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

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

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

We are thankful for the critical review of the manuscript and feel that by addressing the comments the quality of the manuscript was improved significantly.

In this revised manuscript version we carefully addressed the comments of the reviewers.

Thank you very much in advance for your consideration.

With my best wishes from Hannover,

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27/04/2014
Referee 1 M. RAJESWARI:

Major Compulsory Revisions:

Comment 1: “For the study, tissue samples were obtained from nine patients (seven canine tumor and two healthy control), but in section 2.3 authors have mentioned that canine cell line was generated from freshly isolated oral squamous cell carcinoma biopsy (patient 10). It needs clarification.”

Answer to comment 1:

We apologise that we did not state this clearly in the manuscript. We used nine described canine samples for qPCR. The cell line was established from an additional fresh taken biopsy, unfortunately the material amount was limited, thus we could not analyse the bioptic material directly by qPCR.

In order to clarify this point in the manuscript we changed the former paragraph within the manuscript section Methods. (Section: Methods, Page: 08/09)

“The canine cell line was generated from a freshly isolated oral squamous cell carcinoma biopsy. The tumour tissue sample was cut into small pieces with a sterile scalpel and treated with collagenase (0.26%) for 2 hours at 37°C.”

and added the following sentence (Section: Methods, Page: 09/10)

“The canine cell line was generated from a freshly isolated oral squamous cell carcinoma biopsy. Due to the limited amount of bioptic material this sample was not used in the primary tissue screenings. The tumour tissue sample was cut into small pieces with a sterile scalpel and treated with collagenase (0.26%) for 2 hours at 37°C.”

Comment 2: “In section, 2.5 how much was the 250 RNA. Please mention the units consistently.”

Answer to comment 2:

We added the missing information changing the former sentence (Section: Methods, Page: 09)

“On-column DNase digestion was performed with the RNase-Free DNase set (Qiagen, Hilden, Germany). cDNA syntheses was using 250 RNA and the QuantiTect Reverse Transcription Kit following the manufacturer’s protocol (Qiagen, Hilden, Germany).”
On-column DNase digestion was performed with the RNase-Free DNase set (Qiagen, Hilden, Germany). cDNA synthesis was performed using 250 ng RNA and the QuantiTect Reverse Transcription Kit following the manufacturer’s protocol (Qiagen, Hilden, Germany).”

Comment 3: “There are typographical and grammatical mistakes in the manuscript. It would be better to correct the same, like HMGA1 did not showed in abstract, full stop when sentence completes…. in non small cell lung cancer [8]. The whole manuscript is needed to be read thoroughly in terms of English and typographic mistakes.”

Answer to comment 3:
We apologise for the typographical and grammatical mistakes in the manuscript. The complete manuscript was rechecked.

Comment 4: “Reference 33,34, volume, issue and page number are missing”

Answer to comment 4:
We apologise for the missing details and corrected the respective references.

Comment 6: “From the study, authors have shown significant upregulation of HMGA2 while HMGA1 did not show significant deregulation by relative qPCR at gene level. This finding should be corroborated by Western blot result at protein level. It will further strength the study. It would have been better that authors should demonstrate HMGA expression at protein level.”

Answer to comment 6:
We discussed this point intensively while composing the manuscript as we agree with the reviewer that the protein will be the effecting molecule. We did not perform Western Blots in our study additional to the immunohistochemistry due to two major reasons:

- Currently no antibodies are available that detect reliably specifically HMGA proteins in dogs by Western Blots. Unfortunately the antibodies used in immunohistochemistry (IHC) turned out to miss cross-reaction in Western Blots in previous own studies. Thus, a comparison of the protein expression by WB in both species would not have been possible.
- The material of human and veterinary patients was taken during routine clinical care. Thus a histopathologic analysis was non optional exceeding a division of the taken material. In some cases the available material was limited and as we detect the protein by IHC we opted to use the remaining material for the qPCR analyses.
Comment 7: “Is squamous cell carcinomas of the oral cavity in human and canine biased for HMGA1a and HMGA1b?”

Answer to comment 7:
Yes, the assays detect both splicing variants of the HMGA1 transcript.

We added a sentence to the former paragraph in the methods part of our manuscript (Section: Methods, Page: 10)

“Canine relative quantification of HMGA1, HMGA2, GUSB and HPRT genes, were performed as described previously [9, 27]. All human and canine samples were measured in triplicate and for each run non-template controls and non-reverse transcriptase control reactions were included.”

the new paragraph reads as follows (Section: Methods, Page: 11)

“The canine and human HMGA1 qPCR assays detected both splicing variants (HMGA1a and HMGA1b) simultaneously. All human and canine samples were measured in triplicate and for each run non-template controls and non-reverse transcriptase control reactions were included.”

Comment 8: “The legend of Figure 4 is exactly the copy to that of Figure 3. It would be better to reframe the legend of figure 4.”

Answer to comment 8:
As suggested by the reviewer we rephrased the legend of figure 4. The former legend (Section: Legends, Page: 22)

“HMGA2 immunohistochemistry in canine 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.”

the new legend reads as follows (Section: legends, Page: 23/24)

“HMGA2 immunohistochemistry in canine OSCC

A: Immunolabelling of a canine tumor 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.”
Referee 2 Xiaomei Lu:

Major comments:

Comment 1: “In introduction section, the introduction would benefit from shortening, which was wordy and too long to read and contains some redundant information.”

Answer to comment 1:
According to the comment we critically read the introduction of our manuscript. We feel that the reviewers comment was very helpful and shortened the introduction significantly.

Comment 2: “In material and methods section, the number of tissues from human and canine respectively was too insufficient to provide statistical analysis. Moreover, the staging and grading of the tissue samples from canine should be presented as did the tissue samples from human.”

Answer to comment 2:
We agree with the reviewer that the limited number of analysed cases provides a limited base for a powerful statistical analysis. However, we feel that the combination of primary samples of both species provides an interesting sample pool for groups working in human and veterinary cancer research.

According to the reviewers suggestion we added the missing information on grading and staging concerning the canine patients.

The former paragraph (Section: Methods, Page: 07):

“Seven canine tumour and two healthy control samples (five female, four male) were used covering seven breeds: Boxer, Fox Terrier, Irish Terrier, Landseer, Retriever, Sheltie (n=1 respectively), and three Mixed-breeds. Age ranged between a half year and eleven years. Samples derived from the maxilla (4), tongue (2), mandible (1), palate (1), and pharynx (1). All tumours were analysed immunohistologically. All diagnoses were cytologically and histologically confirmed according to the WHO Nomenclature. The tumours were classified as follows: moderate to poor (patient 3 and 7), well (patient 4-6 and 8), and, well to moderate differentiated (patient 9). The non neoplastic control samples were collected from clinically unaltered tongue and palate tissues and the dogs were euthanised due to oral squamous cell carcinoma unrelated diseases. All samples were provided by the Small Animal Clinic, University of Veterinary Medicine, Hannover, Germany.”

was changed as follows adding the requested information (Section: Methods, Page: 08/09):

“Seven canine tumour and two healthy control samples (five female, four male) were used covering seven breeds: Boxer, Fox Terrier, Irish Terrier, Landseer, Retriever, Sheltie (n=1 respectively), and three Mixed-breeds. Age ranged between a half year and eleven years. Samples derived from the maxilla (4), tongue (2), mandible (1), palate (1), and pharynx (1). All tumours were analysed immunohistologically. All diagnoses were cytologically and histologically confirmed according to the WHO Nomenclature. The tumours were staged and graded as follows:
patient 3- grade IV (poor) stage T3bN1bM0, patient 4- grade I (well) stage T2aN0M0, patient 5- grade I (well) stage T3bN1aM0, patient 6- grade I (well) stage T3bN1bM0, patient 7- grade III (moderate) stage T3bN1bM0, patient 8- grade I (well) stage T1aN1bM0, patient 9- grade I (well) stage T2aN0M0. The non neoplastic control samples were collected from clinically unaltered tongue and palate tissues and the dogs were euthanized due to oral squamous cell carcinoma unrelated diseases. All samples were taken and provided by the Small Animal Clinic, University of Veterinary Medicine, Hannover, Germany according to the legislation of the state of Lower Saxony, Germany.”

Comment 3: “In material and methods section, all the real-time RT-PCR primers as well as accession numbers in Gene bank involved in the present study should be provided either in manuscript or in supplementary, to make reproducible and allow for readers' following.”

Answer to comment 3:

We thank the reviewer for this comment. Initially we discussed this when composing the manuscript and spared the information in order to keep the manuscript as short as possible due to the fact that the canine assays were published by us previously and the human assays are commercially available. However, we happily provide the requested information.

The former paragraphs 2.6 and 2.7 were completed as follows: (Section: Methods, Page: 11/12)

“2.6. HMGA1, HMGA2, Lin28, GUSB and HPRT real time PCR

Relative quantification real time PCRs for both species were carried out using the Eppendorf Mastercycler ep realplex real-time PCR System (Eppendorf AG, Hamburg, Germany).

For analysis of the human target genes, 2 µl of each cDNA was amplified in a total volume of 25 µl using universal PCR Mastermix and commercially purchased TaqMan gene Expression Assays (HMGA1– Assay ID: Hs00600784_g1; HMGA2– Assay ID: Hs00971724_m1; Lin28A– Assay ID: Hs04189307_g1; HPRT- Assay ID: Hs02800695_m1; GUSB- Assay ID: Hs99999808_m1; (Applied Biosystems, Darmstadt, Germany)).

For analysis of canine target genes, 2 µl of each cDNA was amplified using universal PCR Mastermix, self-designed TaqMan based Assays ([9, 27] (canine HMGA1 (NM_001003387)- forward primer: 5’ ACCCAGTGAAGTGCCAACACCATAA 3’, reverse primer: 5’ CCTCCTTCTCCAGTTTTTGTTCT 3’, probe: 5’ 6-FAM-AGGGTGCTGCCAACGACACATACCA-TAMRA 3’; canine HMGA2 (DQ316099)- forward primer: 5’ AGTCCTCCCAAAGAGCTCAAAAAG 3’, reverse primer: 5’ GCCATTTTCTAGGTGCTGCTC 3’, probe: 5’ 6-FAM-CGCCCACTACTATGCTACAGCGAC-TAMRA 3’; canine HPRT (NM_001003357)- forward primer: 5’ CCTCTTGGAGGAACTTAA 3’, reverse primer: 5’TGTCTACTACACGCTCAGCTCAGCTCA-TAMRA 3’; canine GUSB (NM_001003191)- forward primer: 5’ TGGTGCTGAGGATTTTGGG 3’, reverse primer: 5’ CTGCCACATGGACCCATTC 3’, probe: 5’ 6-FAM-CGCCCACTACTATGCTACAGCGAC-TAMRA 3’). The canine and human HMGA1 qPCR assays detected both splicing variants (HMGA1a and HMGA1b) simultaneously. PCR conditions were as follows: 10 min at 95°C, followed by 45 cycles with 15 s at 95°C and 1 min at 60°C. All human and canine samples were measured in triplicate and for each run non-template controls and non-reverse transcription control reactions were included.”
2.7. Let-7a, mir-98 and RNU6B real-time PCR

Relative quantification of the human and canine let-7a, mir-98 and RNU6B micro RNA transcript levels were carried out using 1.33 µl of each cDNA amplified in a total volume of 20 µl using TaqMan Universal PCR Master Mix, No AmpErase UNG and TaqMan MicroRNA assays for each gene (Let-7a-Assay ID: 000377; mir-98- Assay ID: 000577; RNU6B- Assay ID: 001093 (Applied Biosystems, Darmstadt, Germany)).

PCR conditions were as follows: 10 min at 95°C, followed by 45 cycles with 15 s at 95°C and 1 min at 60°C. All samples were measured in triplicate and for each run non-template controls and non-reverse transcriptase control reactions were included.

A precedent efficiency analysis of the microRNA PCR assays which were used in this study was performed by applying the same template and dilution steps."

Comment 4: “The relevant results (qRT-PCR and IHC) regarding HMGA1 should be juxtaposed with HMGA2 in results not mentioned and placed in supplementary.”

Answer to comment 4:

We also discussed this point when composing the manuscript opting for the supplementary form as the results do not show a statistical difference in expression. Following the reviewers request we completed the figures 1 and 2 with the data for HMGA1.

We edited the former paragraphs (Section: Results, Page: 11):

“3.1. Real time PCR expression analyses of HMGA1 and HMGA2

3.1.1 Human samples

HMGA1/GUSB expression levels varied from 0.225 to 1.47 within the control samples, and from 0.53 to 2.52 within the tumour samples. The HMGA1/HPRT expression levels ranged from 0.21 to 2.02 within the control samples and from 0.36 to 1.28 within the tumour samples (details supplementary table 1).

HMGA2/GUSB values ranged between 1 and 26.8 in the control samples, 44.4 and 2330 in the tumour samples, and 319 and 1092 within the cell culture samples. HMGA2/HPRT expression levels ranged between 1 and 31.1 within the control group, 24.1 and 3778 within the tumour group, and 226 to 561 within the cell culture samples (figure 1 A, B; supplementary table 1).

3.1.2 Canine samples

HMGA1/GUSB expression levels varied from 1 to 1.67 within the non-neoplastic samples and from 0.152 to 1.69 within the neoplastic samples. HMGA1/HPRT expression ranged from 1 to 1.31 in the non neoplastic and 0.269 to 0.811 within the tumour samples (details supplementary table 2).
HMGA2/GUSB expression levels ranged from 0.383 to 1 within the non neoplastic samples and from 6.45 to 208 within the neoplastic samples. HMGA2/HPRT expression ranged from 0.31 to 1 within the control group, and from 3.69 to 80.7 within the tumours (figure 2, supplementary table 2).”

To be changed as follows adding the requested information (Section: Results, Page: 14):

“3.1. Real time PCR expression analyses of HMGA1 and HMGA2

3.1.1 Human samples

HMGA1/GUSB expression levels varied from 0.225 to 1.47 within the control samples, and from 0.53 to 2.52 within the tumour samples. The HMGA1/HPRT expression levels ranged from 0.21 to 2.02 within the control samples and from 0.36 to 1.28 within the tumour samples (details figure 1 A, B and supplementary table 1).

HMGA2/GUSB values ranged between 1 and 26.8 in the control samples, 44.4 and 2330 in the tumour samples, and 319 and 1092 within the cell culture samples. HMGA2/HPRT expression levels ranged between 1 and 31.1 within the control group, 24.1 and 3778 within the tumour group, and 226 to 561 within the cell culture samples (figure 1 C, D and supplementary table 1).

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HMGA2/GUSB expression levels ranged from 0.383 to 1 within the non neoplastic samples and from 6.45 to 208 within the neoplastic samples. HMGA2/HPRT expression ranged from 0.31 to 1 within the control group, and from 3.69 to 80.7 within the tumours (figure 2 C, D and supplementary table 2).”

Comment 5: “In statistical analysis, the specific statistical methodology employed in the study needs to be provided. Such as student’s test, one-way ANOVA or rank sum test. It was too broad to say p value <0.05 was considered statistically significant.”

Answer to comment 5:

We apologise if we did not stated this point satisfingly in the original manuscript. REST software employs the “Hypothesis Test” for statistical significance calculation. To clarify this we kept the former paragraph (Section: Methods, Page: 13)

“Statistical analysis of the relative real time PCR results applying the Hypothesis Test was performed with the Relative expression software tool REST 2008, version 2.0.7 [29]. A p-value of <0.05 was considered statistically significant.”
Comments 6 & 7: “(6) In discussion part, the conclusion of the study is very likely strongly biased by the choice of so limited cases from both human and dog as it stands. Considering the great variability between individuals, all the trends described here is at best suggestive rather than conclusive, and the correlation between HMGA1/2 and let-7 family is indirect and inferable rather than direct and experimentally solid in light of lack of Luciferase reporter assay on cell line level.

(7) There were some overstated significance of results in the discussion and over-interpretation of existing literatures. The discussion section would also benefit from shortening and succinctness.

Such as
In discussion section, page 17, line 14 “our findings in humans and dogs strongly support.......in both species”, I disagree with these statement. Because, there is no data at all provided in this paper that relates to “prognosis”. So, it would be suggested that the authors should delete the statement.

In discussion section, page 18, line 20-23, the statement that “our immunohistochemical findings in canine ......at the invasive front in canine oral cancer”, is also overstated. In the study, there is no data presented at all that supports the role of HMGA2 in the cellular behaviors in OSCC. Suggest deleting these statements without experimental evidence.

In discussion section, page 19, line 2-4, the statement that “furthermore, miR-98 was ......and miR-98 in OSCC cell line samples” was also lack of evidence results. The correlation between miR-98 and HMGA2 is at best inferable without the biochemical analyses (classical luciferase reporter assay), and may be biased due to rather limited cases in the case of individual variability. So, it would be better to rephrase or delete.”

Answer to comment 6 & 7:

Following the reviewers advice we critically revised the discussion and the conclusions of our manuscript. We agree with the reviewer that by the included number of cases and the experimental design some statements appear overstated. Addressing these points we rephrased the discussion of our manuscript editing several passages. These changes were combined with the requested shortening of the original manuscript. As the changes were substantial we are not listing them here in detail.

Comment 8: “In the reference part, both the volume and page number were missing in the reference [33] and [34], please check it.”

Answer to comment 8:

As stated in the answer “3” addressing the requests of the first reviewer, we corrected the respective references.

Specific comments:

Comment 1: “Figure1. Figure 1A and 1B was alphabetically mislabeled with C and D, please check it.”

Answer to comment 1:

According to the reviewers major comments we introduced the results for HMGA1 in the provided figures. “Figure 1” and “Figure 2” were modified accordingly and the respective sub-figured were renamed.
Comment 2: “Figure 1A and 1B were factually the same results showing the same information, which plotted with different internal control. Suggest choosing one from the two kinds of similar results presented. In the legend to figure 1, the statistical method used should be detailed rather than presented with p value. The same holds true for Figure 2A and 2B.”

Answer to comment 2:

We discussed this point intensively. We feel that the qPCR data in the study benefits from the presentation of both housekeeping genes. However, we would be willing to remove the data as suggested if the Editorial board agrees with the reviewers comment. Taking into account that both other reviewers did not criticise this point we opted to keep the data as provided initially in the original manuscript.

Comment 3: “Figure 3. Without comparison with normal control tissues, whatever human or dog, it is not clear how to interpret the immunohistochemical data. What’s more, regarding Figure 3 and figure 4, the magnification labeling should be uniform, either labeling with scale bars or magnification times. Additionally, The IHC results shown should be quantified, at lest semi-quantified. As stated, the author employed weak, moderate and strong to semi-quantitatively evaluate HMGA2/1 expression, however, there is no mention that the quantitation of HMGA2/1 in figure legend as well as results section.”

Answer to comment 3:

According to the reviewers comment we generated a new “figure 4” uniforming the magnification labelling. Additionally we added approx. percentages of stained cells to the legend of figure 4.

The new legend reads as follows (Section: Figure legends, Page: 24):

“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.”

Comment 4: “Figure 5. In the legend to figure 5, as stated above, the statistical method used should be detailed and provided; moreover the calculated method of HMGA2 and miRNAs should also be given both in legends and manuscript where appropriate. Take, for example, regarding miRNAs 2-##t method was properly used whereas normal mRNA of gene of interest, standard curve method using proper standard to construct calibration curve for real-time RT-PCR may be first of choice. The same holds true for the legend to figure 6.”

Answer to comment 4:

According to the reviewers comment, we added the missing statistical information to the legends of the figures 5 & 6. The new legends of the manuscript were rephrased as follows (Section: Figure legends, Page: 24/25):

“Figure 5: Comparative expression analyses of the HMGA2 and Lin28 genes and the let-7a and mir-98 miRNAs in human OSCC”
The study included 5 non neoplastic control samples (green columns), 6 tumour samples (red columns) and 2 patient derived cell lines (brown columns). A: relative HMGA2/HPRT real time PCR. B: relative Lin28/HPRT real time PCR. C: relative let-7a/RNU6B real time PCR. D: relative mir-98/RNU6B real time PCR.

**Statistical analysis of the relative real time PCR results (Hypothesis Test)** was performed with REST 2008 software tool. A p-value of <0.05 was considered statistically significant.

* indicates a statistical significant expression deregulation of HMGA2 and/or Lin28 and/or let-7a and/or mir-98 when compared to non neoplastic control group; p-value is displayed next to **“**

“Figure 6:
Comparative expression analyses of the HMGA2 and Lin28 genes and the let-7a and mir-98 miRNAs in canine OSCC
The study included 2 non neoplastic control samples (green columns), 7 tumour samples (red columns) and 2 cell line derived samples (brown columns) which derived from patients 1-10. A: relative HMGA2/HPRT real time PCR. B: relative Lin28/HPRT real time PCR. C: relative let-7a/RNU6B real time PCR. D: relative mir-98/RNU6B real time PCR.

**Statistical analysis of the relative real time PCR results (Hypothesis Test)** was performed with REST 2008 software tool. A p-value of <0.05 was considered statistically significant.

* indicates a statistical significant expression deregulation of HMGA2 and/or Lin28 and/or let-7a and/or mir-98 when compared to non neoplastic control group; p-value is displayed next to **“**

**Referee 3, Max Heiland:**

We are happy that the reviewer classified our manuscript as “important to those with closely related research interests”.
We feel that by editing the manuscript according to the reviewers comments, the manuscript quality improved significantly.