Author's response to reviews

Title: HMGA1 and HMGA2 expression and comparative analyses of HMGA2, Lin28 and let-7 miRNAs in oral squamous cell carcinoma

Authors:

Katharina A. Sterenczak (Katharina.Sterenczak@tiho-hannover.de)
Andre Eckardt (Andre.Eckardt@klinikum-bremerhaven.de)
Andreas Kampmann (Kampmann.Andreas@mh-hannover.de)
Saskia Willenbrock (Saskia.Willenbrock@tiho-hannover.de)
Nina Eberle (Nina.Eberle@t-online.de)
Florian Länger (Laenger.Florian@mh-hannover.de)
Sven Kleinschmidt (Sven.Kleinschmidt@laves.niedersachsen.de)
Marion Hewicker-Trautwein (Marion.Hewicker.Trautwein@tiho-hannover.de)
Hans Kreipe (Kreipe.Hans@mh-hannover.de)
Ingo Nolte (ingo.nolte@tiho-hannover.de)
Hugo Murua Escobar (hugo.murua.escobar@med.uni-rostock.de)
Nils C. Gellrich (Gellrich.Nils-Claudius@mh-hannover.de)

Version: 5
Date: 20 June 2014

Author's response to reviews: see over
Dear Editor, dear Editorial Team,

please find enclosed the newly revised version of our BMC Cancer manuscript: 
MS# 1457729371953663 by Sterenczak et al..

We are very glad that the reviewers were in general satisfied with the first revision of 
our manuscript. We carefully addressed the additional comments by the reviewers 
and thank them again for the constructive review of the revised manuscript.

Thank you very much in advance for your consideration.

With my best wishes from Hannover,

H Murua Escobar
List of Changes
BMC Cancer Manuscript: 1457729371953663; Sterenczak et al. revision 2

Referee 1 Xiaomei Lu:

Minor Essential Revision:

Comment 1: “Figure 1 was labeled with A, B, C and D, but in the legend to figure 1, there were only A and B, please check it. Moreover, the specific statistical method used in the study still not mentioned. The same holds true for Figure 2.”

Answer to comment 1:
We apologise for these errors, the legends were corrected regarding the missing description of “C” & “D” as well as the statistical method as follows.

The former legends (Section: Legends, Pages: 23) stating:

“Figure 1:
Expression analyses of HMGA2 in human OSCC
The study included 10 non neoplastic control samples (green columns) and 10 tumour samples (red columns) which derived from patients 1 -11.
A: relative HMGA2/GUSB real time PCR. B: relative HMGA2/HPRT real time PCR.
* indicates a statistical significant expression deregulation of the HMGA2 gene when compared to non neoplastic control group;
p-value is displayed next to *”

and

“Figure 2:
Expression analyses of HMGA2 in canine OSCC.
The study included 2 non neoplastic control samples (green columns) and 7 tumour samples (red columns) which derived from patients 1 - 9.
A: relative HMGA2/GUSB real time PCR. B: relative HMGA2/HPRT real time PCR.
* indicates a statistical significant expression deregulation of the HMGA2 gene when compared to non neoplastic control group; p-value is displayed next to *”

Were replaced by the following (Section: Legends, Page: 23):

“Figure 1:

Expression analyses of HMGA1 and HMGA2 in human OSCC

The study included 10 non neoplastic control samples (green columns) and 10 tumour samples (red columns).

A: relative HMGA1/GUSB real time PCR. B: relative HMGA1/HPRT real time PCR. C: relative HMGA2/GUSB real time PCR. D: relative HMGA2/HPRT real time PCR.

Statistical analysis was performed applying the Hypothesis Test using REST 2008 (version 2.0.7.).

* indicates a statistical significant expression deregulation of the HMGA genes when compared to non neoplastic control group; p-value is displayed next to *”

and

“Figure 2:

Expression analyses of HMGA1 and HMGA2 in canine OSCC.

The study included 2 non neoplastic control samples (green columns) and 7 tumour samples (red columns).

A: relative HMGA1/GUSB real time PCR. B: relative HMGA1/HPRT real time PCR. C: relative HMGA2/GUSB real time PCR. D: relative HMGA2/HPRT real time PCR.

Statistical analysis was performed applying the Hypothesis Test using REST 2008 (version 2.0.7.).

* indicates a statistical significant expression deregulation of the HMGA genes when compared to non neoplastic control group; p-value is displayed next to *”
Comment 2: “Figure 3 and 4 were supposed to detect HMGA2 expression in human and canine OSCC by IHC respectively, the authors should show the statistic result of IHC for human or canine OSCC tissue rather than just put 6 pictures there.”

Answer to comment 2:
We discussed this point intensively while preparing the manuscript. As mentioned by the reviewers the major limitation of the study is the relative small amount of analysed samples for the respective species. Due to this, we feel that statistical analyses based on the limited sample size would not provide the adequate power. Thus, we opted to show representative images of the analysed cases for both species. Composing similar figures of both species in the same way offered us the possibility to visualise that the distribution of the HMGA2 protein is comparable in both species indicating a characteristic pattern.

Thus, we would like to keep the presentation of our figures 3 & 4.

Comment 3: “In Figure 3 and 4, was the magnification times labeling for both B and C the same?”

Answer to comment 3:
In fact the parts “B” and “C” of the figures 3 &4 are presented in the same magnification. The different picture parts represent different areas of the tumour. “B” represents the tumour centre while “C” represents a part of invasive front respectively.

As apparently our presentation did not state this clearly we modified the legend of the respective figures.

The former legends (Section: Legends, Pages: 23/24) stating:

“Figure 3:
HMGA2 immunohistochemistry in human OSCC
A: overview of the immunolabelling including the centre and invasive front of the tumour. In the tumour centre (B) lower numbers of tumour cells with nuclear immunolabelling are present than in the invasive front (C) of the tumour, which has numerous tumour cells exhibiting intense nuclear immunolabelling.”
“Figure 4:
HMGA2 immunohistochemistry in canine OSCC
A: Immunolabelling of a canine tumour grade II showing the centre and invasive front. Staining in the tumour centre (B) revealed approx. 25% tumour cells with nuclear immunolabelling while cells at the invasive front showed approx. 50% staining (C) labelling numerous tumour cells exhibiting intense nuclear immunolabelling.”

Were replaced by the following (Section: Legends, Page: 24):

“Figure 3:
HMGA2 immunohistochemistry in human OSCC
Immunolabelling of a human tumour: overview (A), tumour centre (B) and invasive front (C). In the tumour centre (B) lower numbers of tumour cells with nuclear immunolabelling are present when compared to the respective invasive front (C). The invasive front shows numerous tumour cells exhibiting intense nuclear immunolabelling of HMGA2. Magnification: (A) 50x, (B) and (C) 200x.”

and

“Figure 4:
HMGA2 immunohistochemistry in canine OSCC
Immunolabelling of a canine tumour grade II: overview (A), tumour centre (B) and invasive front (C). HMGA2 staining in the tumour centre (B) revealed approx. 25% tumour cells with nuclear immunolabelling while cells at the invasive front showed approx. 50% staining (C). Magnification: (A) 100x, (B) and (C) 200x.”
Referee 3, Max Heiland:

Comment 1: “How the authors guarantee, that tumor cells obtained for culturing are really squamous carcinoma cells and not e.g. fibroblasts. Did they characterized these cells? Please extend the methods section and discuss.”

Answer to comment 1:

The samples of both origins were taken during routine surgery. The human as well as the respective canine tumour samples were divided in equal representative parts for pathologic analyses and cultivation.

To clarify the sample processing in our manuscript we edited the respective part as follows:

In the former paragraph (Section: Methods, Page: 9):

“Due to the possibility to access fresh neoplastic material of both species we decided to aim at an establishment of OSCC cell lines as tools for further experimental approaches. The successful establishment of new cell lines allowed us to compare the gene expression patterns of the neoplastic primary tissues and the cell lines of both species. Two human cell lines were generated from freshly isolated squamous cell carcinoma biopsies derived from patient 4 and patient 12 (tumour staging see above) ....”

we added the following bold sentences (Section: Methods, Page: 9):

“Due to the possibility to access fresh neoplastic material of both species we decided to aim at an establishment of OSCC cell lines as tools for further experimental approaches. The successful establishment of new cell lines allowed us to compare the gene expression patterns of the neoplastic primary tissues and the cell lines of both species. The respective human and canine tumour samples were verified to be squamous cell carcinomas by routine histopathologic characterisation. The samples were analysed by either a human or veterinary pathologist respectively. Two human cell lines were generated from freshly isolated squamous cell carcinoma biopsies derived from patient 4 and patient 12 (tumour staging see above) ....”
Comment 2: “How many investigators evaluated the immunohistochemical signals? In case of different investigators, how were different results discussed and decided?”

Answer to comment 2:
The respective human and canine samples were analysed by either a human or veterinary pathologist depending on the sample origin. In the respective species all samples were analysed by the same pathologist.

In order to clarify this point we edited the respective parts in our manuscript as follows:

In the former paragraph (Section: Methods, Page: 9):

“Due to the possibility to access fresh neoplastic material of both species we decided to aim at an establishment of OSCC cell lines as tools for further experimental approaches. The successful establishment of new cell lines allowed us to compare the gene expression patterns of the neoplastic primary tissues and the cell lines of both species. Two human cell lines were generated from freshly isolated squamous cell carcinoma biopsies derived from patient 4 and patient 12 (tumour staging see above) ....”

we added the following bold sentence (Section: Methods, Page: 9):

“Due to the possibility to access fresh neoplastic material of both species we decided to aim at an establishment of OSCC cell lines as tools for further experimental approaches. The successful establishment of new cell lines allowed us to compare the gene expression patterns of the neoplastic primary tissues and the cell lines of both species. The respective human and canine tumour samples were verified to be squamous cell carcinomas by routine histopathologic characterisation. The samples were analysed by either a human or veterinary pathologist respectively. Two human cell lines were generated from freshly isolated squamous cell carcinoma biopsies derived from patient 4 and patient 12 (tumour staging see above) ....”
Comment 3: “What was the idea of using Ki-67 antibodies, just to indicate proliferation activity? Is there a relation to the investigated targets?”

Answer to comment 3:

The initial idea to perform a Ki-67 staining in the IHC was to show the proliferative character of the analysed tissue sections. Additionally as stated in the introduction of the manuscript the re-expression of HMGA2 has been widely described to correlate with aggressiveness and malignancy of several tumour entities. Further HMGA2 is strongly expressed in embryonic development and has been shown to stimulate proliferation of different cell types. Thus, the staining of both targets appeared interesting for our study.

(Review on HMGA2 characteristics see e.g. “The HMGA proteins: A myriad of functions” by Isabelle Cleynen and Wim J.M Vand De Ven, 2008, International Journal of Oncology.)

Comment 4: “- Discussion part, paragraph 3: The authors discussed the fact, that multivariate risk factor analysis demonstrated that HMGA2 expression serves as an independent prognostic marker for disease-specific overall survival. Do they mean disease-specific or overall or both, which is totally different..”

Answer to comment 4:

Our paragraph discusses the findings published by Miyazawa et al. 2004. Within this study the identification of prognostic factors associated with oral carcinoma specific death was performed using a multivariate risk factor analysis. As stated by the authors the variables included age, sex, T stage, N status, clinical stage, histological differentiation and HMGA2 staining. T stage and HMGA2 staining were found to be significant independent predictors of death from carcinoma. For overall survival, positive HMGA2 staining was found to be an independent predictor of reduced survival. (All other variables analysed were not associated with survival according to the multivariate analysis).

We do realize that within our paragraph this fact is stated unclear and thus we have edited this part accordingly.
The former paragraph (Section: Discussion, Page: 18):

“HMGA2 was found to be expressed at the invasive front of oral carcinomas leading to the conclusion that –in contrast to HMGA1- HMGA2 immunostaining could be a potential prognostic determinant in stratifying patients into risk groups [11]. Further, multivariate risk factor analysis demonstrated that HMGA2 expression serves as an independent prognostic marker for disease-specific overall survival [11]. Contrary to this HMGA1 expression was also reported to be increased in head and neck carcinomas analysed via semi-quantitative RT-PCR and immunohistochemistry when compared to healthy mucosa samples [12].”

Was changed into the following new paragraph (Section: Discussion, Page: 18):

“HMGA2 was found to be expressed at the invasive front of oral carcinomas leading to the conclusion that –in contrast to HMGA1- HMGA2 immunostaining could be a potential prognostic determinant in stratifying patients into risk groups [11]. Further, multivariate risk factor analysis demonstrated that HMGA2 expression was found to be a significant independent predictor of death of carcinoma and an independent prognostic marker for disease-specific overall survival [11]. Contrary to this HMGA1 expression was also reported to be increased in head and neck carcinomas analysed via semi-quantitative RT-PCR and immunohistochemistry when compared to healthy mucosa samples [12].”

Comment 5: “Did the authors examined the correlation between HMGA1, HMGA2, let-7, mir-98, Lin28 expression and survival on their own data? Was there a difference between the metastasized and non metastasized cases? Please discuss”

Answer to comment 5:

In our study we did not analysed the correlation between the analysed targets and the survival time. The reason for these missing analyses is due to:

A) Especially in veterinary medicine, the patients presented to facilities of Veterinary Universities are in a way pre-selected. This means, that usually only cases in which common veterinaries are incapable to provide accurate treatment options the cases are referred to academic institutions. This implies that the presented patients are often in late stages of the diseases. Following surgical treatment veterinary patients are released with the respective
owners. In many cases these patients are not presented at the University again. Further, some patients get euthanized in the following periods by local veterinarians. In contrast to humans, veterinary cancer cases are not reported to a central register and thus accurate data mining in larger sample set is not possible in dogs.

B) As stated before, the limited number of analysed cases herein would not allow generating powerful analyses for the issues. Thus, we opted to keep our analyses descriptive.

However, to address this point in our manuscript we added the following paragraph in the discussion of our manuscript (Section: Discussion, Page: 21):

“A correlation between the analysed targets and the survival time was not focussed in this study due to the limited number of analysed cases. Additionally, regarding canine patients, an accurate follow up is often hindered by the fact that veterinary patients are frequently not represented at the academic institution as owners follow treatment at local veterinarians. Furthermore, central cancer register for canine patients, as present for human cancer patients, does not exist. However, due to the similarities in canine and human cancer presentation, as reported herein, basic research and the development of clinical regimens in either of the species provide valuable solid data for the respective counterpart.”

**Comment 6:** “- in what sense do the authors consider their targets as prognostic tools? To differentiate between metastasized and non metastasized cases, between limited and advanced tumor size etc? Would their tools be useful during follow up after treatment? Please discuss..”

**Answer to comment 6:**

Within our study we referred to the term “prognostic tool” addressing two major meanings.

On one hand we were interested if the targets could be used as early stage markers to predict the malignant potential of a tumour and thus be of use for the optimisation of therapeutic options. On the other hand we were interested to see if the tumour invasive potential could be characterised by one of the targets. Our results and the results of the groups working in the field indicate that of the analysed targets only HMGA2 showed
potential for risk assessment. To clarify this we stated in our discussion that our results affirm the findings by Miyazawa for HMGA2 and added the sentence (Section: Discussion, Page: 18):

“HMGA2 was found to be expressed at the invasive front of oral carcinomas leading to the conclusion that—in contrast to HMGA1—HMGA2 immunostaining could be a potential prognostic determinant in stratifying patients into risk groups [11]. Further, multivariate risk factor analysis demonstrated that HMGA2 expression was found to be a significant independent predictor of death of carcinoma and an independent prognostic marker for disease-specific overall survival [11]. Contrary to this HMGA1 expression was also reported to be increased in head and neck carcinomas analysed via semi-quantitative RT-PCR and immunohistochemistry when compared to healthy mucosa samples [12].”