Author's response to reviews

Title: Methylation of class II transactivator gene promoter IV is not associated with susceptibility to Multiple Sclerosis

Authors:

Sreeram V Ramagopalan (sramagopalan@gmail.com)
David A Dyment (ddyment@well.ox.ac.uk)
Blanca M Herrera (blanca@well.ox.ac.uk)
Gabriele C DeLuca (gcdeluca@gmail.com)
Matthew R Lincoln (mlincoln@well.ox.ac.uk)
Sarah M Orton (ortons@well.ox.ac.uk)
Lahiru Handunnetthi (lahiru.handunnetthi@green.ox.ac.uk)
Michael J Chao (michael.chao@well.ox.ac.uk)
A Dessa Sadovnick (sadovnik@well.ox.ac.uk)
George C Ebers (george.ebers@clneuro.ox.ac.uk)

Version: 2  Date: 1 May 2008

Author's response to reviews: see over
RE: Methylation of class II transactivator gene promoter IV is not associated with susceptibility to Multiple Sclerosis

Robin Cassidy-Cain
Assistant Editor
*BMC Medical Genetics*

Dear Robin,

Thank you for your e-mail, detailing the comments from the reviewers.

We thank the reviewers for their helpful and constructive comments and respond to their comments in turn (our responses are italicized).

Reviewer 1:

The results presented have to be taken at face value. There are no figures showing the bisulfite PCR specific product. The authors state that PCR was carried out with universal methylated, universal unmethylated and water as positive, negative and blank controls. Including this figure would help tremendously.

*We have now included this figure, please see figure 2.*

This reviewer has some concerns relating to the methodology used for bisulfite sequencing. The authors say that PCR products were sequenced (directly?) using an ABI 3700 sequencer. Under normal bisulfite sequencing, each PCR product should be cloned and ~10 clones should be sequenced for each product. In this instance for this manuscript this would represent 1000 sequencing reactions. This strategy allows a researcher to identify small changes in DNA methylation which would be masked if the PCR product was sequenced directly. The authors do not state if they have sub-cloned and sequenced the PCR product, or if they have used direct sequencing of their bisulfite PCR product. This is something which they need to clarify, as unless specific software was used to measure the peaks the authors may be missing low-level methylation patterns., which might be picked up if sub-cloning of the PCR product was utilised

If the authors can provide details as per methodology in relation to the sequencing, or even include some representative trace files for some of the samples showing complete lack of methylation, this would alleviate this reviewers concerns.
We apologise profusely for the lack of clarity in the methods. There appears to have been a mix up between sequencing of genomic DNA and methylation specific PCR in the methods - neither of which were explained in detail. We apologise for the confusion this may have caused. The methods now reads:

**Sequencing of promoter pIV**

Total genomic DNA was extracted from whole blood as part of the CCPGSMS. PCR was performed using the primers shown in Table 2 under standard conditions [18] with an annealing temperature of 60 degrees Celsius and using AmpliTAQ gold (Applied Biosystems), yielding a PCR amplicon 257 base pairs in size. Sequencing reactions were carried out using BigDye v3.1 after which the DNA was sequenced using an ABI 3700 automated sequencer.

**Table 2 - Primer sequences used for sequencing**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>GGTTGGGACTGAGTTGGAGAGA</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>GGAGCAACCAAGCACCTACT</td>
</tr>
</tbody>
</table>

**Bisulfite treatment and Methylation Specific PCR:**

Genomic DNA was treated using methylSEQr Bisulfite Conversion Kit from Applied BioSystems, following the manufacturer’s protocol. This converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged. Methylation specific PCR [19], using methylated DNA and unmethylated DNA specific primer sets was performed on treated DNA to detect methylation of the CpG island in the MHC2TA promoter. PCR was performed using the primers shown in Table 3 under standard conditions [18] with an annealing temperature of 55.5 degrees Celsius. Each PCR was performed twice for each sample to ensure validity of results. Universal methylated DNA, universal unmethylated DNA (both from CpGenome™) and water was used as positive, negative and blank controls respectively. Amplified fragments were confirmed by a 2.0% agarose gel.

**Table 3 - Primer sequences used for methylation specific PCR**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated Forward</td>
<td>TGTTGGGTGTCTATAGTTGGTTC</td>
<td></td>
</tr>
<tr>
<td>Methylated Reverse</td>
<td>CTACTAATAACCTCTCCCTCCCG</td>
<td>60bp</td>
</tr>
<tr>
<td>Unmethylated Forward</td>
<td>TTGGGTGTTTTATAGTTGGTTC</td>
<td></td>
</tr>
<tr>
<td>Unmethylated Reverse</td>
<td>CTACTAATAACCTCTCCCTCCAC</td>
<td>157bp</td>
</tr>
</tbody>
</table>

We hope the methodology is now clear. We have also added a caveat to the discussion stating that the we may not have been able to detect low level methylation patterns.

Reviewer 2

1. Mean age at blood sample collection for twins should be included in the
manuscript and any differences between sample collection dates for twins should also be described.

*The mean age at blood sampling was 41.1 years, and there was no large differences (standard deviation= 3.7 years). We have included this in the manuscript.*

2. HLA-DRB1 genotype status should also be included. How many pairs were DRB1*15 positive, for example?

*31 twin pairs were HLA-DRB1*15 positive, and again this is now mentioned in the manuscript.*

3. Can authors clarify that the MHC2TA -168A/G (rs3087456) polymorphism was also investigated and not present? It appears this is true, but not explicitly stated. The significance of this finding (given population allele frequency) should be included in the Discussion section.

*rs3087456 is not in the region investigated (promoter IV) but has been investigated in another cohort and not associated with MS (Ramagopalan et al, in press).*

4. Authors should include the size (bp) of the promoter region (pIV) that was fully amplified and sequenced. More information should be included about the experimental design such as use of replicate samples to confirm reproducibility of assay and more information on positive control DNA that was used. The information is critical for other researchers to also investigate and replicate these important findings in larger sample sizes.

*We apologise for the lack of clarity in the methods and hope all is now clear in the revised methods.*

We would be grateful if the Journal would reconsider the manuscript in light of the points raised above.

We look forward to hearing from you,

Prof. George C. Ebers MA MD FRCP FRCP(C) FMedSci
Sreeram Ramagopalan MA