Author's response to reviews

Title: Nuclear hBD-1 accumulation in malignant salivary gland tumours

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Author's response to reviews: see over
Dear Mr Pueblo,

herewith I am returning the revised manuscript of the article:” MS:
2091676334166652 - Nuclear hBD-1 accumulation in malignant salivary gland tumours.” Alterations are highlighted in the revised text. I did reply to all reviewers´ comments separately; my reply is in red text colour:

Mr. Yoshihirio Abiko:

Major:
As the authors state in line 4 of the discussion section, the hBD-1 seems to be shifted to the nucleus. This finding is a key in this article. This data, however, may be lacking objectivity. The authors should demonstrate show much more reliable data using other analyses so that the authors do not have to use the phrase seems to be. This study was conceived as pilot study in which we investigated if there is hBD 1-3 staining in salivary gland tumours at all (which remarkably is). The authors know that by other techniques, as real time PCR a (semi) quantification of hBD 1-3 expression is possible. However we did not obtain tissue for real time PCR, but only paraffin embedded tissue for Immunohistochemistry. For future studies we know that better
now and we are therefore collecting fresh frozen tumour tissue from adenoid cystic carcinomas and pleomorphic adenomas. Pleomorphic adenomas show nuclear as well as cytoplasmatic hBD-1 staining which underlines their biologic behaviour of recurrence and malignant transformation. So the key findings of this study are: 1. there is hBD 1-3 staining in tumours of the salivary glands (which has not been reported yet), 2. the nuclear shift of hBD-1 is connected to malignancy and 3. pleomorphic adenomas show nuclear as well as cytoplasmatic hBD-1 staining. These findings underline real time PCR findings from a study we performed with tissue of squamous cell carcinomas of the oral cavity which has been accepted for publication in the International Journal of Oral and Maxillofacial Surgery. In this study we demonstrated that a decreased gene expression of hBD-1 (as seen in renal clear cell carcinoma and malignant prostate cancer) might be involved in carcinogenesis of these tumours. So altogether this study in combination with our recent studies underlines the idea of hBD-1 as tumour suppressor gene in tumours of the head and neck region.

In figure 1, some of the nuclei show positive staining of hBD-1 even in the healthy salivary ducts. The nuclear staining may not only be in the salivary tumour but also in the healthy salivary gland. How many cells do show nuclear localization of hBD-1 in the malignant salivary tumours? Do all cells show nuclear localization of hBD-1 in the tumour? The authors should show what percentage of nuclear localization is in the tumours.

If you compare the staining of hBD-1 in healthy salivary glands and tumour tissue the staining is different. What could be mistaken as nuclear staining is cytoplasmatic hBD-1 staining interfering with the HE staining, thus, depending on the thickness of the sections, creating the impression of nuclear staining. In the tumours almost all the cells showed nuclear hBD-1 staining, which makes the observation so striking. Figure 4 depicts a representative area of an adenoid cystic carcinoma.

Others:
1. Authors observed immunohistochemical staining for p53 as a tumour suppressor gene. In this case, the wild-type of p53 must be detected. However, immunohistochemical staining for p53 usually shows mutant-type of p53, because the half-life of wild-type p53 protein is too short to detect. The positive staining for p53 probably shows the mutant-type of p53 but not the wild-type. The authors most likely confused mutant type with wild type.
You are right: the p53 section is capable of being misunderstood. I added a sentence as proposed to make it more clearly: P53 immunohistochemistry can only be a screening for potential p53 mutations. The idea of the p53 or bcl-2 screening was to show if there is a clear and obvious connection between both markers and hBD-1. Anyhow I think the small percentage of potentially positive wild-type p53 staining is negligible because there was no striking finding at all.

2. line 12, 13 in the second page of Discussion.
It is too much speculation to say that hBD-1 might play an important role in the malignant progression of salivary gland tumours.
Please see above. In any case I think that there is evidence for a potential role of hBD-1 as tumour suppressor gene.

Is there no need to have an abstract or summary at the beginning?
You are definitively right; the abstract must have been deleted somehow; I added it to the revised manuscript.

Minor:
Table 1. The carzinosarcoma must be carcinosarcoma.
The onkocytoma must be oncocytoma.
I made the corrections as proposed.

Mr. Thomas Pufe:

General comments:
In my pdf version of the manuscript an abstract is missing.
Sorry, the abstract must have been deleted somehow. The revised manuscript has an abstract now.

The work of Varoga et al. was cited as proposed. I additionally added a new work of our group concerning decreased hBD-1 expression in squamous cell carcinomas of the oral cavity (OSCC).

The study would benefit from a fusion of the single figures. It would be more easy
to compare the shift from the cytoplasm to the nucleus. As proposed we provided a fusion of the single figures in the revised manuscript.

The authors depict hBD-1, -2 and -3 in healthy salivary glands but only hBD-1 in salivary gland tumours. The authors should add pictures from hBD-2 and -3 from salivary gland tumours. As suggested, we also provided pictures of hBD-2 and 3 in salivary gland tumours.

The authors should discuss the expression of human beta defensins in other diseases like osteoarthritis (Varoga et al. J Pathol. 2006 Jun;209(2):166-73) or Rheumatoid Arthritis (Paulsen et al. J Pathol. 2002 Nov;198(3):369-77). We included the works of Varoga and Paulsen in our discussion. Our new work concerning hBD-1 in OSCC has also been included in the discussion.

Minor comments:
There are some typographical errors.
The manuscript has carefully been checked for mistakes.

Mr. Geovanni Cassali:

1) The sample size is too small.
First aim of this study was to show that hBDs are traceable in salivary gland tumours at all. Therefor we obtained tissue samples of 21 salivary glands, including 7 benign and 7 malignant tumours. Until now there is no study which showed that hBDs are traceable in these tumours at all. Observations concerning the distribution of hBD-1 in malignant salivary gland tumours, our own findings concerning hBD-1 in squamous cell carcinomas of the oral cavity combined with findings of other authors in different epithelial tumours of the urinary tract (Donald CD, Sun CQ, Lim SD, Macoska J, Cohen C, Amin MB, Young AN, Ganz TA, Marshall FF, Petros JA. Cancer-specific loss of beta-defensin 1 in renal and prostatic carcinomas. Lab Invest. 2003 Apr;83(4):501-5.) gave us the idea that hBD-1 is a tumour suppressor gene. In
present studies we investigate bigger subsets of pleomorphic adenomas and adenoid cystic carcinomas.

2) The immunohistochemistry analysis is superficial, a quantitative or semi-quantitative method is preferred. This point might be discussed: I do not think that a quantification would help much for a better understanding of the key findings of this study because the main observation, that there is a nuclear shift of hBD-1 in salivary gland tumours, could be made clear by a descriptive approach. If you insist on a quantification we could perform the quantification for instance by the RGB method (Matkowskyj KA, Schonfeld D, Benya RV. Quantitative immunohistochemistry by measuring cumulative signal strength using commercially available software photoshop and matlab. J Histochem Cytochem. 2000 Feb;48(2):303-12.).


4) It is necessary a statistic analysis. A better description of tumors is necessary. Ex.: There are three major variant histologic growth patterns of ACC: cribriform, tubular and solid. The solid pattern is associated with a more aggressive disease course. The expression of markers could be different. We included 3 ACCs with a solid growth pattern.

Mr. Katsuhiro Uzawa

Major Compulsory Revisions:
1. It may be difficult to obtain clinical samples of malignant salivary gland tumours. It is desirable to increase the number of the cases of the immunohistochemistry if it can be obtained in a clinical specimen. Please see my reply to Mr. Geovanni Casalis comment 1).
2. If you extracted the RNA of the clinical specimens, you had better confirm the expression of the hBD-1 gene in malignant salivary gland tumors in RT-PCR or real-time RT-PCR. Unfortunately we did not obtain tissue for real time PCR, but only paraffin embedded tissue for Immunohistochemistry because we were not aware of what we will find. For our present studies we are collecting fresh frozen tumour tissue from adenoid cystic carcinomas and pleomorphic adenomas. In another study we investigated the expression of hBD-1 in different lesions of the oral mucosa, including OSCC by real time PCR. This study is mentioned in the discussion now.

**Minor Essential Revisions:**

1. You should refer to Instructions for BMC Cancer authors and must reconstitute an article.

Abstract section is deleted.

*Sorry for that, the abstract is included now.*

Please change it in a Background section not an Introduction section.

*The Introduction is renamed in Background.*

Please change it in a Methods section not a Materials and Methods section.

*Materials and Methods was changed into Methods as proposed*

Conclusions section is deleted.

*A Conclusions section is included now.*

Competing interests section is deleted.

*The authors declare that they do not have competing interests.*

Authors’ contributions section is deleted.

*To be honest this one I do not understand. Contributions to what?*

Yours sincerely

Dr. Dr. M. Wenghoefer