greek population. The authors should discuss this and also describe the SNPs in context with previous studies and their location within MAPT (eg rs242562 is in LD with rs242557).”

We agree with the reviewer that our genetic analysis of the MAPT locus presented in our original manuscript appeared to be somewhat incomplete. The objective of our study was to add new data on the previous two contradictory studies in Greek PD patients with regard to the H1H1 genotype effect and the relevance of H1 sub-haplotypes.

As previously described by Pittman et al. in 2005, six haplotype-tagging SNPs can capture more than 95% of haplotype diversity of the MAPT locus (rs1467967, rs242557, rs3785883, rs2471738, deletion-insertion in intron 9, rs7521). In response to the reviewer’s comment we have in the revised manuscript included SNP rs3785883, which was moderately associated with PD in Greek patients as reported by Fung et al. in 2006. In addition, we also genotyped our patients for SNP rs242557 in response to another reviewer’s comment. However, it turned
out, that SNP rs242557 was in complete LD with rs242562 among our H1H1 subgroup of patients. We added this information into the Results section of our revised manuscript. Overall, we have analyzed 5 polymorphisms at MAPT including the H1/H2 polymorphism, rs242562, rs2435207, rs242557, and rs3785883. We did not genotype rs2471738, because it is known that this SNP is in strong LD with rs2435207 according to the study by Zabetian et al. in 2007, who reported D’=0.98 in PD patients and D’=0.97 in controls. Consequently, we feel that it was not necessary to genotype rs2471738. Taking together, we have improved our analysis of the MAPT locus and feel that the analysis of additional SNPs would not significantly affect our results and conclusions.

In the revised manuscript we added in response to the reviewer’s comments the following changes:

In the Methods section we added the information on the two additionally analysed SNPs rs3785883 and rs242557. In addition, we added the following new paragraph on the genotype selection in the Methods section of the revised manuscript:

**Selection of polymorphisms**

Our primary objective was to select MAPT polymorphisms which were previously suggested to contribute to the risk of developing idiopathic PD in Greek subjects. Therefore, we focussed on the H1/H2 insertion/deletion polymorphism, rs242562, rs2435207, and rs3785883 and their sub-haplotypes, which were associated with PD in Greek patients.[16-17] Furthermore, we aimed to provide more data on MAPT variants with a potential functional role on the regulation of MAPT in tauopathies, since increased expression of H1 haplotype has been suggested as a mechanism of PD susceptibility.[20] SNP rs242557 has been implicated as a functional variant affecting transcriptional activity of tau in patients with Alzheimer’s disease.[34] The SNP rs242557 was reported to be strongly associated with rs242562 (r²= 0.96) by Zabetian et al.[27] Nevertheless, taking into account its potential functional role we analyzed
rs242557 in our study. It turned out that rs242557 was in complete LD with rs242562 among our H1H1 subgroup of patients. The latter SNP is included in a three-locus clade containing also rs 3785883 and rs2471738, which has been strongly associated with the tauopathies in genetic studies [35] and in a quantitative trait analysis in Alzheimer’s disease.[34] The strongest two-locus haplotype identified having a significant effect on tau levels in the Alzheimer’s disease study was the one containing both rs242557 and rs 3785883.

Overall, we have analyzed 5 polymorphisms at MAPT including the H1/H2 polymorphism, rs242562, rs2435207, rs242557, and rs3785883. We did not genotype rs2471738 which is also included in the three-locus haplotype analysis previously referred, because it is known that this SNP is in strong LD with rs2435207 according to the study by Zabetian et al. who reported D´=0.98 in PD patients and D´=0.97 in controls.[27] Taken together we were able to represent the previously referred two and three-locus functional sub-haplotypes by the already selected SNPs.

2. “Noting that the PD cases are all clinical, the authors should discuss the possible inclusion of misdiagnosed PSP cases”.

This is another valid issue. In our study idiopathic Parkinson's disease (PD) was diagnosed according to the criteria of the UK Parkinson's Disease Society Brain Bank. The use of the UKPDS standard diagnostic criteria has been shown to increase diagnostic accuracy reaching levels of up to 90%. (Hughes AJ, Daniel SE, Lees AJ. Improved accuracy of clinical diagnosis of Lewy body Parkinson’s disease. Neurology 2001 Oct 23;57(8):1497-9, and Gibb WRG, Lees AJ. The significance of the Lewy body in the diagnosis of idiopathic Parkinson’s disease. Neuropathol Appl Neurobiol 1989;15:27-44).

We added this information into the Methods section related to Subjects of the revised manuscript:
The process of sample collection did not include any intervention that is not part of any common clinical practice. Idiopathic Parkinson's disease (PD) was diagnosed according to the criteria of the UK Parkinson's Disease Society Brain Bank. The use of the UKPDS standard diagnostic criteria has been shown to increase diagnostic accuracy reaching levels of up to 90%. [32] The PD symptoms were quantified by applying Part III of the Unified Parkinson’s Disease Rating Scale (UPDRS) [33] score.

Nevertheless, we agree with the reviewer that misclassification of PD patients represents a potential limitation in our study. We added a brief comment on this issue in the Discussion of the revised manuscript and quoted the important paper by Vandrovcova et al. (Neurobiol Aging doi:10.1016/j.neurobiolaging.2007.11.019) demonstrating indeed that in pathologically confirmed PD cases the association of MAPT with the disease is chiefly driven by the H1/H2 division alone. The related added part in the Discussion, concerning limitations of the study reads now:

A limitation of our study results from the small sample size and limited statistical power. As a consequence we examined, however, the consistency of our results by genotyping the H1H2 subjects of our cohorts (data not shown), namely 118 patients and 119 control individuals as previously suggested.[35] In this analysis we still did not identify any effect of single SNPs or SNP sub-haplotypes. In addition, although we used a well established standardized clinical scoring system for PD diagnosis misclassification of PD patients by lack of histological confirmation represents a potential other limitation of our study. Nevertheless, recently it was shown [43] in pathologically confirmed PD cases that the association of MAPT with the disease is chiefly driven by the H1/H2 division alone, which is in agreement with our results.
3. “Page 6, line 12: H1H1 > H1H2.

We changed the corresponding statement in the Methods section relating to statistical analysis:

Fisher’s exact test was used to compare H1H1, H1H2, and H2H2 genotype distribution between cases and control, respectively.

4. Use the rs numbers for SNPs 1 and 2 throughout the text and tables.

We used the rs numbers for all SNPs throughout the entire revised manuscript including text and tables.

**Reviewer #2:**

1. “The use of appropriate H1 subhaplotype markers. The authors used rs242562 and rs243207 to tag H1 subhaplotypes as originally described in Skipper et al, 2004 paper. However, as the authors have discussed, the results for these two markers were contradictory for almost all subsequent reports, even after the analysis of the third Greek population as reported by this paper. I would suggest that a more productive way to select candidate polymorphisms would be to identify polymorphisms that have been shown to have a regulatory function in gene expression. For example fine mapping of the MAPT locus revealed that the polymorphism rs242557 within the H1c subhaplotype was the probable functional variant associated with Tau levels in cerebrospinal fluids [Laws SM et al. Fine mapping of the MAPT locus using quantitative trait analysis identifies possible
causal variants in Alzheimer’s disease. Mol Psychiatry 2007; 12: 510-517]. The use of this polymorphism may resolve some of the inherent contradictions of the multiple studies examining the effect of H1 subhaplotypes.”

We agree that the H1c subhaplotype including SNP rs242557 has been implicated as a functional subhaplotype affecting activity of tau in patients with Alzheimer´s disease as stated by the reviewer.

The SNP rs242557 was reported to be strongly associated with rs242562 ($r^2= 0.96$) by Zabetian et al. in 2007. Nevertheless, in response to the reviewer’s comment and its potential functional role we analyzed rs242557 in our study. It turned out that rs242557 was in complete LD with rs242562 among our H1H1 subgroup of patients. We added this information into the Results section of our revised manuscript.

In addition, in response to the reviewer’s comment we analyzed rs3785883 in our study. This SNP is included in a three-locus clade containing rs242557, rs 3785883, and rs2471738, which has been associated with both tauopathies (Pittman et al., 2005) and Alzheimer’s disease (Laws et al. 2007). The strongest two-locus haplotype identified having a significant effect on tau levels in the Laws et al. study in 2007, was the one containing both rs242557 and rs 3785883. Overall, we have now analyzed 5 polymorphisms at MAPT including the H1/H2 polymorphism, rs242562 and rs 242557, rs242562, rs2435207, rs242557, and rs3785883. We did not genotype rs2471738, which is also included in a three-locus haplotype analysis previously reported, because it is known that this SNP is in strong LD with rs2435207 according to the study by Zabetian et al. (2007) who reported $D^\prime=0.98$ in PD patients and $D^\prime=0.97$ in controls. Taken together we were able to represent the previously referred two and three-locus functional sub-haplotypes by adding rs3785883 in our revised analysis.

We mentioned the additional genetic analysis in the following new paragraph on the genotype selection in the Methods section and Results section, of the revised manuscript, accordingly.
Selection of polymorphisms

Our primary objective was to select *MAPT* polymorphisms which were previously suggested to contribute to the risk of developing idiopathic PD in Greek subjects. Therefore, we focussed on the H1/H2 insertion/deletion polymorphism, rs242562, rs2435207, and rs3785883 and their sub-haplotypes, which were associated with PD in Greek patients.[16-17] Furthermore, we aimed to provide more data on *MAPT* variants with a potential functional role on the regulation of *MAPT* in tauopathies, since increased expression of H1 haplotype has been suggested as a mechanism of PD susceptibility.[20] SNP rs242557 has been implicated as a functional variant affecting transcriptional activity of tau in patients with Alzheimer’s disease.[34]

The SNP rs242557 was reported to be strongly associated with rs242562 (r²= 0.96) by Zabetian et al.[27] Nevertheless, taking into account its potential functional role we analyzed rs242557 in our study. It turned out that rs242557 was in complete LD with rs242562 among our H1H1 subgroup of patients. The latter SNP is included in a three-locus clade containing also rs 3785883 and rs2471738, which has been strongly associated with the tauopathies in genetic studies [35] and in a quantitative trait analysis in Alzheimer’s disease.[34] The strongest two-locus haplotype identified having a significant effect on tau levels in the Alzheimer’s disease study was the one containing both rs242557 and rs 3785883.

Overall, we have analyzed 5 polymorphisms at *MAPT* including the H1/H2 polymorphism, rs242562, rs2435207, rs242557, and rs3785883. We did not genotype rs2471738 which is also included in the three-locus haplotype analysis previously referred, because it is known that this SNP is in strong LD with rs2435207 according to the study by Zabetian et al. who reported D’=0.98 in PD patients and D’=0.97 in controls.[27] Taken together we were able to
represent the previously referred two and three-locus functional sub-haplotypes by the already selected SNPs.

2. “The use of statistical method as described in RESULTS section (paragraph 1, line 6) to look at the effect of sex on the association study. The method used by the authors is not a statistical adjustment, but rather, a stratification of the cohort by sex. This reduces the cohort size and statistical power for detecting association. Logistic regression analysis should be used to adjust for the effect of sex in their cohort.”

We completely agree with the reviewer. We performed logistic regression analysis to account for sex differences. After adjustment for sex the comparison for H1H1 vs. H1H2 and H2H2 genotypes between cases and controls remained significant (p=0.005).

In the revised manuscript we changed in Methods in the statistical analysis section and the Results section including Table 2, accordingly.

3. “There is no discussion of the apparent sex effect on the association between MAPT H1 haplotypes and disease risk, even it was observed by the authors and by the Fidani L. et al, 2006 study.”

We addressed this issue in the Discussion of the revised manuscript as follows:

In the previous study in Greek patients a role of gender effects on the association between MAPT and PD was suggested.[16] The authors found in separate analysis according to sex a significant association between H1H1 and PD in men but not in women. This could point to a potential sex specific effect of genetic MAPT variation to PD susceptibility. However, it may be also related to a reduced statistical power in subgroup analysis. Hence, when we performed
a separate statistical analysis for men and women in our cohort we also found a significant effect in 72 male patients but no significant effect in 50 female patients. In contrast, in logistic regression analysis we found no significant effect of sex status affecting the significant association between H1H1 genotype in the overall cohort. Our latter finding is in agreement with a large US case-control cohort study that did not support a sex specific effect.[27]

4. “Clarification of the in DISCUSSION section (paragraph 2, line 13): “Since the association between the overall MAPT locus and PD has been well established, [18,27,31,35] it appears possible that MAPT interacts differently with other genes of the MAPT region in different populations”. Could the authors clarify this statement? Do they mean that there are separate functional polymorphisms within the other genes within the extended H1 haplotype? This issue is particularly relevant for the Q7R polymorphism in Saitohin gene is in linkage disequilibrium with the MAPT H2 haplotype and would be difficult to separate the effect of the Saitohin polymorphism and that of the MAPT H2 haplotype [Clark LN et al. The Saitohin ‘Q7R’ polymorphism and tau haplotype in multi-ethnic Alzheimer disease and Parkinson’s disease cohorts. Neurosci Lett 2003; 347: 17-20].

We agree that our previous statement on this issue was misleading. In fact, we did not intend to discuss a role of potential functional polymorphisms within other genes within the extended H1 haplotype, because of the complexity of this genomic region. The latter is a result of both the complex haplotype structure and the fact that the MAPT locus contains other genes which are yet not fully characterized.

As reported, other genes have been implicated with PD either within the MAPT locus or within the extended H1 haplotype, e.g. Saitohin and KIAA1267. The importance of these genes could vary among different ethnicities due to different allele and haplotype frequencies.
Therefore, we have changed the misleading sentence, as follows:

The genetic association between the overall MAPT locus and PD has been well established [18,31,41]. Other genes of the MAPT region either within the MAPT locus (e.g. Saitohin) or within the extended H1 haplotype (e.g. KIAA1267), have been also implicated in PD [31, 42]. The genetic dissection of the MAPT locus and its role in PD is still a major challenge, because of the complexity of this genomic region. The latter is a result of both the complex haplotype structure and the fact that the MAPT locus contains other genes which are yet not fully characterized. Moreover, it is currently unclear whether the influence of these genes could vary among different ethnicities due to varying allele and haplotype frequencies within the MAPT locus.

Reviewer #3:

1. “it would be useful to include p values for association in table 2”

A correction in Table 2 was made, accordingly.

2. “the issue of power is of genuine concern for the sub-haplotyping - this analysis is only performed in H1 carriers and thus the authors are performing a comparison between ~80 patients and ~60 controls; I suspect that the authors do not have power to exclude an effect of the magnitude one might suspect for the sub-haplotype; a power calculation would be informative in this context.
The reviewer is right that the sample size in our study is not large and the power therefore somewhat limited. A power calculation revealed that we had a limited power of 60% to exclude a possible effect.

In response to the comment, we examined the consistency of our results by genotyping H1H2 samples of our cohorts (data not shown), namely 118 patients and 119 control individuals as previously suggested (Pittman et al., 2005). In this analysis we still did not identify any effect of single SNPs or SNP sub-haplotypes. In addition, Skipper et al. 2004 and Fidani et al. 2006 analyzed cohorts of similar size, respectively, when they identified rs242562-rs2435207 as risk sub-haplotypes.

In conclusion, the power of our sub-haplotyping analysis is limited and we definitely cannot exclude a possible effect. Therefore we were somewhat more cautious in our phrasing in discussing this issue in the Conclusion part of the revised manuscript:

Our data show strong evidence of an association between the H1/H1 genotype and PD in Greek population, however the SNPs rs242562, rs2435207 and rs3785883 within the H1 haplotype do not seem to alter susceptibility for PD.

However, we think that our data are still of interest and useful for the evaluation of previous reports. Our data are also in agreement with the study reported by Vandrovcova et al., in 2007, in which pathologically proven PD cases were included. These authors identified some weak and inconsistent SNP effects while confirmed the significant effect of the H1H1 genotype.

We addressed this issue in the Discussion in the following paragraph:

A limitation of our study results from the small sample size and limited statistical power. As a consequence we examined, however, the consistency of our results by genotyping the H1H2 subjects of our cohorts (data not shown), namely 118 patients and 119 control individuals as
previously suggested.[35] In this analysis we still did not identify any effect of single SNPs or SNP sub-haplotypes. In addition, although we used a well established standardized clinical scoring system for PD diagnosis misclassification of PD patients by lack of histological confirmation represents a potential other limitation of our study. Nevertheless, recently it was shown [43] in pathologically confirmed PD cases that the association of MAPT with the disease is chiefly driven by the H1/H2 division alone, which is in agreement with our results.

3. In the discussion the authors talk about interaction of MAPT with other genes on the extended MAPT haplotype (such as saitohin) - I don't follow the reasoning in this section, perhaps you could clarify.

We agree that our previous statement on this issue was misleading. In fact, we did not intend to discuss a role of potential functional polymorphisms within other genes within the extended H1 haplotype, because of the complexity of this genomic region. The latter is a result of both the complex haplotype structure and the fact that the MAPT locus contains other genes which are yet not fully characterized.

As reported, other genes have been implicated with PD either within the MAPT locus or within the extended H1 haplotype, e.g. Saitohin and KIAA1267. The importance of these genes could vary among different ethnicities due to different allele and haplotype frequencies. Therefore, we have changed the misleading sentence, as follows:

The genetic association between the overall MAPT locus and PD has been well established [18,31,41]. Other genes of the MAPT region either within the MAPT locus (e.g. Saitohin) or within the extended H1 haplotype (e.g. KIAA1267), have been also implicated in PD [31, 42]. The genetic dissection of the MAPT locus and its role in PD is still a major challenge, because of the complexity of this genomic region. The latter is a result of both the complex haplotype
structure and the fact that the MAPT locus contains other genes which are yet not fully characterized. Moreover, it is currently unclear whether the influence of these genes could vary among different ethnicities due to varying allele and haplotype frequencies within the MAPT locus.