Author's response to reviews

Title: The Int7G24A variant of transforming growth factor-beta receptor type I is a risk factor for colorectal cancer in the male Spanish population: a case-control study

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Author’s response to reviews: see over
Dear Editors,

We thank you for your prompt response to our submission. The reviewers’ comments were very constructive and have resulted in a vastly improved manuscript. We have addressed all comments as follows.

<table>
<thead>
<tr>
<th>Italic and underlined:</th>
<th>referees’ comments.</th>
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</thead>
<tbody>
<tr>
<td>Italic:</td>
<td>original text.</td>
</tr>
<tr>
<td>Bold and italic:</td>
<td>text added in response to the referees’ suggestions.</td>
</tr>
</tbody>
</table>

**Referee 1**

- **Major Compulsory Revisions**

  1. *There were totally 504 cases and 504 controls enrolled in this work. But in the section of stratification analysis according to age and sex, the total number in each group is less than 504 (See Table1). Why?*

Data for some of the cases and controls in this study were unavailable:

- Age: 64 cases
- Sex: 7 cases
- Age and sex: 1 control case

We added the following sentence to the *Methods* section:

“The numbers of cases and controls for whom information was unavailable for stratification were 64 and one, respectively”.

2. *As described in “Methods” part, the DNA samples from patients were extracted from the non-cancerous colorectal tissues. Were these samples fresh, frozen or paraffin-embedded tissues? Were they confirmed as “non-cancerous” by pathologists?*

The non-cancerous colorectal tissues were frozen and stored at −80 °C in *RNA later* (Ambion). They were cryopreserved within 30 minutes of resection and
were verified as non-cancerous by a pathologist according to the standard operating procedures for sample collection of both Hospital Tumour Banks.
We added the following phrase to the Methods section:
“non-cancerous frozen colorectal tissue”.

- **Minor Essential Revisions**
1. **Did the controls have history of chronic intestinal diseases, such as inflammatory bowel diseases?**
The controls were patients who attended the emergency room, and their diagnoses (e.g., hernia, bone fracture, hydrocele and appendicitis) were unrelated to the disease of interest. We added the following phrase to the Methods section:
“selected according to diagnoses unrelated to the disease of interest”.

2. **Please provide the sequence of primers used in real-time PCR.**
We used TaqMan SNP Genotyping Assay C_1413390_20 from Applied Biosystems for the genotyping experiments on the rs334354 polymorphism.
This company did not disclose the sequences of the primers and probes. We have included the identification number of the assay in the text as follows:
“(TaqMan SNP Genotyping Assay; ID: C_1413390_20; Applied Biosystems)”. 

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**Referee 2**

- **Minor Essential Revisions**
..... Nonetheless, there is still a question: a recent study has shown that TGFBR1 variants Int7G24A is not associated with an increased familial colorectal cancer risk. How do you explain the result which is different from others?

The results reported by Professor A Lindblom’s group (Br J Cancer, 2009; 100; 1674–1679) do not contradict our results.

- First, they analysed data from a selected subgroup of cases with familial histories of colorectal cancer. We excluded patients with a familial cancer
syndrome from our study and only analysed data from “apparent” sporadic colorectal cancer cases.

The low-penetrance effect of this putative susceptibility allele would be very difficult to detect in cases with high penetrance alleles. The “noise effect” of high-penetrance alleles in familial cases is unavoidable, and such cases are not ideal for detecting low risk levels for cancer.

- Second, although the results of Skoglund-Lundin et al. were suggestive of a trend for this polymorphism, they were not statistically significant (see Table 3A and B, page 1677). Their cases and controls were not matched and the statistical power of their analysis was low because of the number of cancer cases and controls (214 and 856, respectively).

- Third, in our manuscript we emphasized that the colorectal cancer susceptibility induced by the Int7G24A allele is gender dependent, as only males are affected. As Skoglund-Lundin et al. did not stratify their data according to sex, the gender-dependent effect would not have been evident to them.

Referee 3

**Major concerns**

1. Authors should well refer relevant published papers. They claimed that “there are no published reports on the association of this genetic variant with CRC”. Actually that is not true. Lindblom A group have reported relevant data on Br. J Cancer (2007 Oct 22;97(8):1175–9. Epub 2007 Sep 11). In contrast, this study showed Int7G24A are not associated with familial CRC, hereditary non-polyposis colorectal cancer (HNPCC) and non-HNPCC. Although these two studies were performed in different populations, these controversial data would lead readers to doubt the relevance between Int7G24A and CRC.

The cited article (Br J Cancer 2007 Oct 22;97(8):1175–9. Epub 2007 Sep 11) is about sporadic and familial breast cancer and not colorectal cancer. The recent report of Lindblom’s group regarding the Int7G24A polymorphism and CRC (Br J Cancer, 2009; 100; 1674–1679) was discussed in our response to referee #2.
We have referred to Lindblom’s article in the Background, Discussion and References as follows:

Background:
To our knowledge, there is only one published report on the association between this genetic variant and CRC [11]. In that study, there was no evidence of an association between this polymorphism and colorectal cancer risk in familial cases.

Discussion:
Skoglund-Lundin et al. [11] did not detect a significant association between CRC risk and the Int7G24A variant when they analysed data from cases with a familial history of colorectal cancer. The expectedly low-penetrance effect of this putative susceptibility allele would be very difficult to detect in cases with high-penetrance alleles. Moreover, the authors did not stratify their data according to sex.

References:

2. In this manuscript, authors concluded that a statistically significant association of the Int7G24A variant with CRC susceptibility in the patients younger than 70 years. How did they give this threshold level? I can not imagine that the significance of association if they change the threshold into 55 or 65 years old. I would like to see the results after re-analysis based on different thresholds of age.

When a series is stratified by age, it is very common to use the median age as the threshold for establishing groups (younger and older than the median age). We used 70 years as the threshold for the analysis because it was the median age on diagnosis of CRC. This was mentioned in the original text (Unconditional
logistic regression adjusted for age, considering the median age for cases (70 y) as the threshold level).

Nonetheless, we re-analysed the data according to thresholds of 55 and 65 years (see the following table). With a threshold of 65 years, there was a significant association between the Int7G24A variant and CRC in the younger group but not the older group. With a threshold of 55 years, the very low number of cases and controls under 55 years of age detracted from the statistical power of the analysis, even when there was a clear trend between cases and controls. The number of young patients was low because known hereditary cancer cases were excluded from our study and only “apparent” sporadic colorectal cancer cases were included.

3. one more question about the statistics of age groups (<70 or >70). Total number of control groups is 504(284+226), however 64 patients were excluded in the experimental groups. Authors need clarify it.
This point was discussed in our response to referee #1.

4. Suggestion: some results of genotyping should be added as a figure.
Thank you for this suggestion; we have included a figure (Figure 1) with panels A and B that show representative examples of genotyping of the Int7G24G>A polymorphism using real-time PCR and direct sequencing, respectively.
<table>
<thead>
<tr>
<th>Int7G24A genotype</th>
<th>Cases (n=504)</th>
<th>Controls (n=504)</th>
<th>OR [95% CI]</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤65 yrs (n=141)</td>
<td>≤65 yrs (n=150)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G, number (%)</td>
<td>75 (53.19)</td>
<td>99 (66)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>G/A, number (%)</td>
<td>58 (41.13)</td>
<td>45 (30)</td>
<td>1.701 [1.041–2.781]</td>
<td>p*=0.035</td>
</tr>
<tr>
<td>A/A, number (%)</td>
<td>8 (5.67)</td>
<td>6 (4)</td>
<td>1.760 [0.586–5.288]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;65 yrs (n=299)</td>
<td>&gt;65 yrs (n=353)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G, number (%)</td>
<td>188 (62.88)</td>
<td>233 (66.01)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>G/A, number (%)</td>
<td>94 (31.44)</td>
<td>111 (31.44)</td>
<td>1.050 [0.751–1.468]</td>
<td>p*=0.016</td>
</tr>
<tr>
<td>A/A, number (%)</td>
<td>17 (5.69)</td>
<td>9 (2.55)</td>
<td>2.341 [1.020–5.371]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤55 yrs (n=48)</td>
<td>≤55 yrs (n=22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G, number (%)</td>
<td>25 (52.08)</td>
<td>14 (63.64)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>G/A, number (%)</td>
<td>21 (43.75)</td>
<td>7 (31.82)</td>
<td>1.680 [0.572–4.932]</td>
<td>p*=0.454</td>
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<tr>
<td>A/A, number (%)</td>
<td>2 (4.17)</td>
<td>1 (4.55)</td>
<td>1.12 [0.093–13.482]</td>
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</tr>
<tr>
<td></td>
<td>&gt;55 yrs (n=392)</td>
<td>&gt;55 yrs (n=481)</td>
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<tr>
<td>G/G, number (%)</td>
<td>238 (60.71)</td>
<td>318 (66.11)</td>
<td>1 (ref)</td>
<td></td>
</tr>
<tr>
<td>G/A, number (%)</td>
<td>131 (33.42)</td>
<td>149 (30.98)</td>
<td>1.175 [0.880–1.568]</td>
<td></td>
</tr>
<tr>
<td>A/A, number (%)</td>
<td>23 (5.87)</td>
<td>14 (2.91)</td>
<td>2.195 [1.106–4.356]</td>
<td>p*=0.031</td>
</tr>
</tbody>
</table>

p*: p for trend (Armitage’s trend test)