Author's response to reviews

Title: Common Variants in Mismatch Repair Genes Associated with Increased Risk of Sperm DNA Damage and Male Infertility

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Author's response to reviews: see over
Dear Dr Gu,

Your manuscript has now been peer reviewed and the comments are accessible in PDF format from the link below. Do let us know if you have any problems opening the file.
Referee 1:
http://www.biomedcentral.com/imedia/7608569846478180_comment.pdf
Referee 2:
http://www.biomedcentral.com/imedia/1370417096652582_comment.pdf
Referee 3:
http://www.biomedcentral.com/imedia/1506030082660000_comment.pdf

We would be grateful if you could address the comments in a revised manuscript and provide a cover letter giving a point-by-point response to the concerns.

In addition to the referees' comments, there are a number of necessary editorial revisions, which are outlined below:

1. Authors' contributions: in this section, "HZ" does not seem to be on the author list. Please clarify whether this should be "YZ". Also, please clarify whether YL and CH were involved in the drafting or revising of the manuscript. Please note that to qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; and 3) have given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.

2. Please confirm whether written informed consent was obtained from patients in your study. This information should be included in the Methods section.


4. We recommend that you ask a native English speaking colleague to help you copyedit the paper. If this is not possible, you may need to use a professional language editing service. For authors who wish to have the language in their manuscript edited by a native-English speaker with scientific expertise, BioMed Central recommends Edanz (www.edanzediting.com/bmc1). BioMed Central has negotiated a 10% discount to the fee charged to BioMed Central authors by Edanz. Use of an editing service is neither a requirement nor a guarantee of acceptance for publication. For more information, see our frequently asked questions on language editing services (http://www.biomedcentral.com/authors/authorfaq).
Dear Editors and Reviewers,

We highly appreciate the detailed valuable comments of the referees on our manuscript of ‘MS 1175193129623197 (Common Variants in Mismatch Repair Genes Associated with Increased Risk of Sperm DNA Damage and Male Infertility)’. The suggestions are quite helpful for us, and these comments will greatly enhance the quality of this manuscript. We have modified the manuscript accordingly, and the detailed corrections are listed below point by point.

The major changes are highlighted in the revised manuscript. We hope the Reviewers and the Editors will be satisfied with our responses to the ‘comments’ and the revisions for the original manuscript. Thank you again for your consideration and we await a favorable response to the revision.

Thanks and Best Regards!

Yours sincerely,

Aihua Gu
Responses to the quests:

Reviewer 1: Dr. Doug Carrell

Reviewer's report:
This is an interesting and well written manuscript. The study is nicely reported. However, it has one potential major flaw, and I would also make one significant suggestion.

1) The data do not appear to have undergone a Bonferroni correction, which may make the data not significantly different from controls. This must be done.

Many thanks for the comments and we agree with your suggestion. By performing the Bonferroni correction as suggested, we found these three SNPs (rs4647269, rs1059060 and rs2075789) was also significant. Considering the False Discovery Rate (FDR) is a new approach to the multiple comparisons problem, which controls the expected proportion of false positives among suprathreshold voxels\textsuperscript{ref}, instead of controlling the chance of any false positives (as Bonferroni methods do), we applied the FDR method to reduce the potential for spurious findings due to multiple testing. For example, in an experiment with 1000 genes, we would expect on average one of them to have a $P$-Value as small as 0.001. FDR is the fraction of the genes at or below a given $P$-Value that are expected to have such small $P$-Values by chance. It is numerically equal to the Bonferroni-corrected $P$-Value divided by the number of genes with $P$-Values that small or smaller.


2) Although the SNP rates are significantly higher for some variants in the patient group, the actual rates are quite low and would potentially account for a low percentage of infertility. This needs to be emphasized and better discussed.

We deeply appreciate the reviewer for the wonderful suggestion, which is undoubtedly helpful to our manuscript. We have emphasized and discussed it in the conclusions section of our manuscript (in page 17) accordingly.

Quality of written English: Not suitable for publication unless extensively edited. Thanks for the reviewer’s advice. We have invited a professor (in Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, USA) for language editing. All the minor grammatical errors have been modified.
Reviewer 2: Dr. JOAQUIN GADEA

Reviewer's report:

Authors evaluated the relationship between SNP in mismatch repair genes and infertility. They studied 21 SNPs in 5 MMR genes. They reported 3 different SNPs that looks like to be related to infertility. Two of them are also related to an increase in sperm DNA damage.

This reviewer considers this manuscript is relevant and offers original information that could help to understand the genetic basis of some male infertility cases. My main concern is related to the spermatology area of this manuscript and the design of the study that will have importance in the possible clinical application of this valuable information.

First at all, the 3 groups of study are based in fertility (control and idiopathic infertility) and sperm concentration (<20 or >20 x 10^6 spermatozoa/mL). However, the control group was not divided according the same criteria of sperm concentration. So I suggest to evaluate your data from 4 groups (fertile <20 x 10^6 spermatozoa/mL; fertile >20 x 10^6 spermatozoa/mL, infertile <20 x 10^6 spermatozoa/mL; infertile >20 x 10^6 spermatozoa/mL). So, it would be possible to relate the SNP and sperm concentration and SNP and fertility in a more adequate way. 

Thanks for the reviewer’s careful insights and good suggestion.

In the presents study, all the controls have normal semen parameters and had fathered at least one child without assisted reproductive technologies. Those controls with the semen parameters below the WHO reference (e.g. sperm concentration < 20 x 10^6 spermatozoa/mL) were excluded. So the study subjects were divided into three groups (controls, infertile patients with sperm concentration < 20 x 10^6 spermatozoa/mL and patients with sperm concentration >20 x 10^6 spermatozoa/mL).

Sperm concentration is only one of the parameters to evaluate the normal semen quality and spermatogenesis process. Other sperm parameters as motility, viability, morphology and total number of sperm in the ejaculate could be of interest to study. Many thanks for the reviewer’s wonderful suggestion.

Considering that MMR gene knockout mice displayed poor sperm production owing to complete or partial meiotic arrest, we hypothesized that genetic variants in MMR genes had more effects on genetic susceptibility to azoospermia or oligozoosperma than on other sperm parameters in male infertility. Therefore, the phenotype ‘sperm concentration’ is preferred in this study. However, other parameters as motility, viability, morphology and total number of sperm may also be influenced. We will take them into consideration for future projects.

In the text, concentration values (low or high) are assimilated to impaired or normal spermatogenesis. In opinion of this reviewer it is not a precise an accurate language. Please review this concept.
We appreciate the correction from the reviewer. In our manuscript, ‘Impaired spermatogenesis’ has been revised to “azoospermia or oligozoospermia” and ‘Normal spermatogenesis’ has been revised to “normal sperm count” accordingly.

Some specific questions
Introduction
I suggest including some words related to the relationship between SNP and infertility, and supported by some references. For example:
Carrell DT, Aston KI. The search for SNPs, CNVs, and epigenetic variants associated with the complex disease of male infertility. Systems Biology in Reproductive Medicine 2011: 17-26.
Thanks for the reviewer’s advice. We have added some words and references related to the relationship between SNP and infertility in the background of our manuscript (page 4).

Methods
Subjects and sample collection
How many azoospermic cases are in the case 1 group?
Thanks for the reviewer’s question. In the method part of our manuscript (page 5), we added one sentence “In the final analysis, 1,292 idiopathic infertility patients aged 24 to 42 years old were included, and were divided into three subgroups: 268 infertility patients with non-obstructive azoospermia, 256 infertility patients with oligozoospermia (sperm counts <20 × 10⁶/ml) and 768 infertility patients with normal count (sperm counts ≥20 × 10⁶/ml).”

What criteria were used to classify as “normal semen quality”? According to the WHO reference (WHO 1999), those controls with sperm concentration ≥20 × 10⁶/ml, motility (grade a + b) ≥ 50% or grade a ≥ 25% and teratozoospermia index (TZI) ≤ 1.6 were classified as subjects with “normal semen quality”.

Reference:

All patients has data from at least two semen assessments, what value are you using for classify by concentration, mean value, the best one, the worst one?
Thanks for the wonderful comments.
In order to reduce the variation of assessment of sperm characteristics, each sample was assessed twice in parallel. For instance, sperm concentration was detected twice and the difference (D) between two results was calculated according to a formula (D (%) = (Max–Min)/Min×100%). If D ≤15%, use the mean value of two results to express the final value; if D ≥15%, test the sample three times and use the median value of three results as the final value.

DNA fragmentation analysis
Semen samples were frozen – 70ºC. Please, give more information about this process. After a period of 48-72 h of sexual abstinence, semen samples were collected by masturbation into wide-mouthed sterile containers and were delivered to the laboratory within 1 h of ejaculation. The diluted samples were cooled gradually at 5 ºC for 2 h, frozen at -70 ºC for further evaluation. We have added this information in the “DNA fragmentation analysis” section in our revised manuscript (page 6).

It is well known that cryopreservation resulted in a significant increase in percentage sperm DNA fragmentation (Thomson et al. Hum Reprod 2009;24: 2061-2070). Did the authors evaluate the cryopreservation effect on DNA fragmentation in this experiment?

Thanks for this comments. We agree with the referee that cryopreservation resulted in a significant increase in percentage sperm DNA fragmentation.

In order to determine whether the results of the TUNEL analyses were profoundly influenced by cryopreservation, ten semen samples were pre-treated with or without cryopreservation prior to TUNEL analyses. As showed in Table 1, modest but significant elevated levels of sperm DNA fragmentation induced by cryopreservation (P = 0.001). However, all the semen samples undergo the same cryopreservation process, thus we believe that the cryopreservation, if any, is unlikely to be substantial.

Table 1. Effect of cryopreservation on sperm DNA fragmentation.

<table>
<thead>
<tr>
<th>Sperm DNA fragmentation</th>
<th>No. 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>mean±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>cryopreservation</td>
<td>35.8</td>
<td>24.8</td>
<td>15.2</td>
<td>26.7</td>
<td>35.8</td>
<td>43.7</td>
<td>32.5</td>
<td>19.2</td>
<td>27.7</td>
<td>17.8</td>
<td>27.9±2.9</td>
</tr>
<tr>
<td>Fresh sample</td>
<td>32.5</td>
<td>23.9</td>
<td>15.1</td>
<td>23.3</td>
<td>31.7</td>
<td>38.3</td>
<td>31.5</td>
<td>17.1</td>
<td>25.5</td>
<td>16.0</td>
<td>25.5±2.5</td>
</tr>
</tbody>
</table>

Results
Please show in a table the sperm parameters from the samples (control and patients) as mean±sem (motility, concentration, volume, viability, morphology, TUNEL, etc).

We appreciated reviewer’s suggestion. The information about the semen parameters is indeed important for results recognition. We have added this information in Table 1 and the related information in the result section in page 12.

Please show the DNA fragmentation values in percentage not in logarithmic values in the text and figure 1. (for example 11.82 % not 2.47, 26.57% not 3.28).
Discussion

Smoking effect reported in table 1 is not discussed.

Thanks for the reviewer’s comments.

Another interesting finding was that smoking was associated with increased risk of male infertility. Although tobacco cigarette smoke on male reproduction are somewhat inconclusive, a number of studies have shown higher incidences of abnormal morphology [1,2], decreased motility, and sperm density in men who smoke [3,4]. A meta-analysis [5], including 27 studies indicated that cigarette smoking is associated with 13% reduction in sperm concentration, 10% reduction of sperm motility, and 3% reduction of morphologically normal sperm. Furthermore, fluctuation in reproductive hormone levels have been documented in male smokers [6,7]. However, the mechanism(s) of these changes, if any, remains unclear.

This part has been added in last paragraph of the discussion part.


Discussion must improve. The relation between SNPs and infertility must be reevaluated.

Thanks for the reviewer’s comments. In the conclusion section, we have deleted some redundant information in the manuscript and reevaluated the relation between SNPs and infertility accordingly.

Quality of written English: Needs some language corrections before being published.

Thanks for the reviewer’s advice. We have invited a professor (Thomas Jefferson University, Philadelphia, Pennsylvania, USA) for language editing. All the minor grammatical errors have been modified, which were marked in red in the revised manuscript.
Reviewer 3: Dr. Tom Brown
Reviewer's report:
This paper describes a study on genetic variations in MMR genes to determine whether they are associated with an increased risk of DNA damage and male infertility. The results indicate that MLH1 rs4647269, PMS2 rs1059060, PMS2 775Asn and MSH5 rs2075789 are associated with significantly increased risk. These results improve the understanding of the role of genetic variation in sperm DNA integrity and might have implications for improved reproduction success rates. They also point to a possible diagnostic test. Overall the methodology and the fluorescent experiments appear to be sound and reliable and the paper contains potentially important results with broad implications.
This referee has a good understanding of the fluorescence techniques involved in this work but is not an expert on reproductive biology, so cannot accurately assess the novelty of the work.
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
None
We thank the reviewer for the positive comments on our manuscript.

Necessary editorial revisions
In addition to the referees' comments, there are a number of necessary editorial revisions, which are outlined below:

1. Authors' contributions: in this section, "HZ" does not seem to be on the author list. Please clarify whether this should be "YZ". Also, please clarify whether YL and CH were involved in the drafting or revising of the manuscript. Please note that to qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; and 3) have given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.
Thanks for the editor’s correction. We have revised this section, and “HZ” has been changed into “YZ”. Please find them in the revision. We also clarified that YL and CH were involved in the drafting of the manuscript.

2. Please confirm whether written informed consent was obtained from patients in your study. This information should be included in the Methods section. Thanks for the wonderful suggestion. We confirmed that written informed consent was obtained from patients in our study. It was described in detail in our revised manuscript (in the methods section). “All participants completed an informed consent and a questionnaire including detailed information, such as age, cigarette smoking, alcohol drinking, tea and vitamin consumption, and abstinence time.”

3. Please ensure that your manuscript adheres to the STROBE guidelines for reporting cross-sectional studies (http://www.strobe-statement.org/fileadmin/Strobe/uploads/checklists/STROBE_checklist_v4_cross-sectional.pdf). Thanks for the editor’s suggestion. We make sure that our manuscript conforms with the STROBE guidelines.

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Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals ). It is important that your files are correctly formatted. Thanks for the editor’s remind. We have read all the list of points about the journal style, and changed all style mistakes throughout the manuscript.

We deeply hope our responses are satisfactory. If you have further suggestion, please kindly give us the instruction.