Author's response to reviews

Title: Interaction between polymorphisms of the Human Leukocyte Antigen and HPV-16 Variants on the risk of invasive cervical cancer

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Dear Madam or Sir,

I enclose herewith a revised version of an original manuscript entitled “Interaction between polymorphisms of the Human Leukocyte Antigen and HPV-16 variants on the risk of invasive cervical cancer” by myself, Maciag, Ribeiro, Petzl-Erler, Franco and Villa.
Regarding the comments and questions addressed by the referees, I provide a list of answers below.

Sincerely yours,

Patrícia Savio de Araujo Souza, PhD
Responses to Dr Zehbe’s comments:

1. As suggested, we included more up-to-date references on page 3 and 9;
2. Regarding HPV typing, DNA was extracted, purified by spin column chromatography, and amplified by polymerase chain reaction (PCR) using the MY09/11 and PGMY protocols for the presence of HPV DNA. A negative control tube including all PCR reagents except DNA was included in all PCR reactions. Typing of amplified products was performed by hybridization with individual oligonucleotide probes and by restriction fragment length polymorphism (RFLP) analysis, which identified more than 40 HPV genital types. We included more than 30 type-specific positive controls in hybridization membranes. DNA specimen quality was checked by amplification of a 268-bp human β-globin gene region. Specimens were tested blindly and precautions were taken to prevent contamination. Samples that were negative for both HPV and β-globin were considered inadequate for analysis. It is the routine of our laboratory to use several controls in PCR reactions that are then transferred to nylon membranes to be hybridized with type-specific probes. These include both recombinant plasmids containing the genomes of several HPVs, both LR and HR types, as well as DNAs extracted from cervical samples previously shown to be HPV-positive to different types. Each time we change batches of primers or probes, a quality control is performed to check the performance of the new sets of oligonucleotides and reagents. In addition, we regularly use Roche’s Line Blot assay to confirm cases with multiple HPV types. In the majority of cases, we find the same types in both assays that use exactly the same primers (PGMY09/11), the discrepancies being explained by levels of the different HPVs present in the sample which often are detected more easily by a more sensitive method such as dot blot hybridization. Indeed, with the experience accumulated over a decade, we consider that the most robust, sensitive, and reproducible assay to detect multiple infections is still type-specific hybridization of PCR products.

Considering the determination of HV-16 variants, we characterized E6 and L1 genes by nested PCR, followed by hybridization with oligonucleotide probes as described by Wheeler et al., 1997. Samples that originate hybridization patterns diverse from that obtained by described variants were submitted to cloning and to identify infections by multiple variants, five clones from each transformation were selected for sequencing.
analysis. Samples were classified as positive for multiple infections only if clones from different HPV-16 branches were identified in one gene fragment and confirmed in the other (E6 and L1). We excluded samples with unmatched patterns in E6 and L1 analysis.

3. We redesigned the sentences in the 1st paragraph of page 7 and page 9;

4. Although we find the inverse association between 83L and DRB1*04, the 83V was not associated with DRB1*04. Other reports that described the association between DR4 and 83V cases, but the 83V group was mainly composed by European variants carrying the 83V. As our 83V group included mainly AA variants, it is possible that the other positions influence this result.

5. We also corrected the sentence in conclusion.
Responses to Dr. Madeleine’s comments:

1. We improved our description of HPV16 variants and its association with ICC in the introduction;

Methods:

1. Unfortunately, we have no data about responses in case or control group;
2. DNA source used in HLA typing was the same used in HPV detection, and we add it to the text, in methods, in the end of samples section.
3. We present in the paper all the association found, and we add this information in the methods section;

Results

1. We redesign the table 1, but we decided to maintain the HPV-16 distribution in all cases, to permit comparison with the distribution of variants In ICC cases from other studies;
2. The N/C prevalence of controls is the second column of table 2; and we also included these data on tables 3 and 4.
3. We grouped HLA-DQA1*0101 and HLA-DQA1*0104 alleles because using our set of probes we could not discriminate between them. On the other hand, we have probes to discriminate the HLA-DQA1*0102 allele.
4. In the group E-P of table 3 we also included one sample that is referred as E-6994A in table 1, because the only difference between them is the synonymous substitution A6994G.
5. In table 4 we decided to include all the variants according to the 83 position. To do so, we grouped the E-P variants (n=23), one E-6994A, one E-G131T and 4 AF2 variants. The description of the 83L and 83V groups is on the 8th paragraph of results section.

Discussion

1. As suggested, we rewrote the sentence, but the meaning is that we found that the number women carrying AA variants was similar to the number of women harboring E-P cases.
2. We add the data concerning to DRB1*1302 absence in AA cases to the abstract. We also add the DQB1*0301 ORs and CIs to the tables 3 and 4, but we did not find associations of this allele with cases.
Responses to Dr. Xu’s comments:

1. Due to the large number of studies of HLA class II /HPV related diseases association we decided to include just one review citation in the third paragraph, and some findings (references 11, 12, 13, 14 15 and 16 on the fourth paragraph) in the background of previous version. But in this version we add a few others to comply with your suggestion.

2. We redesign the first session of results;

3. As suggested, we added the P value of the associations reported in the results section; however, information about statistical inference was already available via the confidence intervals.

4. In our analysis, we first calculated the crude OR and 95% CIs, and then, confounding by age and ethnic group was ascertained using a change-in-estimate criterion for the crude observations. Adjustment for potential confounders was made in all comparisons, but in most of them, results were similar. The difference in values of crude and adjusted OR, like the observed for the DRB1*08041-DQB1*0301 in table 2, reveals that one of the variables considered in the adjusted analysis is a confounder, or, the result of crude analysis might be biased. Results in table 2 were shown for comparison with those in tables 3 and 4. The number of carriers of this haplotype is small in our groups, but it seems interesting that it was not found in cases positive for AA or 83V variants.

5. We redesign the tables;

6. As suggested, we add the reference group in tables 3 and 4;

7. We decided to show just the results for the alleles and haplotypes previously associated with HPV-related diseases or statistical significant ones, and to summarize them in the tables, just to reduce their size. Because of that, we presented the results for DRB1*04 group, but not for the haplotypes including DR4 alleles. The “data not shown” cited for DRB1*04-DQB1*0302 and 83L is OR= 0.28; 95% CI: 0.06-1.27.

8. We corrected “CRPV” and used “cottontail rabbit papillomavirus” instead.