Author's response to reviews

Title: No role for Estrogen Receptor 1 Gene Intron 1 Pvu II and Exon 4 C325G Polymorphisms in Migraine Susceptibility

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Author's response to reviews: see over
The Editorial Team
BMC Medical Genetics

Dear Members of the Editorial Team

We hereby submit a revised manuscript with the amended title of “No role for Estrogen Receptor 1 Gene Intron 1 Pvu II and Exon 4 C325G Polymorphisms in Migraine Susceptibility” by Natalie J Colson, Rod A Lea, Sharon Quinlan, and Lyn R Griffiths.

We have now addressed all reviewers’ comments below and made the required changes to our paper. We believe that the paper is now ready for publication in BMC Medical Genetics.

RESPONSE TO REVIEWERS’ COMMENTS

Reviewer: Amanda Shearman
Reviewer's report:
General
The authors previously reported an association of the Estrogen Receptor alpha (ESR1) exon 8 G594A polymorphism and migraine susceptibility in two independent cohorts. Here they report negative results for two further ESR1 polymorphisms: a T/C PvuII polymorphism in intron 1 and the C325G polymorphisms in exon 4. The manuscript is well written and carefully presented.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

The SNP database contains hundreds of ESR1 SNPs in multiple linkage disequilibrium blocks. Thus the current title and conclusions that migraine susceptibility is restricted to the 3 prime end of the gene should be worded more specifically. The need for studies with a high density of polymorphisms to follow your previous important findings of ESR1 association with migraine might be mentioned.

Response: We have now amended the title to
“No role for Estrogen Receptor 1 Gene Intron 1 Pvu II and Exon 4 C325G Polymorphisms in Migraine Susceptibility”.

We have amended the Abstract Conclusion on page 2 to
“We have found no role for the polymorphisms in intron 1 and exon 4 with migraine susceptibility. To further investigate our previously implicated exon 8 marker, we suggest the need for studies with a high density of polymorphisms be undertaken, with particular focus on markers in LD with the exon 8 marker.”
We have also amended the Conclusion on page 9 paragraph 3 to
“The fact that alleles of the two SNPs tested in the present study showed no
association with migraine and were not in LD with alleles at the exon 8 SNP
highlights the need for further studies with a high density of polymorphisms
spanning the estrogen receptor to further investigate our previously reported
susceptibility locus at exon 8. In particular, such studies should focus on markers
that are in LD blocks with the G594A polymorphism. Additionally, we believe
further investigation of the exon 8 locus for a potential functional variant is
clearly warranted, perhaps utilising allele specific gene expression methods.”

The context of your work/Discussion may be strengthened by consideration of prior reports of
the polymorphisms you studied with other phenotypes with established relationships with
migraine, for example, estrogen level and stroke (Schuit SC, de Jong FH, Stolk L, Koek WN,
van Meurs JB, Schoofs MW, Zillikens MC, Hofman A, van Leeuwen JP, Pols HA,
Uitterlinden AG. Estrogen receptor alpha gene polymorphisms are associated with estradiol
levels in postmenopausal women.
SE, Mendelsohn ME, Housman DE, Miller GJ. Estrogen Receptor a gene variation and risk of

Response:
As recommended, we have considered prior reports of the polymorphisms under study
with other phenotypes with established relationships with migraine, such as estrogen
levels and stroke.

We have included in the Background paragraph 3 on page 4
“The Pvu II locus has been associated with variation in estradiol levels in post
menopausal women [19] and with an increased risk of stroke in men [20].
Interestingly both estrogen withdrawal and high estrogen concentrations have
been implicated in migraine susceptibility in women [16], and there is evidence
for an increased risk of stroke in MA sufferers [21].”

You should discuss your ESR1 PvuII CC frequencies, for example, 27% in males with
migraine, and 19% in control males, 30 % in migraine without aura (MO) and 20% in
migraine with aura (MA). Test specifically for association of PvuII CC with migraine in men.

Consider your results in the context of ESR1 variation providing a common basis for the
relationship between migraine and stroke.

Response: As recommended we have discussed our ESR1 PvuII CC frequencies in males
and in MO and MA. Please see (i) below

We have also considered our results in the context of ESR1 variation providing a
common basis for the relationship between migraine and stroke. Please see (ii) below

(i) We have included in the Results and Discussion paragraph 1, pages 6 & 7
“Furthermore, no significant difference was seen when the migraine population was subdivided into MA (genotype frequencies $X^2 = 3.53, P = 0.17$, allele frequencies $X^2 = 0.32, P = 0.57$) and MO (genotype frequencies $X^2 = 1.66, P = 0.44$, allele frequencies $X^2 = 1.52, P = 0.22$), although the increased frequency of the CC genotype in MO (30%) compared to MA (20%) may warrant follow-up in a larger study group. There was no statistically significant difference in the migraine and control groups with regard to males (genotype frequencies $X^2 = 2.36, P = 0.31$, allele frequencies $X^2 = 2.36, P = 0.12$) and females (genotype frequencies $X^2 = 0.65, P = 0.72$, allele frequencies $X^2 = 0.03, P = 0.86$). With regard to male frequencies, it was interesting to note that there was a higher frequency of the CC genotype in male migraineurs (27%) compared to the male control group (19%). While this analysis did not reach statistical significance due to small numbers in the male subgroup, it may warrant further investigation in a larger study group, particularly in view of the previously reported role of the CC genotype in increased stroke risk in males [20] and the potential relationship between migraine and stroke [21, 32-35].”

(ii) We have included in the Results and Discussion paragraph 1, pages 6 & 7

“While this analysis did not reach statistical significance due to small numbers in the male subgroup, it may warrant further investigation in a larger study group, particularly in view of the previously reported role of the CC genotype in increased stroke risk in males [20] and the potential relationship between migraine and stroke [21, 32-35].”

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
None

Discretionary Revisions (which the author can choose to ignore)
None

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No
Declaration of competing interests:
I am an MIT employee. A patent application titled "Estrogen Receptor Alpha Gene Variation and Disease" was filed through the MIT Technology Licensing Office and listed my name among others. I have received no compensation for this. Otherwise I have no competing interests.
Reviewer: Maria Del Zombo

Reviewer's report:
General
The authors have previously reported an association between the estrogen receptor 1 (ESR1) gene exon 8 G594A polymorphism (SNP rs2228480) and migraine susceptibility in two independent Australian cohorts. This SNP is synonymous with no associated amino acid change, consequently it is unlikely that this polymorphism is causative, but may be in linkage disequilibrium (LD) with an unknown causative variant. In this paper the authors report results of the analysis of two further SNPs in the ESR1 gene in the same study group, rs8179176 in intron 1 and ss12568596 in exon 4.

The results showed no evidence for association of the polymorphisms and migraine susceptibility and no evidence for LD between these two SNPs and the previously implicated exon 8 SNP. They conclude that the previously reported migraine susceptibility locus appears to be restricted to the 3’ region of ESR1 with no association to markers located toward the 5’ end of the gene. The question posed by the authors is well defined. The methods are appropriately and thoroughly described, allowing other researcher to replicate the work. The discussion and conclusions are adequately supported by the data.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
I suggest the authors to simplify Tables 1 and 2 and explain what the “Both” group represents.

Response: We have amended “Both” to “MA & MO” in both tables on page 13.

Discretionary Revisions (which the author can choose to ignore)

What next?: Accept after minor essential revisions

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests:
I declare that I have no competing interests.

Reviewer:
Jane Worthington

Reviewer's report:
General
Review of article:
The estrogen receptor 1 locus associated with migraine susceptibility is restricted to the 3’ end of the gene. NJ Colson et al.

1) Background:
In this paper the authors have attempted to further investigate the association of the estrogen receptor alpha locus and migraine. A previous publication from this group had identified association to a synonymous SNP in exon 8. They here explore the possibility that other SNPs in LD with the SNP initially investigated, are the responsible for the association.
Major compulsory revisions
2) Study Design
The authors state that the previously published association with the exon 8 SNP (rs2228480) may be due to linkage disequilibrium (LD) with a causative variant. However, the SNPs chosen for investigation by the authors, in an attempt to refine the region of association, reside 155 kb and 257 kb upstream, and are thus unlikely to be in LD with this SNP. In this case, the HapMap data in the CEU sample may have highlighted SNPs showing LD with the exon 8 polymorphism in this population, and would have better informed the researchers about the SNPs that are more likely to explain the previously identified association. The authors state in the Conclusions (Paragraph 2, line 5) that an absence of LD between the exon 4 and exon 8 SNPs has previously been reported, thus raising the question of why the exon 4 and intron 1 SNPs were chosen as candidate polymorphisms.

Response: As recommended we have now included a more detailed explanation of why these particular SNPs were analysed. Pvu II has been implicated in stroke and estradiol levels and C325G has been implicated in breast cancer, a disease in which hormones play a role. We agree with this reviewer and have now indicated the need for further studies with a high density of polymorphisms, with particular focus on markers in LD with the exon 8 marker.

We have amended the Background paragraph 3 on page 4 to
“In this study we have analysed two further single nucleotide polymorphisms (SNPs) in ESR1 in the same study group, the Pvu II C/T SNP in intron 1 (rs2234693,) and the C325G SNP in exon 4 (rs1801132) which is located in the hormone binding region. The Pvu II locus has been associated with variation in estradiol levels in post menopausal women [19] and with an increased risk of stroke in men [20]. Interestingly both estrogen withdrawal and high estrogen concentrations have been implicated in migraine susceptibility in women [16], and there is evidence for an increased risk of stroke in MA sufferers [21]. It has been reported that the C325G SNP may play a role in calcium metabolism [22] and susceptibility to breast cancer [23, 24] a disease in which hormones play a role.”

We have amended the Abstract Conclusion on page 2 to
“We have found no role for the polymorphisms in intron 1 and exon 4 with migraine susceptibility. To further investigate our previously implicated exon 8 marker, we suggest the need for studies with a high density of polymorphisms be undertaken, with particular focus on markers in LD with the exon 8 marker.”

We have also amended the Conclusion on page 8 paragraph 3 to
“The fact that alleles of the two SNPs tested in the present study showed no association with migraine and were not in LD with alleles at the exon 8 SNP highlights the need for further studies with a high density of polymorphisms spanning the estrogen receptor to further investigate our previously reported susceptibility locus at exon 8. In particular, such studies should focus on markers that are in LD blocks with the G594A polymorphism. Additionally, we believe further investigation of the exon 8 locus for a potential functional variant is clearly warranted, perhaps utilising allele specific gene expression methods.”
3)Title:
The statement made in the title is too specific. The authors have genotyped two polymorphisms within the 295.7kb of the ESR1 gene, one in intron 1 and another in intron 4, further to the exon 8 SNP investigated in the previous publication. It does not follow from the lack of association of these two SNPs with susceptibility to migraine that the association is restricted to the 3’ region of the gene. No more variants in the 3’ region have been genotyped, and many other SNPs in the upstream 5’ region have not been investigated. The data reported in this article do not further our understanding of the role of polymorphism in ESR1 in susceptibility to migraine.

Response: We agree with this suggestion and have thus amended the title to “No role for Estrogen Receptor 1 Gene Intron 1 Pvu II and Exon 4 C325G Polymorphisms in Migraine Susceptibility” and recommend the need for studies with a high density of polymorphisms and a focus on markers in LD with the exon 8 marker (as outlined in the previous response).

Minor essential revisions
1.)Third paragraph, line 12: The ‘rs’ number provided for the PvuII polymorphism is incorrect. The ‘rs’ number for this SNP is rs2234693 (Johansson et al. Ann Rheum Dis 2005;64:1611-1617).

Response: We have amended this to the correct ‘rs’ number. Please see page 4 paragraph 1.

2.)Third paragraph, first sentence: Some background regarding the role of hormones such as oestrogen in the biology of migraine should be given. Although this has been clarified in the Conclusion section, it should be provided in the Introduction also, as this information is essential to the understanding of why ESR1 was chosen as a candidate for a migraine susceptibility gene.

Response: As advised, we have now included some background regarding the role of hormones in migraine.

We have included in the Background paragraph 3 page 3 “Migraines in women frequently occur during the childbearing years and are often influenced by significant hormonal milestones. The fluctuating hormone levels of the menstrual cycle have been implicated in migraine but a definitive role is yet to be established [16]. It has been suggested that factors additional to the circulating hormone levels may be at play [17]. Thus, we considered that variation in the ESR1 gene may confer increased migraine risk.”

3)Third paragraph, final sentence: This sentence should read “The minor allele at each of these SNPs has been shown to have a frequency of >20%”, and the population in which this was demonstrated should be given.
Response: As advised, we have amended this sentence and shown the populations in which this was demonstrated. Please see page 4

“The minor allele at each of these SNPs has been shown to have a frequency of >20% as determined in Australian and other Caucasian populations [25, 26]”

Methods:
4.) Genotyping: RFLP analysis for SNP genotyping is a rather out-dated technique, recognised to be error prone. It is advisable to include samples of known genotype (determined by sequencing) on each gel and to check for Hardy Weinberg Equilibrium in control samples. The volume of DNA polymerase used in the PCR should be stated.

Response: We acknowledge that RFLP methods are now less frequently used in favour of other methods however we took numerous precautions to reduce the risk of error and for both markers we followed previously reported protocols (Sasaki et al, 2003 and Curran et al, 2001). For each marker, both our case and control groups were within Hardy Weinberg Equilibrium, and our allele frequencies were similar to other published frequencies. In addition, internal controls using random repeat samples and negative controls were run to rule out contamination and to reduce the possibility of genotyping errors. Both enzymes used for these assays were obtained fresh from the supplier and stored in optimal conditions.

We have included in the Genotyping subsection of Methods in page 6

“To reduce the likelihood of genotyping error occurring, random repeat samples and negative controls were included in both assays.”

We have included in the Case Control Analysis subsection of the Results and Discussion in page 7 paragraph 1

“All allele frequencies did not deviate from Hardy Weinberg Equilibrium in both case and control groups (at $P = 0.4, P = 0.6$) and were similar to previously reported frequencies [26].”

and paragraph 2

“All allele frequencies did not deviate from Hardy Weinberg Equilibrium in both case and control groups (at $P = 0.1, P = 0.3$) and were similar to frequencies previously reported in an Australian study group [25].”

We have included the volume of DNA polymerase used in the PCRs in the Genotyping subsection of Methods in page 5 paragraph 1

“The 20 µl PCR reaction mix contained 40 ng genomic DNA, 0.2µM of each primer, 1 x PCR buffer, 2mM MgCl$^2$, 0.2mM dNTPs and 0.2 µl Taq polymerase (5U/µl).”

and paragraph 2

“The 20 µl PCR reaction mix contained 50 ng genomic DNA, 0.3 µM of each primer, 1 x PCR buffer, 2.25 mM MgCl$^2$, 0.2mM dNTPs and 0.2 µl Taq polymerase (5U/µl).”

Discretionary revisions:
7) Background:
Second paragraph, last sentence: Examples of those loci showing association with susceptibility to migraine could be cited.
Response: As suggested, we have now cited examples of those loci showing association with susceptibility to migraine. See Background paragraph 2 page 3

“Several loci have shown promise, although these need to be followed up by both replication and functional studies to determine a definitive causative role [5-15].”

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What next?: Reject because scientifically unsound
Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No
Declaration of competing interests:
'I declare that I have no competing interests'

We respectively request that you now consider publishing this manuscript in “BMC Medical Genetics” as a research article.

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We thank you in advance for your consideration and await your response.

Yours sincerely

Professor Lyn Griffiths
Director, Genomics Research Centre