Author's response to reviews

Title: HFE H63D mutation frequency shows an increase in Turkish women with breast cancer.

Authors:

Aysen Gunel-Ozcan (agozcan@yahoo.com)
Sibel Alyilmaz-Bekmez (alyilmazs@mynet.com)
Nilufer E Guler (nguler@hacettepe.edu.tr)
Dicle Guc (dguc@hacettepe.edu.tr)

Version: 3 Date: 9 December 2005

Author's response to reviews: see over
Re: HFE H63D mutation frequency shows an increase in Turkish women with breast cancer

Dear Editor,
Thank you very much for your letter of November 28, 2005 and the detailed comments of the reviewer. Enclosed please find author’s reply to the comments of the reviewer and the manuscript altered accordingly.

We hope that the revised manuscript will meet the requirements for publication

Yours sincerely,

Aysen Gunel-Ozcan, MD, Ph.D.
Authors’ reply to the comments of the reviewer:

We gratefully acknowledge the precision of the reviewer clear and concise expression of the main points of our manuscript.

In Specific:

To Referee 1 (Ernest Beutler)

Major Compulsory Revisions

1. The results of the comparison that gave statistical significant difference (\( P=0.02, \ OR=2.05,\ 95\ \%\ CI=1.12\ to\ 3.75 \)) were between all breast cancer cases and controls. But the subgroup analysis showed that the difference between the cases and control groups is based on the sporadic cases (\( P=0.0001,\ OR=3.13,\ 95\%\ CI=1.65\ to\ 5.94 \)). This point has been clarified by incorporation of the related sentences in the Results section, paragraph 2, paragraph line 10-12.

2. According to the reviewer’s recommendation we have referred the studies that show significant increases in both transferrin saturation and serum ferritin levels in H63D heterozygotes. However, there are studies that could not find any effect in homozygotes and heterozygotes rather than compound heterozygotes. Related references are given in the manuscript. These contradictory observations indicate that the role of H63D in iron homeostasis is not well established. Necessary revisions have been made in the Discussion section, paragraph 4, paragraph line 4-8.

3. To comply with the comments of the reviewer the style of the text is altered accordingly; the first paragraph of the Background section moved to the third paragraph of the Background section.

4. Negative studies re: association of HFE mutations with cancer is added at the end of the second paragraph of the Background section (paragraph line 8-9).

5. According to the reviewer’s rightful comment pointing to the stratification, this point is incorporated into the text (Discussion fifth paragraph).

6. We have been referred the recent article by Kondrashova TV et al in Biochim Biophys Acta, 2005. To compare with our results we have also stratified groups according to ages and done the statistical analysis. We have chosen 20-45 ages as young group and 45-70 ages as old group.
Table of the Group between the age of 20 and 45.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Cases (N)</th>
<th>Control (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H63D</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>WT</td>
<td>34</td>
<td>67</td>
</tr>
</tbody>
</table>

Table of the Group between the age of 45 and 70.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Cases (N)</th>
<th>Control (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H63D</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

Age information for the 7 of the cases and for the 2 of the controls was absent. We have found statistically significant association of H63D with breast cancer among women < 45 years old ($P= 0.049$, OR= 1.9, 95% CI: 1.00 to 3.77) but not among women > 45 years old ($P= 0.24$, OR= 4, 95% CI: 0.38-41.7). This preliminary result seems to be a controversial to Kondrashova TV et al study wherein a positive association of H63D with breast cancer was found among women > 57 years old. For the > 45 years old women, the estimation could be made in wide range because of the insufficient number. Under these conditions, the analysis based on age stratification in our study may cause wrong interpretations. Therefore, we do not give the analysis from age groups in our article.

Necessary revisions under this scope were made in the second paragraph of Discussion (paragraph line 5-11)

7. Since the four-subgroup analyses are not independent from each other, the reviewer suggests multiplying $P$ value (0.02), which has been found for the general comparison by a factor 4. However, if an adjustment like this would be made the type I error ($\alpha$) value 5%, which was accepted for the general comparison, should be changed. Four-subgroup analyses are related with general comparison. Therefore, error for each comparison should be divided by 4 ($\alpha/4=0.05/4=0.0125$). This means that in the subgroup comparisons, statistical significance of $P$ value was determined by according to 0.0125 instead of 0.05. As is known Bonferroni adjustment. As far as we understand, the reviewer asks for this adjustment from us and accordingly we repeated the statistical analysis, but we did not observe any changes in the results.

The explanation, ‘In general group comparisons $P$ value $< 0.05$ was accepted as statistically significant whereas in subgroup analysis after making Bonferroni adjustment $P$ value $< 0.0125$ value was accepted as statistically significant’, was added to the statistical analyses paragraph under the methods section (Paragraph line 7-10)
Minor Essential Revisions

1. Suggested revisions have been made in the Table 2 (Statistical Analysis of Cases with H63D mutation). But, under the view of second reviewer’s recommendation, we changed the place of Table 3 with Table 2. Finally, Table 2 named as ‘Statistical Analysis of Cases with H63D mutation’ has become Table 3 in the revised version of our manuscript.

2. Necessary revisions have been made in the Table 3 (Characteristics of breast cancer patients). But, under the view of second reviewer’s recommendation, we changed the place of Table 3 with Table 2. Finally, Table 3 named as ‘Characteristics of breast cancer patients’ has become Table 2 in the revised version of our manuscript.

3. To comply with the comments of the reviewer Table 4 has been removed and necessary changes were made in the Discussion (second paragraph, line 3-5).
To Referee 2 (Ronald Acton)

Major Compulsory Revisions

- We agree with the reviewer and to prevent confusion, we have clarified the phenotype frequency comparison in the results under the abstract section. We have also used the H63D allele frequencies to compare cases and controls, and found the $P$ value 0.039 and OR as 1.75. According to this, allele frequencies comparison as well as phenotype comparison shows a significant difference for a positive association of H63D with breast cancer.

- To comply with the comments of the reviewer correction has been made in regard to ‘have a role’ (Discussion section, paragraph line 5).

- According to the reviewer’s recommendation the study of Kondrashova et al has been referred and necessary correction has been made (Discussion section, second paragraph first line, and lines between 5 and 8).

- ‘the’ has been inserted before HLA complex (Discussion section, last paragraph, line 8).

Minor Essential Revisions

- Unfortunately, we could not find C283Y positive individual in our study group. But, we strongly believe that the methodology is robust. It is a standard procedure that has been previously described and used in many other C282Y typing studies (references 1 and 28 in the article). We used Rsal enzyme from Roche diagnostics, which has high specific activity. Additionally, upon digestion with Rsal, the 387 bp PCR product of C282Y region gave two fragments of 247 bp and 140 bp in wild-type DNA as expected which was also the clue of the reliability of the enzyme.

Discretionary Revisions

- We have changed the place of Table 3 (Characteristics of breast cancer patients) with Table 2 as suggested by the reviewer.
To Referee 3 (Gregor Weirich)

Major Compulsory Revisions

1. We agree with the reviewer. Initially, we had planned the study according to 108 archived DNA samples. However, we could not amplify 20 samples. A larger study group would have yielded a more robust estimate of the frequency and eliminated the chance factor if we had had the resources to achieve.

2. Being a major reference center, Hacettepe University Oncology Institute has the potential to comprise patients from different regions of Turkey. Below, we give the table showing the origins of patients according to their birth of place. Accordingly, percentages of these distributions are incorporated into the text (Results first paragraph, line 1-5)

<table>
<thead>
<tr>
<th>Origin according to the Birth of Place</th>
<th>No of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Turkey</td>
<td>N= 26</td>
</tr>
<tr>
<td>South and West of Turkey (Mediterranean Region)</td>
<td>N= 19</td>
</tr>
<tr>
<td>East of Turkey</td>
<td>N= 14</td>
</tr>
<tr>
<td>North of Turkey (Black-Sea Region)</td>
<td>N= 10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>N= 69</strong></td>
</tr>
</tbody>
</table>

Medical records on ‘Birth of Place’ information was not available for 19 patients.

3. According to the reviewer’s recommendation, we have added the information about cancer type and stage in the first paragraph of results (line 4-7). Overall, ninety-four percent of the women had limited-stage (stages I-III) and thirty-one percent of the limited stage was stage III. 3 women (6 %) had distant metastases at diagnosis.