**Author's response to reviews**

**Title:** Cyclin A1 shows age-related expression in benign tonsils, HPV16-dependent overexpression in HNSCC and predicts lower recurrence rate in HNSCC independently of HPV16

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**Author's response to reviews:** see over
Dear Sir or Madam,

Please find enclosed the revised manuscript entitled “Cyclin A1 shows age-related expression in benign tonsils, HPV16-dependent overexpression in HNSCC and predicts lower recurrence rate in HNSCC independently of HPV16” which we would like to re-submit for possible publication in the Journal “BMC Cancer”.

Please find the point-to-point response to the comments of reviewers listed below.

Herewith, I state that the manuscript has not been submitted for publication elsewhere. There is no conflict of interest to declare. All authors have read the final manuscript and approved its submission.

Thanking you in advance,

Sincerely yours,

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Point-to-point response to comments of reviewers

Reviewer 1

Background: this section is hard to read and should be re-written more clearly. L. 12: "and" is doubled.

> The background section was re-written.

Results: tables may be re-ordered since methods section is after results.

> Tables were re-ordered in accordance to the order in which they are mentioned.

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

> The whole manuscript has been carefully proof-read for quality of written English and necessary corrections have been made.

Reviewer 2

Major compulsory revisions

HPV related SCC of the oropharynx are biologically different to their ‘non-HPV related’ SCC counterparts and are also different from SCC at other sites in the head and neck. The current study includes 63 cases from the oropharynx and 18 cases from other sites. It would be useful to indicate which subsites harboured HPV infection - probably the oropharyngeal cases based on established literature.

> A new table (Table 2) shows the frequency of HPV16 and p16 overexpression in HNSCC with respect to primary site. However, it has to be mentioned that the group “Oropharynx” only includes the tumors arising from the tonsil and the base of the tongue (N=61). The two tumors of the soft palate were localized anterior the frontal arch of the palate and therefore not assigned to the oropharyngeal site. The results shown in Table 2 are also presented in the text (“Results - Prevalence of HPV16 DNA, overexpression of p16 or p53, and p53 mutation in HNSCC and controls”). Since we were able to demonstrate a linkage between oropharyngeal site and a higher frequency of HPV16 and p16 overexpression in previous investigations (Ref. 25: Weiss D et al, Head Neck 2011) we did not focus on correlations between HPV16 and clinicopathological characteristics this time.

Increased Cyclin A1 expression correlates with HPV16 infection (DNA and p16 expression) and improved local control. Perhaps by restricting the study to just the oropharyngeal SCC similar trends would be observed, but this would underscore the clinical utility of the test in this specific disease setting. The inclusion of a small number of
cases (n=18) from other subsites (oral cavity, hypopharynx and larynx) potentially detracts from what could be a stronger message: ‘increased Cyclin A1 expression can be used as a marker of loco-regional control in oropharyngeal SCC irrespective of HPV status’?

> The following table shows the statistic results of oropharyngeal SCC (N=61).

<table>
<thead>
<tr>
<th>Clinicopathological parameter</th>
<th>N</th>
<th>Performed Statistic</th>
<th>Subgroups</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [y]</td>
<td>61</td>
<td>Spearman Rank Order</td>
<td>σ = 0.0281</td>
<td>0.829</td>
</tr>
<tr>
<td>Sex</td>
<td>61</td>
<td>Mann-Whitney Rank Sum</td>
<td>male vs. female</td>
<td>0.729</td>
</tr>
<tr>
<td>Recurrence rate</td>
<td>26</td>
<td>Mann-Whitney Rank Sum</td>
<td>Recurrence vs. no recurrence</td>
<td>0.041</td>
</tr>
<tr>
<td>Progression free survival</td>
<td>61</td>
<td>Kaplan-Meier</td>
<td>CCNA1 &lt;4 vs. ≥4</td>
<td>0.814</td>
</tr>
<tr>
<td>Overall survival</td>
<td>61</td>
<td>Kaplan-Meier</td>
<td>CCNA1 &lt;4 vs. ≥4</td>
<td>0.883</td>
</tr>
<tr>
<td>HPV16 DNA status</td>
<td>61</td>
<td>Mann-Whitney Rank Sum</td>
<td>HPV16+ vs. HPV16-</td>
<td>0.042</td>
</tr>
<tr>
<td>E6 quantitative</td>
<td>36</td>
<td>Spearman Rank Order</td>
<td>σ = 0.216</td>
<td>0.205</td>
</tr>
<tr>
<td>E7 quantitative</td>
<td>36</td>
<td>Spearman Rank Order</td>
<td>σ = 0.241</td>
<td>0.156</td>
</tr>
<tr>
<td>P16 Expression</td>
<td>59</td>
<td>Spearman Rank Order</td>
<td>σ = 0.341</td>
<td>0.008</td>
</tr>
<tr>
<td>P53 Expression</td>
<td>60</td>
<td>Spearman Rank Order</td>
<td>σ = 0.120</td>
<td>0.360</td>
</tr>
<tr>
<td>Cyclin A1 Methylation</td>
<td>33</td>
<td>Mann-Whitney Rank Sum</td>
<td>methylated vs. non-methylated</td>
<td>0.775</td>
</tr>
</tbody>
</table>

The results shown in the table above demonstrate that the statistical relations between Cyclin A1 expression and clinicopathological parameters, especially HPV16 status, p16 expression and recurrence rate, are getting less if only the tumors from oropharyngeal site are included. For this reason we would rather keep these results for non-noteworthy.

For the immunohistochemical analysis the majority of the cases were analysed providing full data for the cohort. Where laboratory analysis was restricted by availability of tissue (p53 mutations n=20 and Cyclin A1 methylation n=44) the interpretation of the results are limited by the small sample size.

> DNA fragmentation in formalin-fixed paraffin embedded tissue limited the number of usable results regarding p53 mutation analysis as well as Cyclin A1 methylation analysis. Since we also collected fresh-frozen tissue sections of some tumors we were able to do further p53 mutation analysis in 21 tumor samples (N=41 in total).
Again, there is no relevant relationship between p53 mutational status and methylation or expression of Cyclin A1 (p=0.491 and p=0.920). We additionally screened 10 benign tonsils for p53 mutation but could not find any mutation (N=30 in total). The corresponding data have been revised accordingly. With regard to Cyclin A1 methylation we already analyzed 58 tumor samples in total. Yet, due to DNA fragmentation in formalin-fixed paraffin embedded tissue, we only get useful results in 44 cases. The usable results were mainly deduced from fresh-frozen tissue sections. Therefore, we think that inclusion of more samples (which would only be FFPE samples) would not lead to much better results. The limitation of results by the small sample size and its reason are mentioned in the “Methods” (“Patients and Controls”) and “Discussion” section. The fact that we also used fresh-frozen tissue sections is mentioned in “Methods – Patients and Controls”.

The immunohistochemical tests were assessed by ‘at least 3 pathologist’. Immunohistochemistry scoring is typified by inter-observer variation and it is not clear how discordant scores were resolved to achieve a ‘consensus’ score for each case.

The section “Methods - Immunohistochemical analysis of Cyclin A1, CDKN2A/p16 and p53” was re-written to demonstrate the precise process for evaluation of immunohistochemical staining used in this work.

Whilst the ‘cut off’ for positive staining are justified in the text, it must be acknowledged that these values are essentially empirical. p16 overexpression is widely used in clinical practice and much of the published work is based on a nominal ‘cut off’ of strong nuclear and cytoplasmic staining in >70% of the tumour (Singhi and Westra, 2010), 10% in this study seems rather low.

After intensive study of the literature we decided to readjust our cut-off for considering p16 overexpression at ≥20% (≥ category 4). This is mainly due to the published work of Klaes et al (Int. J. Cancer, 2001), Klussmann et al (Am J Pathol, 2003) and Begum et al (Clin Cancer Res, 2003), where the cut off is set at >25% positive cells. An even higher limit value from our point of view makes no sense, because otherwise we would get very discrepant results regarding HPV16-positivity and p16 overexpression (e.g. cut off >70%: HPV16+ but no p16 overexpression: N=31 (84%)!). It should also be noted that we, unlike other investigators, compared p16 expression of tumors with those of tonsils and in tonsils the expression of p16 did not exceed the level of 10% in any case. We believe that it makes more sense to align the limit value according to the expression level in healthy tissue, as to simply set it arbitrarily. O’Regan and colleagues also adjusted their cut-off for p16 overexpression by correlating the expression in HNSCC with that of normal (benign) tissue. They found a p16 expression rate in normal tissue of 0-5% (O’Regan et al, Human Pathology, 2008). A threshold of >10% for
accepting *p16* and *p53* overexpression, respectively, was used by Nemes et al (Nemes et al, Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2006). Other research groups just categorized the staining intensity without setting a threshold for overexpression (Kumar et al, J Clin Oncol, 2008; Sano et al, Am J Pathol, 1998; Reed et al, Cancer Res, 1996). As we did, most of the above-cited studies used the CINtec® kit (mtm laboratories, Heidelberg, Germany) for evaluation of immunohistochemistry.

Regardless of a limit value, the main messages of our work, such as the correlation between *Cyclin A1* expression and *p16* expression in tonsils as well as in tumors, remain unchanged, since we used Spearman Correlation for these analyses.

All the changes regarding statistical data and their interpretation arising from the readjustment of *p16* cut-off were made accordingly in tables and manuscript. We also changed the passage “Staining for all three gene products was localized to the nucleus of tumor cells” (“Methods - Immunohistochemical analysis of *Cyclin A1*, *CDKN2A/p16* and *p53*”) into “Strong nuclear staining was considered positive for *Cyclin A1* and *p53* expression. *p16* expression was scored as positive if strong and diffuse nuclear and cytoplasmic staining was present (Figure 1 and 2)”.

However, we maintained the cut off for *p53* at ≥10 %, because this value is widely used by others (Lingen et al, Head&Neck, 2000; Khadem et al, Cancer Letters, 2002; de Vicente et al, Head&Neck, 2004; Farhadieh RD et al, ANZ J Surg, 2009).

There are no photomicrographs included to allow the reader to visualise the staining localisation or intensity. This is particularly important for *Cyclin A1*, which is the novel aspect of this study, as other researchers will not be able to repeat the work if there are no photomicrographs to ‘benchmark’ further studies.

> The manuscript now includes photomicrographs demonstrating staining results of *Cyclin A1*, *p16* and *p53* in benign tonsils and HNSCC (Figure 1 and 2).

The correlative nature of the study makes the discussion rather speculative and the mechanisms are not clear. The clinical utility of *Cyclin A1* requires validation in larger studies and the functional consequences of *Cyclin A1* overexpression are unclear.

> We are fully aware that the results presented here do not clarify the cause and effect relationship between HPV16 infection of oral mucosa cells and *Cyclin A1* methylation and *Cyclin A1* overexpression in any way. This is already mentioned in the “Discussion” section. However, the fact that different study groups found a relationship between the two factors in two different HPV-induced tumor entities makes a causal link very likely.
We have set ourselves the target to be able to answer two important questions soon. First, we want to clarify the causal link between HPV infection and *Cyclin A1* methylation and expression by in vitro studies. Second, we aim to examine the clinical relevance of *Cyclin A1* in a larger group of patients. But to even have a starting point for further projects, we were and are primarily dependent on the results of formalin-fixed sections.