Author’s response to reviews

Title: Eyebrow hairs from actinic keratosis patients harbor the highest number of cutaneous human papillomaviruses

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

Thank you for the valuable comments on our submission (MS: 1384423676897003) to BMC INFECTIOUS DISEASES.

We have modified the manuscript according to the referees’ comments.

All changes made are underlined in the revised manuscript. Comments to the referees’ are listed on separate pages by a point-by-point discussion attached.

We hope that our manuscript is now suitable for publication in BMC INFECTIOUS DISEASES and would like to thank you for your consideration.

Yours sincerely

Ingo Nindl, PhD

On behalf of all authors

Corresponding author
Responses to Reviewers’ Comments:

Reviewer 1

Major points

Comment 1: Several previous studies have shown that cutaneous HPV types are highly prevalent in eyebrow hairs of healthy individuals (without EV, AK or cutaneous SCC). Thus, it would be crucial to include in this study a control group of age and sex-matched healthy individuals, test their eyebrow hairs for the presence of cutaneous HPV types and get an idea about »background« prevalence and distribution of cutaneous HPV types in eyebrow hairs of healthy individuals. Only direct comparison of the presence and distribution of HPV genotypes in eyebrow hairs from healthy individuals with the presence of HPV genotypes in eyebrow hairs from patients with AK would then enable grounded conclusions.

Answer: We agree that the number of cutaneous HPV types in eyebrow hairs of healthy individuals is very high. This is included in our discussion section and clearer expressed in our revised version.

Discussion section: “...De Koning and colleagues examined eyebrow hairs from 845 healthy individuals (SCC-free) from six different countries and cutaneous HPV infections were detected in 84-91% [22]. Another study analyzed eyebrow hairs of 845 healthy humans without SCC from three countries and cutaneous HPV types were found in 89% [7]. Thus, the percentage of cutaneous HPV types in eyebrow hairs of healthy individuals was high and comparable with AK patients...

The prevalence of cutaneous HPV types in hairs of healthy individuals was (i) in the same range and (ii) similar in two large epidemiological studies. We do not think that we can generate more value and novel information by including 75 healthy individuals. Moreover, the objective of our study was to compare HPV prevalence of different specimens from AK patients comparable with a study analyzing different samples of SCC patients [Plasmeijer et al., Int J Cancer 126: 2614-2621 (2010)]. However, based on this comment, we have additionally revised our conclusions. See also our answers to comment 2 below, and comment 1 of reviewer 3.

Comment 2: I don’t agree with the conclusion that eyebrow hairs are an appropriate indicator of cutaneous HPV in AK patients and are useful non-invasive marker. The prevalence of HPV types in eyebrow hairs was almost two times higher than in AK lesions and the presence of more than one HPV type was found in more than three quarters of eyebrow hairs. Thus despite the fact that at least one HPV type in common was found in 91% of patients with HPV-positive AK lesions and eyebrow hairs it is not possible to predict which of the HPV types from eyebrow hairs is also present in AK lesion.

Answer: We thank the reviewer for this comment and have revised our conclusions in our manuscript as indicated:
(i) Abstract, last sentence: "... Thus, eyebrow hairs revealed the highest number of cutaneous HPV infections, are easy to collect and are an appropriate screening tool in order to identify a possible association of HPV and AK."

(ii) Discussion, last sentence: "... Thus, eyebrow hairs seem to be an appropriate marker to examine the role of cutaneous HPV and AK."

See also our answer to comment 1 of reviewer 3.

Minor points

Comment 3: A table listing all patients and HPV types detected in each of the sampling sites would be very helpful and perhaps more informative than figures 3 and 4.

Answer: Based on this comment, we have included a table with all patients and HPV results of each specimen (eyebrow hairs, AK, and normal skin) in our revised manuscript (see additional file 1: Table S1).

Comment 4: It would be interesting to know if a particular combination of HPV types was linked to AK lesions.

Answer: No specific HPV type or particular combinations of HPV were associated with AK lesions. This is mentioned in our revised manuscript:

Result sections; "...Thus, no specific HPV type seems to be associated with AK or the control group (hairs and/or normal skin)."

Discussion section, last paragraph conclusions; "... In our study, not a specific type or particular combinations of HPV types were detected in AK lesions compared to normal skin and eyebrow hairs."

Comment 5: I suggest to use »the presence of cutaneous HPV types« instead of »infections of cutaneous HPV typ"es in eyebrow hairs and the presence of a single HPV type instead of single infections at least where suitable (e.g. abstract background line 29; abstract results; results line 159).

Answer: Based on this comment, we have changed “presence of HPV types...” instead of infections of HPV types and “the presence of a single HPV type...” instead of single infections where suitable in our revised manuscript.

Comment 6: Try to avoid the expression »overlapping infections« and instead use at least one HPV type in common or concordant HPV types,

Answer: We agree and based on this comment, we have revised >>overlapping infections>> to >>concordant infections>> in our present manuscript.

Comment 7: Abstract, results: What is meant as »the highest number of HPV infections« and the highest number of multiple infections of HPV positive specimens? Either define the highest or rewrite the results.
Answer: Based on this comment, we have revised both sentences in the abstract of our present manuscript to:

>>HPV prevalence>> and >>multiple types of HPV positive specimens.>>

Comment 8: The first sentence of the Results (page 6) should be rewritten. Better would be: A significant higher number of cutaneous HPV infections (betaPV and gammaPV) were detected in eyebrow hairs (63/75; 84%) compared to AK lesions (35/75; 47%) and normal skin (28/75; 37%) (p<0.001), respectively (Fig. 1).

Answer: We thank the reviewer and have changed the first sentence of the Results as suggested.

Comment 9: Lines 168 and 201 consider adding different to HPV types.

Answer: We agree and have added >>different HPV types>> instead of >>HPV types>> in both lines (lines 168 and 201 of our first version) as indicated in our revised manuscript.

Comment 10: Figure 2, correct the title of y axis: No. of HPV positive samples instead of Total number of infections

Answer: Based on this comment, we have corrected our title of the y-axis of figure 2 first version; currently figure 1 of our revised manuscript, respectively to: "Number of HPV positives."

REVIEWER 2

Minor Essential Revisions

Comment 1: Title. I suggest to modify it as follows “Eyebrow hair from actinic keratosis patients harbor the highest number of cutaneous human papillomaviruses”

Answer: We agree and changed the title as suggested to “Eyebrow hairs from actinic keratosis patients harbor the highest number of cutaneous human papillomaviruses”.

Comment 2: The authors made some statements in the introduction claiming that beta 1 and beta 2 species display different pathogenic activity and reads “The molecular mechanisms of beta1PV (e.g., HPV5, HPV8, and HPV20) are different from beta2PV (e.g. HPV23 and HPV38). At present, HPV38 is the only cutaneous type, which is able to immortalize human primary keratinocytes indicating that the oncogenic potential of the beta2PV seems to be higher compared to the beta1PV types.” Although some differences are emerging, I think we do not have enough evidence yet to fully support these statements. Based on that, I suggest to change ‘are different’ with ‘may be different’

Answer: We agree and changed this sentence in our present manuscript to “The molecular mechanisms of beta1PV (e.g., HPV5, HPV8, and HPV20) are very likely different from beta2PV (e.g. HPV23 and HPV38) [8, 10, 15].”
HPV38 is so far the only HPV type, which is able to immortalize human primary keratinocytes. Moreover, only beta2PV types (HPV23 and HPV38) are able to physically bind HIPK2 and inhibit p53 phosphorylation at Ser46, and thus are anti-apoptotic. We think that these observations show important differences of beta1PV versus beta2PV types.

**Comment 3:** Same for the second sentence…We do not actually know whether beta 2 species are more pathogenic than beta1. I agree that HPV38 is the only beta type for which an in vitro transforming activity has been demonstrated, but, if we look at EV or more precisely the patients with primary immunodeficiencies (PIDs), beta 1 species have been primarily demonstrated in active infections. Based on this background, I would recommend to modify these sentences because this part of the introduction is also a bit controversial when compared to the second part mentioning EV skin cancer with beta1 species and the same distribution of beta 1and beta 2 found in other reports. While mentioning EV skin cancer, please cite more recent publications demonstrating transcriptionally active beta 1 infection in skin cancers (Dell’Oste et al JID 2009, and Borgogna et al Virology 2012).

**Answer:** See also our answer above to comment 2. Moreover, we have included in our background section the transcriptional activity of cutaneous HPV in skin tumors of EV patients.

“…In the SCC lesions of EV patients, cutaneous HPV types (predominantly beta1PV types especially HPV5 and HPV8) have been detected and are very likely etiologically linked with skin cancer in this specific genetic background [19], which is supported by HPV expression in skin tumors of EV patients [Borgogna et al Virology 2012]…”

**Comment 4:** The study population was already used for other purposes in a previous report (Nindl et al , B J Dermatol 2009). In this manuscript, as well as the previous one, it is not indicated whether the patients were immunocompetent or immunosuppressed. Since we are dealing with viruses which latently infect the host and can be reactivated under condition of immunosuppression, details about that must be included in the paper.

**Answer:** We have examined immunocompetent AK patients and have indicated this in our revised version as indicated both in the abstract and methods section.

**Comment 5:** Figure 1 can be omitted because: i) prevalence of HPV infection is reported in the first paragraph of the results section, therefore, the graph is redundant and not necessary, and ii) concordance is reported in more details in Figure 4 and it is not clear (at least for this referee) about how it was calculated and represented in figure 1.

**Answer:** We thank the reviewer for this valuable comment and have omitted Figure 1.

**Comment 6:** The discussion is a bit too long, most of it is dedicated to the comparison between the results obtained in this work and previous reports, and sometimes also a bit confusing. Because of interest, these comparisons should be expressed in a clearer way. I understand it won’t be easy, but efforts should be put on that, for instance including a table.
and describing better the results obtained first and then the comparison. For some cohorts from previous reports it is again not indicated whether they were from immunocompetent or immunosuppressed populations. As mentioned above, this is a crucial point which must be displayed and may be distinction between the two groups also help in making the comparison clearer and interesting for the reader. Another aspect that need to be discussed is the difference between AK and SCC samples, also since the precancerous AK lesions are known to harbor higher multiplicity of infection and higher viral loads in comparison to overt SCC.

**Answer:** We agree and based on this comment, we have revised and shortened our discussion section by approximately 20% (all changes are underlined).

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**REVIEWER 3**

**Minor Points**

**Comment 1:** The authors state correctly that to date there is no evidence that a specific carcinogenic HPV type is associated with AK and thus I do disagree with the last sentence of the final conclusion: "Thus, eyebrow hairs are an appropriate indicator of cutaneous HPV in AK patients and are a useful non-invasive marker especially in large epidemiological studies." Based on the presented results it should be more pointed out that eyebrow hairs are an appropriate screening tool in order to identify a possible association of cutaneous HPV in AK patients. That they are useful as non-invasive markers for large epidemiological studies is not substantiated by data in the present study because also here no significant association between certain HPV types and AK was observed.

**Answer:** We agree and have revised our conclusions based on this comment in the present manuscript.

(i) Abstract, last sentence: "... Thus, eyebrow hairs revealed the highest number of cutaneous HPV infections, are easy to collect and are an appropriate screening tool in order to identify a possible association of HPV and AK..."

(ii) Discussion, last sentence: "...Thus, eyebrow hairs seem to be an appropriate marker to examine the role of cutaneous HPV and AK..."

**Comment 2:** How do the authors know that the frozen material of AK used for DNA isolation and HPV typing really did contain AK? The presence of AK in the other half of the tissue does certainly not mean that AK is also present in the frozen tissue. Did the authors perform frozen sections ("sandwich")? If yes they should mention this in Materials and Methods, if not the authors should explain why.

**Answer:** We thank the reviewer for this comment, and have included the following sentence in our Methods section, study population first paragraph:

"...We have performed frozen sections of all divided AK lesions to confirm the presence of dysplastic cells before we have isolated the DNA."