Author's response to reviews

Title: Associations of Prostate Cancer Risk with Viral Infection and the RNASEL R462Q variant.

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Author's response to reviews: see over
Mrs. Jenny Leigh  
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Subject: MS: 1903186081317681- Associations of Prostate Cancer Risk with the RNASEL R462Q Polymorphism and Viral Infection.

Dear Mrs. Leigh:

Enclosed you will find the revised version of the manuscript “Associations of Prostate Cancer Risk with the RNASEL R462Q Polymorphism and Viral Infection” written by Martinez-Fierro ML., et al. As suggested, we edited the language of the article, and corrected gene nomenclature and abbreviation spelling for better comprehension. We also followed reviewers’ recommendations, as described in the attached list of changes.

We hope that you will find the article satisfactory for its publication.

Thanks a lot for all your attention. I remain

Sincerely yours,

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Reviewer's report:

Major Compulsory Revisions
1. The aim and hypothesis of the study are ambiguous. In the abstract, the aim of this study seems to be to compare viral infection and PC risk. In the main text, however, the hypothesis is stated as "The RNaseL variant R462Q increases susceptibility of PC". Therefore, the conclusion in the abstract is completely different from that in the main text, and the result of comparison between the genotypes and PC risk is not addressed in the abstract. The authors should clearly state the aim of the study and make consistency between the abstract and the main text.

Answer: Abstract was restructured as recommended (page 3). The new abstract states that the aim of the study is to analyze possible associations among prostate cancer, prostate viral infections, and the RNASEL 462Q allele in PC patients and controls, as further developed in the main text.

2. The authors state that the association between the R462Q genotype and PC risk could not be determined due to the absence of PC patient with the Q/Q genotype. The chi-square test is available even in such case, and a dominant model (RR vs. R/Q and Q/Q) is also applicable, although the odds ratio is not able to be calculated.

Answer: As suggested, we recalculate the association as recommended (using the dominant model) and added: “We also tested the association between PC or viral infection and R462Q genotypes identified in this study. For this, frequencies of the R/Q and Q/Q genotypes were grouped and compared with the R/R frequencies. No associations with PC (P=0.48) or viral infection (P=0.34) were found in this subset analysis”, in the Results section (page 10, line 164).

3. The cancer detection rate reported in this study is quite low in patients with PSA level 4.1 – 8.0 ng/ml. Since the smaller number of biopsy specimens leads to higher contamination rate of PC patients in the control group. The number of biopsy specimens obtained should be described.

Answer: In the Methods section was stated that the octant technique was performed to obtain the histological diagnosis (page 5, line 90). After this, we added: “and 8 cylinders were taken for histological diagnosis”. Considering this clarification, we consider that subject diagnosis and classification were correctly performed, as allowed by our clinical
guidelines; but we acknowledge that the number of cylinders used for cancer detection may constitute a limitation of the study. We also indicate that we used two additional cylinders for viral screening (Results section, page 11, line 193).

4. What is a definition of "benign prostatic tissue"? Does it mean normal prostate tissue without hyperplasia or prostatitis? Since "benign" is used as the opposite of "malignant", "benign" usually includes hyperplasia, prostatitis, and any other non-malignant condition. "Benign prostatic tissue" used in this article seems to be a confusing expression.

**Answer:** Normal prostate tissue without other histologic finding is referred like “benign prostatic tissue” in this article. As suggested, the sentence “benign prostatic tissue” was replaced by “normal prostate tissue” in the Results section (page 9, lines 152 and 153), and in the Discussion section (line 215).

5. Table of multivariate analysis (and univariate analysis, if possible) should be needed. At least, variables included in the model and odds ratio of each variable should be indicated.

**Answer:** Since the association between PC and HPV infection was the only positive finding of the multivariate analysis for this study, we considered that the suggested table is not relevant for the article and decided not include it; but we can include the table if requested again.

**Discretionary Revisions.**
1. The content of the last paragraph of the Discussion section should be moved to the result section.

**Answer:** As suggested, the last paragraph of Discussion section was moved to Results section (page 11, line 192).

2. Figure 1 (also figure 2) could be combined with table 2.

**Answer:** As recommended by the other reviewers, both figures were eliminated.

**Minor Essential Revisions.**
1. Nomenclatures of the gene name and genotypes should be unified. (RNase L or RNASEL? R/R or G/G, R/Q or G/A, and Q/Q or A/A?)

**Answer:** This was also recommended by the other reviewers. Accordingly, gene and genotype nomenclatures were unified to RNASEL and to R/R, R/Q, and Q/Q genotypes.

2. BTR in the last paragraph of the discussion section should be spelled out.

**Answer:** “BTR” was changed to “TRB” in the Discussion section (page 11, line 198).
Level of interest: An article whose findings are important to those with closely related research interests.

Quality of written English: Acceptable.

Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests:
I declare that I have no competing interests.
REFEREE 2.

Reviewer: Nicole Fischer.

- Major Compulsory Revisions:

1. The title of the manuscript is misleading and should be changed: The current study does not provide information about the Prostate cancer risk and RNaseL SNP R462Q.

   Answer: As recommended by Reviewers 2 and 3, the manuscript title was replaced by “Associations of Prostate Cancer Risk with Viral Infection and the RNASEL R462Q variant”.

2. The assortment of viruses included is not clear to me; why was there only CMV as a human herpesvirus analyzed, HHV8, HSV and most of all EBV should have been included as well especially with regard to Table 5 (which was mislabelled as Table 6) where the authors compare the results of their study to studies published earlier.

   Answer: HCMV was the main Herpesvirus member associated to prostate infection when we designed the study (2005) and considering the amount of biopsy material required for the viral screening, we decided to focus on this virus, despite reports implicating other viruses as EVB or HHV8. “Table 6” was relabeled as “Table 4” (lines 235, 249, and 386 respectively).

3. The authors should provide more detailed information about the cylinder biopsies used for the study. Those used for DNA extraction were not identical cylinder biopsies that underwent pathological examination, how can the authors be sure that the biopsy that was included in DNA extraction contained tumor material?

   Answer: As referred before in the response of Question 3 of Referee 1, 10 cylinders (8 for pathology and 2 for viral screening) were obtained from each participant, and this was detailed in the reviewed article. Biopsy materials were not cross evaluated for PC and viral infection. We assume that the number of cylinders sent to Pathology is adequate for PC diagnosis, as standardized in the Hospital; but cannot exclude the possibility of tumor tissue in a cylinder used for virus screening, as suggested by the Referee.

4. The control group should be defined more precisely: were multiple biopsies taken and considered as tumor free? Were those patients monitored over time (watch and wait)?

   Answer: We stated that the octant technique was performed to obtain the histological diagnosis in the Methods section, and added the number of cylinders sent for histological diagnosis (page 5, line 90). We did not follow the subjects after the study; therefore, the sentence “Study subjects were not followed thereafter” was inserted in page 6, line 94.

5. Detection limits (range of PCR) for polyomaviruses is not defined.
Answer: We specified that the detection limits of nested PCR protocols were established at 30 copies for polyomaviruses and 60 copies for HPV, XMRV, and HCMV in the Results section and explained the experiment to establish de detection limit (page 10, lines 170 and 171, respectively).

6. Discussion should include the possibility that other mutations (than R462Q) within the RNaseL gene (which have been described to be associated with increased PC risk) could be present in the samples.

Answer: As suggested, genetic factors or DNA mutations other than RNASEL R462Q may influence PC risk and/or infection susceptibility, but they were not aimed in this work as indicated in the Discussion section (page 13, line 231).

7. Genotype frequency difference of the current study QQ: 0% (versus expected values of 10-15% in other studies) should be discussed.

Answer: We state that genotype frequencies found in our study may be explained by ethnical variations in the Discussion section (page 12, line 211).

8. The paper by Hohn et al., 2009 Retrovirology should be included by the authors.

Answer: As suggested, this reference was commented and included (reference 22) in the Discussion section (page 12, line 224).

- Minor Essential Revisions:

1. Abstract: last sentence should be rephrased

Answer: The abstract was modified according to Referee 1, and the last sentence was eliminated.

2. Figure 2 does not provide any useful information. The information is already given within the text of the manuscript.

Answer: As mentioned before, Figures 1 and 2 were eliminated.

3. Table 6 should be Table 5 (also in the manuscript text).

Answer: As referred above, “Table 6” was relabeled as “Table 4” and in the main text (lines 235, 249, and 386 respectively).

4. Table 5, abbreviations of viruses: E.B.? Epstein Bar? Epstein Barr Virus or EBV.
**Answer:** Abbreviations were replaced for “Epstein-Barr Virus” in table 4.

5. Description of the positive control plasmids pHCMV and pXMRVm is missing in Table 2, supplementary data.

**Answer:** In supplementy material Table 2 the sentence “pHCMV from HCMV genome (ATCC number: VR-977), and pXMRVm from DNA of 22Rv1 cellular line respectively” was added as additional information about these control plasmids.
Reviewer: Oleg Alexeyev

Reviewer's report:
In this reviewer opinion the manuscript title is ambiguous and gives impression that there is association between RNASEL R462Q polymorphism and viral Infection and prostate cancer. In fact the only association to have been demonstrated is for HPV and PC.

Answer: As suggested by the other reviewers, the manuscript title was modified, as mentioned before.

Abstract
1. The second sentence in the background section is more suitable for introduction.

Answer:
2. The abstract lacks consistency in presentation. For example, genotyping of the R462Q alleles is mentioned in the methods section but not in the results and conclusions.

Answer: As referred above, the Abstract was restructured and genotyping of R462Q is mentioned in Methods, Results, and Discussion.

3. The last sentence in the conclusion section is used to indicate the major finding of the paper. Is it so for the current paper?

Answer: The Conclusion was modified to emphasize that our study found a positive association between PC and HPV infection, and that we could not establish interactions among RNASEL 462Q/Q, PC cases and prostate viral infections, as explained in the main text.

Methods
4. Selection criteria for TRP are vague. The authors should elaborate on how many patients fulfilled each of the stated criteria: abnormal DRE, TURP. Which TURP findings were used as a criterion for TRP?

Answer: Description of TURP criteria guidelines followed in this study were included in the reviewed article (page 5, line 78).

5. It is not clear how case patients were matched with control patients? Definitely not by age, as can be seen from the fig 1.

Answer: We anticipated the possibility to match study groups by age, since prostate pathologies usually affect men above 50 years old, and recruitment efforts were focused
in men attending the Urology Service with confirmed biopsy results and who were willing to participate. Although men in the control group were above 50 years old, matching cases and controls by age was not possible, however we did the necessary adjustments in the logistic regression model, as referred in page 10, line 179 (we added “Due to the significant differences between ages in the study groups, adjustments for age were included in the multivariate logistic regression model; the odds ratio for HPV infection and PC was calculated in 3.98 (95% CI: 1.17 to 13.56, P=0.027)”. We also included a P value to indicate the differences in ages between groups in line 147.

6. Description of equipment for PSA determination, ultrasound investigation as well as the regime of antibiotic prophylaxis/enema is not pertinent to the scope of the paper and can be omitted.

**Answer:** This description was eliminated.

Results

7. The section on clinical characteristics of the patients would suit Material and Methods.

**Answer:** We decided to leave the clinical characteristics of the patients in the Results section. We consider that description of the strategy and criteria for patient enrollment suits well in the Methods section and clinical characteristics of the study subjects suits better in the Results section, because is a result of the study design.

8. The figures 1 and 2 to a large extent duplicate the text and can be omitted. The same for figure 2.

**Answer:** Both figures were eliminated.

R462Q genotyping

9. Units for measuring frequencies of genotypes should be clearly stated.

**Answer:** We measure R462Q genotype frequencies as the proportions of genotypes in the studied population sample.

Viral sequence detection

10. The sentence “The presence of HPV sequences was detected in 15 subjects (11.5%) of which 11 (20.0%) of them were cases and 4 (5.33%) controls” should be re-written. From the mathematical viewpoint it should be 11 (73%)...and 4 (27%).

**Answer:** We rewrite the sentence to attend Referee’s suggestion, as follows: “The presence of HPV sequences was detected in 15 subjects (11.5%) of which 11 (73.0%) of them were cases and 4 (27.0%) controls” (page 10, line 172).
Discussion

11. The authors have utilized 2 biopsy cores for virus detection (page 5). This is a major limitation of the study which may explain low viral detection. The point should be addressed.

**Answer:** As indicated, the number of cylinders used to perform the viral screening is a limitation of the study. We address this limitation in the Results section (page 11, line 193) by adding: “By other hand, in the most of the cases we used a low quantity of prostate tissue (two TRB cylinders) for the virus screening and it could to enhance the differences in virus detection between studies.”

12. A disproportionally large space is devoted to XMRV, given the single positive sample in the control cohort.

**Answer:** We devote attention to the XMRV because of the growing amount of information linking PC with this virus infection and we stated that this agent lacks association with the tumor in our study, as reported by some authors in Europe.

13. “Absence of HCMV, polyomaviruses, and XMRV in tumor prostate tissue suggests that they are not associated with PC in Mexican population” page 12 is clearly an overstatement, given the size of the case cohort.

**Answer:** We insert a paragraph in the Results section to address the limitations of the study, and mention that an important limitation of this study is the “n” of the study sample (page 11, line 192).

**Level of interest:** An article of limited interest.

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

**Answer:** We performed several language corrections. These changes are included in “Format corrections” at the end of reviewer’s comments.

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:** I declare that I have no competing interests.
FORMAT CORRECTIONS.

1. Page 3 (line 11) two new affiliations were added:


2. In line 17 “School of Medicine, Universidad Autonoma de Nuevo Leon. Av. F. I. Madero S/N, Col. Mitras Centro. Monterrey, N.L., C.P. 64460” was replaced by “Centro de Investigación y Desarrollo en Ciencias de la Salud, Universidad Autonoma de Nuevo Leon Av. Gonzalitos S/N, Col. Mitras Centro, C.P. 64460 Monterrey, Mexico”

3. In page 4 (line 52) the text “The inflammatory etiology of prostate cancer (PC) is supported by gene mutations in important antiviral effector proteins involved in hereditary PC, such as the RNASEL gene (involved in antiviral and antiproliferative roles of interferons) at the HPC1 locus [7-10]” was replaced by “The inflammatory etiology of prostate cancer (PC) is supported by the fact that the candidate gene for hereditary PC at the HPC1 locus is RNASEL, which is involved in antiviral and antiproliferative roles of interferons [7-10]”

4. In page 4 (line 64) the text “The RNase L variant R462Q is suggested to increase susceptibility for PC, but this hypothesis has only been tested for the XMRV virus [12, 15, 16]. No study has reported relationships among the polymorphism, other viral infections, and PC. We assessed these possible associations in the present study” was replaced by “The RNASEL variant R462Q is suggested to increase susceptibility for PC. In addition, this increase in susceptibility has been associated with an increase in prevalence of the Xenotropic Murine Leukemia Virus-related gammaretrovirus virus (XMRV) [12, 15, 16]. No study has reported relationships among the variant, other viral infections, and PC. We assessed these possible associations in the present study.

5. In page 5 (line 71) the title “Patients and samples origin” was changed by “Patients and samples collection”

6. In page 5 (line 83) the text “The control group was constituted by subjects who underwent a TRB or TURP but had no pathological evidence for PC” was replaced by “The control group constituted of subjects who underwent a TRB or TURP but had no pathological evidence for PC”.

7. In page 5 (line 86), “subject before TRB or TURP procedures” was replaced by “subject before their procedure”.
8. In the line 96 the text “DNA of blood samples was obtained using a standard phenol/chloroform protocol, and DNA/RNA extraction of snap-frozen tissue samples was performed using the Trizol reagent”, was replaced by “DNA from blood samples was isolated using a standard phenol/chloroform protocol, and DNA/RNA extraction of snap-frozen tissue samples was performed using the Trizol reagent (Invitrogen, Carlsbad, CA)”.

9. In the line 113, the paragraph “Viral sequences were identified in prostate tissue by nested PCR using previously described protocols [4, 12, 17]. Briefly, 500 ng of prostate DNA were subjected to amplification for polyomaviruses and HPV screenings [4, 12, 17]. For XMRV screening, 1 µg of RNA was reverse transcribed using the SSIII system (Invitrogen) and 2 µL of cDNA were used in a specific PCR protocol described by Urisman et al. [12]” was replaced by “Viral sequences were identified in prostate tissue by nested PCR as previously described [4, 12, 17]. Briefly, 500 ng of DNA isolated from the prostate were subjected to amplification for polyomaviruses and HPV screenings [4, 12, 17]. For XMRV screening, 1 µg of RNA was reverse transcribed using the SSIII system (Invitrogen) and 2 µL of the cDNA reaction were used in the protocol described by Urisman et al. [12].”

10. In the line 130, the text “HPV DNA genotypes of positive samples were discriminated using the Linear Array HPV Genotyping Test (Roche Diagnostics, Basel, Switzerland),” was changed by “HPV DNA genotypes were discriminated using the Linear Array HPV Genotyping Test (Roche Diagnostics, Basel, Switzerland),”.

11. In Results section (line 143) the text “A total of 130 Mexican men were entered into the study, 55 constituted the case group and 75 were admitted to the control group” was replaced by “A total of 130 Mexican men (55 prostate cancer cases and 75 controls) were entered into the study.”

12. In line 173, “Infections by XMRV and HCMV were detected in one (1.33%) and six (8.0%) control samples respectively, but not in tumoral prostate tissue” was replaced by “Viral genomes for XMRV and HCMV were detected in one (1.33%) and six (8.0%) control tissues respectively, but not in the prostate tumors”.

13. In line 186, the paragraph “Given the limited amount of sample, HPV typing was restricted to 6 samples (5 cases and one control) as shown in Table 4. Ten different types of HPV were determined” was replaced by “Given the limited size of the specimens, HPV sub-typing was performed on only 6 samples (5 cases and one control) as shown in Table 4. The samples were screened for ten different types of HPV;”
14. In line 223, the sentence “Differences between the reported XMRV prevalences suggest that the high susceptibility observed in Urisman’s study might be influenced by hereditary factors typical of familiar PC but absent in sporadic PC cases” was deleted.

15. In line 230, the text “Our results, based on studies on RNA from fresh frozen tissues, reflected the viral replication and transcription activities in infected tissues. Since we no detect XMRV activity in PC tissues, we discard its association with PC” was replaced by “Our results, based on studies on RNA from fresh frozen tissues, could not demonstrate its association with PC.”