Author's response to reviews

Title: Analysis of angiogenic markers in oral squamous cell carcinoma - gene and protein expression

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Author's response to reviews: see over
Dear Editor and Reviewers,

We would like to thank you and the reviewers of our manuscript entitled "Analysis of angiogenic markers in oral squamous cell carcinoma - gene and protein expression" (MS: 1003841322142338) for recognizing the potential contribution of our study to the research field and the constructive points discussed. Most of the helpful comments and suggestions for improving the manuscript have been now incorporated into the revised version. In this letter, we provide a point-by-point response to each addressed comment; the corresponding alterations in the text have been highlighted. Therefore, we hope that the manuscript is now acceptable for publication in Head & Face Medicine.

Sincerely yours,

Sonja Sielker, Ph.D.

Review 1:

Abstract:
*The total number of healthy mucosa samples is not given as well as the total number and names of angiogenic genes under examination (VEGF, ANGPT and EFNB).*

We included the total numbers of healthy mucosa and the genes under investigation as well as their names on page 2.

.... by comparison to 30 samples of healthy oral mucosa....

A distinctive expression profile of VEGFA, EFNB2, PECAM1/CD31, ANGPT1 and ANGPT2 was revealed.
Immunohistochemistry is abbreviated as “IHC” in the text without explanation.

Based on the suggestion of the reviewer all abbreviations were checked.

p.2 (e.g.)

Immunohistochemistry (IHC) was performed to trace the signalling cascade …

In the abstract, oral squamous cell carcinoma is addressed. These two entities should be differentiated and not mixed as seen within the text.

According to the suggestion of the reviewer, Beginning with the title throughout the text head and neck squamous carcinoma (HNSCC) has been changed to oral squamous cell carcinoma (OSSC).

p.3 (e.g.)

Oral squamous cell carcinoma (OSSC) is one of the most commonly diagnosed cancer entities

Introduction:
The study of Kämmerer et al. 2014, should be added and discussed.

The study of Kämmerer et al., Angiogenesis-related prognosis in patients with oral squamous cell carcinoma – role of the VEGF +936C/T polymorphism; J Oral Pathol Med 2014, underlines the significance of angiogenesis affecting local and distant tumour growth as well as patients’ prognosis and disease-free survival. Following the suggestion of the reviewer, we included and discussed the interesting results on p. 4.

Kämmerer et al. were able to show that higher microvessel density was associated not only with higher tumour stage as well as earlier relapse, but also with a higher rate of metastasis and significantly decreased overall and disease-free survival [6]. These findings provide the base for and strongly suggest further elucidation of VEGF expression and regulation in malignant tissues.

Material and Methods:
Did the study have a prospective or a retrospective approach?

We now made clear on p.6 that the study at hand is a retrospective one.

For this retrospective analysis, 83 tissue samples…

Immunohistochemistry was assessed on deep frozen samples?
From which region was the sample taken?
Was there a histopathological evaluation before stating that it was OSCC in the respective samples?

We included the lacking information on p. 6
Healthy tissue controls (n = 30) were taken from oral vestibular mucosa samples during orthognathic or traumatologic surgery after informed consent.

and p. 7
A selection of 14 paraffin embedded tumours samples providing a representative amount of tissue for immunohistochemical staining was analysed in this investigation. IHC was performed after histopathological confirmation of OSCC.
**Why did the authors chose CD31?**

Basically, we chose CD31 for the same reasons as Kaemmerer et al.; CD31 (PECAM1) is described in the literature as a marker of mature endothelial cells whereas CD34 is found additionally on endothelial progenitor cells (Pubmed gene). Our aim was to determine if an increase in angiogenic activity on gene level really leads to an increase in mature endothelial structures on protein level, which seems not to be true for our data.

The reasoning is included in the introduction, p. 5.

…to the mature endothelial cells marked by CD31.

**Please indicate the localisations as well as the TMN (or even UICC) stadia of the tumors in a separate table.**

In order to comply with the reviewer’s suggestion, we inserted a new table containing clinicopathological features (Tab.1) and deleted redundant information from the text on page 7. Table 1 gives an overview of localisation and stage of the OSCC samples.

**Tab.1: Clinicopathological features of the included OSCC samples.**

**Why did the authors examine only 14/83 samples with immunohistochemistry?**

For a more conclusive message and a strong support of our microarray data, immunohistochemical analysis of all 83 samples would have been preferable. Unfortunately, upon histological and RNA analysis only 14 of the 83 tissue samples provided enough tumour material for a convincing immunohistochemical staining of five proteins. Therefore, we decided to present these results as hint/ supportive information without the claim to present a significant meaning on their own.

p.7

After examination only 14 of the 83 tissue samples provided enough tumour tissue to allow for a decent analysis, these were included in the study.

**What did the authors mean with “CD31 is not detected in the analysed samples”?**

In our immunohistochemical analysis, we were not able to demonstrate a strong CD31 staining. That even, if there is a strong translational angiogenic impact; the result is not necessarily a mature vascular structure with mature endothelial cells that are characterised by CD31. With other words the vasculature of the tumour is still in the process of development and endothelial cells are not fully differentiated yet and therefore lack expression of CD31.

**Data of prognosis are not given within the manuscript at all. Please compare with similar publications and adapt.**

The latest tumour samples have been taken in 2012. We prefer to correlate our microarray data to a long-term survival at a future date so that we can make a sound statement concerning recurrent disease or survival over five years. Therefore, currently, we do not dare to make such an analysis yet.
Results:
*When talking about prognosis, were all OSCC-samples resected with safety margins? Which ones?*

According to current standard surgical principles of tumour resection, the malignant tissue was resected with a safety margin of 1 cm in all directions. Therefore this information was not included in the manuscript.

*Did the authors also try to calculate with different nodal involvements (N1, N2) or invasion of lymphatic vessels (L1)?*

A more concise analysis of nodal or lymphatic involvement is clearly preferable. However, our data does not allow such a fine classification so we had to confine ourselves to a simple differentiation of nodal involvement, N+, and no nodal involvement, N-.

*Why are patients’ habits reported as the authors did not correlate them with their findings?*

According to the suggestion of the reviewer, we analyses gene expression and patients’ habits. Subsequently, one way ANOVA was accomplished and differences in expression were analysed on a level of significance of $p < 0.05$. In PostHoc analysis with testing parameter ‘patients’ habits’, there was no significant correlation. So we deleted the information from the text.

Discussion:
*Lacks addressing the clinical relevance.*

Our idea was to determine whether or not OSCC might be accessible for anti-angiogenic therapy via antibody therapy attacking endothelial cells. Such an approach provides the presence of a mature endothelial structure such as epitopes.

A corresponding passage has been included on p. 9

The prognosis of patients suffering from OSCC has been unalteredly poor despite much scientific effort to improve the therapy. Tumour-related neo-angiogenesis is an important prerogative for tumour growth and spread; in different tumour entities, i.e. colon carcinoma, anti-angiogenic therapy has proven its effect. Therefore, in this study, we examined the expression of key regulators in the angiogenic and vasculogenic cascade in 83 OSCC tumour samples as the overexpression of single factors during the angiogenic cascade might provide novel points of attack.

Tables:
*Giving percentages is not recommended in sample sizes <100.*

In order to prevent misinterpretation the description of tumour percentage was changed to number of tumour samples in different stage groups; this was performed for tables 2 to 5.

… samples mean number of tumour samples in…..
Did the authors try to state descriptive p-values after Bonferroni correction as well? These values should also base on t-tests correlating the single stages with the gene expression profile. By the way, p-values are sufficiently stated as p<0.05 or even p<0.001. For one way ANOVA we used a modified Levene testing with no further correction. Additionally we used PostHoc analysis with a Bonferroni-Holm correction for further group analysis. P-value information was reduced to p<0.05 (e.g.) in the text.

Statistical analysis of expression factors and IRS score according to tumour stage and grading was carried out by one way ANOVA using a modified Levene testing and p<0.05, and a PostHoc analysis with Bonferroni-Holm testing.....

PostHoc analysis was performed with testing parameters a) T stage, b) grading, and c) genes. PostHoc analysis with the first two parameters showed no significant correlation. However, a direct comparison of the different genes via PostHoc analysis showed significance with p<0.05, with the exception of VEGF vs. EFN2 and VEGF vs. ANGPT1 which demonstrated no significance. A direct comparison of ANGPT1 and ANGPT2 proved a significant correlation with p<0.0001.

Figures:
The figures for revision are way too small and not of a good quality. The figures were resubmitted now with a better resolution.

Review 2:
Page 3 - OSCC is not explained, EC is not explained.
We included the missing explanations on page 3 and page 4, respectively. Oral squamous cell carcinoma (OSCC) is one of the most commonly diagnosed cancer entities. ...that is expressed on endothelial cells (ECs), exclusively.

Page 4 - EFN2 and after this must be a space, also on page 9. The missing spaces were included.