Author's response to reviews

Title: Analysis of mutations in the E6 oncogene of Human Papillomavirus 16 in cervical cancer isolates from Moroccan women

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Author's response to reviews: see over
Responses to the referee 1 report: Elena Burroni

Title: Analysis of mutations in the E6 oncogene of Human Papillomavirus 16 in cervical cancer isolates from Moroccan women

Reviewer's report:

The manuscript is well presented, written and discussed. This topic is really interesting because there are few data on HPV16 variants in North Africa, in particular in Morocco.

First of all, many thanks you for your interest to our manuscript.

Minor essential revisions:

1) Many references are cited and possible differences among already published and present results are discussed, except for one point, authors have found a very high percentage of HPV16 positive samples in this population (87.29%), that is not in line with what reported in 2 Moroccan studies that they have cited (Lalaoui 2003 and Meftah El Khair 2010), so please add this in the discussion.

As suggested, the discussion part was revisited to compare obtained results with reported data from previous Moroccan studies. Indeed, Lalaoui et al. (2003) have found HPV 16 in 48% of HPV positive cases, whereas in the study of Meftah El Khair et al. (2009) HPV16 was found in 71% of HPV positive cases, including mono-infected cases (37%) and co-infected cases (34%). This difference may be related to the sampling bias but also could be due to the molecular technique used for the HPV genotyping. In the previous studies, HPV genotyping was done by combining consensus PCR and dot blot hybridization with specific probes. In our study, the HPV genotyping was performed by DNA sequencing of hyper-variable region in L1 fragment. Many studies have reported that genotyping by sequencing is more accurate and give a high sensitivity to (Lee et al, BMC Women's Health 2009, 9:8).

2) Background, page 3, line 7: the term “Human papillomavirus” has already been explained in the previous sentence so, please use only HPV

We agree with you and sorry for this mistake. The entire document has been checked to use only HPV instead of “Human papillomavirus”

3) Some English errors, methods, page 4, line 19 change “Genecology” in “Gynaecology”; discussion, page 12, line 17 correct “somme” in “some” and page 12, line 18 “carnigenesis” in “carcinogenesis”

Many thanks. All these errors have been corrected in the revised version

4) Methods, page 5, line 15, delete “specimens”, “fresh frozen tissue” is sufficient
As suggested, “specimens” was deleted in the Methods section.

5) Methods, page 5, line 20 please specify the quantity of the volume used to re-suspend DNA.

DNA was re-suspended in 25 – 30 µL of sterile distilled water according to the amount of the pellet.

6) Table 1: GP5+/6+, MY09/11 and PC04 and GH20 primer sequences can be omitted from table 1 and references included in the text.

Primer sequences were taken from different references. To facilitate the use of these sequences by the readers, we think that the main option is to give the complete sequences in the table. However, if you prefer to delete them from the main document, we can give the corresponding references in the text.

7) Methods: authors assess that they performed nested PCR to all samples, since the limits of nested PCR are well known (the major is the possibility of false positive results) I ask to the authors to explain why they didn’t sequence the fragment amplified by MY09/11 and didn’t perform nested PCR just in case of negative result for MY09/11.

We agree with you, DNA sequencing of fragment amplified by MY09/11 would be very interesting. Nested PCR is time and budget consuming and can generate false negative. In our case, DNA amplification with MY09/11 generated non sequencable bands. Moreover, to avoid false positive results, DNA amplification and sequencing were performed twice.

8) Moreover, I appreciate the authors have performed twice the PCR amplification and DNA sequencing, but why didn’t they confirm the results obtained with GP6+ sequencing primer also with GP5+ primer?

We agree with you. HPV genotyping is mainly determined by sequencing the hyper-variable 34–50 bp DNA sequence which is downstream of the GP5+ primer site, and usually this region is sequenced twice by GP6+ and GP5+ primers. In our study, and due to limited budget, we have used only GP6+ as it gave a good matching with reference sequences. All sequences were made twice for more accuracy.

9) Results: page 8, line 17 change GP5+/GP6+” in “GP5+/6+”

The main document was checked and GP5+/GP6+ was replaced by GP5+/6+ and MY09/MY11 by MY09/11.

9) I suggest to the authors to adapt the classification showed on table 2 to the last classification of HPV16 variants (Cornet et al. 2013 Journal of Virology) (i.e. Af1 reported on table 2 is better identified by Af1b) that they have mentioned several times in the manuscript.
Thank you for your suggestion. Cornet *et al.* has reported Af1b as a sublineage. Worldwide, the main classification used is the last global classification of E6 HPV16 variants published by Huertas-Salgado *et al.* (“E6 molecular variants of human papillomavirus (HPV) type 16: An updated and unified criterion for clustering and nomenclature”. Virology 410 (2011) 201–215). In this classification, Af1 is considered as a lineage whereas Af1-d is a considered as a class which is subdivided in 2 sub-classes (Table2). The table 2 was rearranged to avoid any misunderstanding.

Level of interest: An article of importance in its field

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

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

**Responses to the Referee 2 report: Heather Cubie**

Title: Analysis of mutations in the E6 oncogene of Human Papillomavirus 16 in cervical cancer isolates from Moroccan women

Reviewer's report:

1. Major compulsory revisions

There are one or two statements which are too sweeping and not backed up by statistical evidence or power. In the Results section on p10, paragraphs 2+3, the statement that Af variants are mostly found in advanced cancers requires statistical comparison since the numbers are relatively small.

All correlations were subjects to statistical analysis, and as reported in the last paragraph of page 10, no significant statistical difference between HPV variants and cancer stage was detected. However, it seems that, according to the cancer stage, percentage of E variants reduce and conversely the percentage of Af variant increase. There’s tendency to a possible association between Af variants and advanced stages. Therefore, to avoid any misunderstanding, the statement was replaced by “Af variants seems to be most likely detected in advanced cancer stages”

Surprisingly, statistical analysis is only provided in relation to the association of different cancers and associated HPV16v with age. Such analyses are needed elsewhere.

As we have reported above, statistical analysis was done for all comparisons, but significant association was found only for the association of different cancers and associated HPV16v with age.
Regarding the association between HPV variants and malignant phenotype, no significant difference was found. However, as for the association with cancer stage, there’s tendency to a possible association. Indeed, only 41.18% of poorly differentiated cases have E variants versus 66.67% and 61.29% of moderated and well differentiated cases respectively. Conversely, Af variants were found among 47.06% of poorly differentiated cases and only in 28.20% and 22.58% of moderated and well differentiated cases respectively.

In addition, there might be other reasons for more advanced disease e.g. poor health and nutrition. How comparable are the groups of women with European, African and North American variants?

We agree with you, many studies have highlighted the impact of some environmental factors like poor health, nutrition and hormonal factors in the progression of the disease. Unfortunately we don’t have enough information about the women participating in this study to compare them with E, Af and NA variants. The only available criteria were age, type of carcinoma, stage and differentiation. Comparison of the groups of women with E, Af and NA variants would be of great interest to evaluate factors involved in the disease progression for better management of cervical cancer.

2. Minor essential revisions

2.1 P3 para 1: clarify whether cervical cancer is top or second most common cancer in women in Morocco – both are mentioned in the same paragraph.

Sorry for this mistake, all reported data indicate that cervical cancer is the second most common cancer among women in Morocco. Thus, the sentence “where the incidence of cervical cancer ranks top among cancer in women” was deleted.

2.2 P4 Clinical Specimens: spelling of Gynaecology/Gynecology

It was done.

2.3 Materials and Methods are too detailed and could be cut (especially established PCR techniques for HPV genotyping and detection of variants where published methods can be referred to with only modifications detailed).

As requested, the Material and Methods section was re-edited to be limited to the methodological approaches. All details were cut to give only references of the published methods.

3. Discretionary Revisions
The Tables are helpful, but would be more readily followed if the percentages are either removed. Alternatively, linking them to proportions within a group of cancers (i.e. European or African) would match better with the text.

As requested, percentages was removed from the tables and reported in the main text.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

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.

Responses to the Referee 3 report: Alyssa Cornall

Title: Analysis of mutations in the E6 oncogene of Human Papillomavirus 16 in cervical cancer isolates from Moroccan women

Reviewer's report:

Major compulsory revisions

Page 7, first paragraph: 'In accordance with established guidelines, a nucleotide sequence was assigned to an HPV type if it corresponded with a known HPV genotype by >95%.' The authors have referenced Wu, et al which is incorrect, and there is some doubt as to where this guideline actually originated - if the origin of this guideline cannot be accurately established, the interpretation of these results is also on doubt. Please clarify with the correct reference.

We agree with you, there’s mention of these guidelines in Wu et al., 2006 but referenced as Gravitt et al., (J. Virol. Methods. 2003. 112:23-33). However, after close research, we have found that the paper of Gravitt et al., don’t give mention of this statement. Further research allowed us to find other references that define HPV genotypes. In this regards, Lee et al. (2009) and De Villiers et al. (2004) have reported that a genotype of HPV differs in the L1 gene DNA sequence by at least 10% from every other known HPV type. Thus, we have replaced 95% by 90%.
Moreover, in our study, and as reported by Lee et al. (2009), an exclusive 100%"identities" match between the "query" sequence and the"subject" sequence was required for accurate genotyping. A 34-base sequence downstream of the GP5+ primer site excised from the electropherogram of the sample, which is fully matched with a standard HPV signature sequence stored in the GenBank validated the HPV genotype, except for some variants of HPV-16, HPV-
31 and HPV-33, for which BLAST algorithms of a 46–50 base sequence in this region were needed for unequivocal genotyping (Lee et al, BMC Women's Health 2009, 9:8).

Minor essential revisions

It is good that the authors have used only single 16 positive samples for E6 variant analysis, please indicate this in the methods.

We agree with you. Thus, in the paragraph “Determination of HPV16 E6 variants” of the Methods section, we have added this sentence in the beginning of the paragraph: “Samples with a single HPV16 genotype were selected for E6 variants investigation”.

Page 8, Results, Subtype distribution, second paragraph, the following sentence is unclear as to meaning: 'Sequence analysis of the HPV amplimers allowed the identification of 5 carcinogenic HPV genotypes: 16, 18, 31, 33 and 35.' Do the authors mean that the primers they used only amplified these types, or only these five types we're observed; where there sequences which could not be identified because satisfactory matches could not be found in the database?

We mean that only these five types were observed. Thus, to avoid any misunderstanding, the sentence was revised and was replaced by: “A total of 5 carcinogenic HPV genotypes: 16, 18, 31, 33 and 35 were identified”.

There are some inconsistencies in the references section, please review carefully and correct.

Many thanks for this remark. The entire references section was checked and corrected.

Minor grammatical and spelling corrections are required.

Discretionary revisions

Could the relationship between variant and disease stage be influenced by the age of the patient, rather than the aggressiveness of the virus? It appears that the later stage cancers are seen most often in the older patients, which mostly seem to have been infected with non-European subtypes.

Many thanks for this relevant remark. Due to limited number of cases especially in younger women, it was very difficult to perform multi-parameter statistical analysis to evaluate the impact of age on the association between HPV 16 variants and the cancer stage. In this study, we found that the majority of cervical neoplasia with HPV16 E variant lesions presented at stage I and II and conversely, Af variants seem to be most likely detected in advanced cancer stages, with no significant difference. There’s tendency to a possible association between Af variants and advanced stages.
We believe that the age of patient is key factor in the progression of the disease. Indeed, several studies have suggested that HPV16 E6 variants are involved in determining persistence of the viral infection and the development of cervical lesions (Grodzki et al. 2006). Moreover, non-European HPV16 variants were found to be more associated with disease progression than the European variants (Xi et al, Cancer Epidemiol Biomarkers Prev, 2002, 11)

Page 14: the relationships between particular E6 subtypes and the cellular signaling pathways mentioned are interesting but could be expanded on slightly so that the non-expert does not need to find another reference to realize the significance of these interactions.

As requested, the discussion part was re-edited to give more information about the relationships between particular E6 subtypes and the cellular signaling pathways

Discussion on pages 13 and 14 could be arranged better so that the delineation between each discussion point is more clear; at the moment, several topics seem to have been thrown together with little distinction between them.

We agree, the discussion part was rearranged and restructured.

Level of interest: An article whose findings are important to those with closely related research interests

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

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.