Peer Review File

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Reviewer A

COMMENT 1. This study focuses on microRNA in pleomorphic adenomas highlighting those related to apoptosis. It is important and could contribute to the literature as there are little papers about that. English revision is recommended.

REPLY 1. Thank You very much for careful revisions of our manuscript and useful suggestions.

CHANGES IN THE TEXT 1. N/A.

COMMENT 2. Introduction – There are a few short paragraphs. I suggest to gather each one.

REPLY 2. That is correct. We have modified our text as advised.

CHANGES IN THE TEXT 2. Page 3-5, lines 67-117. Paragraphs are gathered.

COMMENT 3. Results – I suggest briefly introducing each table

REPLY 3. We have modified our text as advised.

CHANGES IN THE TEXT 3 – lines 191-192, for Table 1 were added. Detailed description of Tables 3, 4, 5, was given not in the place of the first mention of the tables but later. Now these descriptions are present in the lines 220-228 and 235-238.

COMMENT 4. Methodology- which was scored as up or downregulation?

REPLY 4. This consideration is added to the text now.

CHANGES IN THE TEXT 4. Lines 173-175. “Up- or downregulation of the microRNAs expression level was considered as increase or decrease of the microRNA level revealed in tumor tissue comparing to the intact tissue”.

COMMENT 5 - I suggest that tissue salivary gland adjacent to tumor should be changed for salivary gland adjacent

REPLY 5. We are completely agreed with this suggestion.

CHANGES IN THE TEXT 5 - all together 17 changes were done through over the

text including figure legends.

COMMENT 6. Discussion – all statistical results should be discussed
REPLY 6. All the results are discussed now.
CHANGES IN THE TEXT 6. Lines 303-306 and 307-317 were added.

COMMENT 7. Clinical aspects x MicroRNAs?
REPLY 7. Whenever there was a sufficiently high level of miRNA-34a and miRNA-29a expressions in the tissues of the large salivary glands pleomorphic adenomas compared with the intact salivary gland tissue evaluated as 10:1, these molecules can be used as a genetic markers for the verification (identification) of this type of tumor. This was changed in the Conclusion 1. Blood, showed levels of expression of miRNA-34a and miRNA-29a lower than normal (intact gland), and this was not statistically significant; therefore, it cannot be a diagnostic criterion for the genetic verification of pleomorphic adenomas at the preoperative stage.
CHANGES IN THE TEXT 7. Lines 307-309 and 351-354.

COMMENT 8. 3rd paragraph -The authors should get clearer : If one …
REPLY 8. The sentence is changed to “The level of miRNA-34a is in 1.95 times greater (1052.02 ± 367.20 versus 539.09 ± 158.70; $p<0.05$) and miRNA-29a is in 1.37 times greater (111.93 ± 56.97 versus 80.58 ± 37.49; $p<0.05$) in the tissue of the salivary gland adjacent to the tumor comparing to the tumor tissue”.
CHANGES IN THE TEXT 8. Lines 280-283.

COMMENT 9. 3rd paragraph - Please, let me know how this sentence was based on: However, an increase in the proliferation and differentiation of cells in the salivary gland adjacent to the tumor was the manifestation of increased activity (metabolism) of the gland cells, and this was a manifestation of the degeneration and transformation into the tumor component.
REPLY 9. Yes, it was strange sentence… Now it is changed to “However, an increase in the proliferation and differentiation of the cells in the salivary gland tissue adjacent to the tumor may be considered as a manifestation of transformation into the tumor component.”.

CHANGES IN THE TEXT 9. Lines 288-290.

COMMENT 10. 4th paragraph- I think it is tough to choose a surgical removal except for barrier function, isn't? and the authors should clear the relation between apoptosis and proliferation in this study as this has been reported in this study.

REPLY 10. The apoptotic and proliferation assays were not performed in the present study. Still it is known from literature that both studied microRNAs have proaptotic abilities. However, an increase in the expressions of miRNA-34 in the adjacent tissue of the salivary gland led to increased processes of cell proliferation and differentiation. Thus, the question arises, why do the antitumor mechanisms of humans, including apoptosis, not lead to tumor regression when the microRNA content in the tumor is 10 times higher than normal? A possible answer to this question is the presence of additional mechanisms of protection in the tumor itself, which provokes further investigation. In particular the presence of HPV infection may block apoptosis even during increased expression of proapoptotic microRNAs through p53 sequestering and degradation in cytoplasm. Based only on the data obtained in the present study we can propose a surgical removal of the border zone despite the high expression of proapoptotic microRNAs and possible barrier functions of apoptosis. Now this sentence is changed to "This study has once again confirmed at a genetic level, the need not only for the excision of the tumor (partial parotidectomy) but also for the performance of a subtotal resection, with the removal of the salivary gland adjacent to the tumor, where proliferative changes can occur despite the barrier function of possibly increased apoptosis in this area."

CHANGES IN THE TEXT 10. Lines 291-295.

COMMENT 11. 5th paragraph – .. in this area of the salivary gland. Which area?

REPLY 11. Changed to "…in the salivary gland tissue adjacent to tumor".

CHANGES IN THE TEXT 11. Line 313.

COMMENT 12. The authors should better discuss immunohistochemical results. p16 positivity is indicative of HPV at all? Why did you study these markers in this study with apoptotic MicroRNAs?

REPLY 12. p16 is an inhibitor of cyclin-dependent kinases 4 and 6 which activate the

negative cell cycle regulator protein pRB. pRB in turn downregulates p16 expression in a loop mechanism. HPVs can interfere with this regulatory circuit by its virtue to inactivate pRB and thus lead to the overexpession of p16. Thus increase in p16 protein level is a non-direct marker of all types of HPVs infections. P16 considered as an HPV marker in many publications and was shown to be present in a lot of head and neck tumors (DOI: 10.1097/PAI.0b013e3182936ea7, DOI: 10.1159/000082474). To reveal etiologic factors of the tumors in our study we have performed immunohistochemical analysis of this marker as well as a marker for another virus – EBV. Finally, we have added to this manuscript the results of PLAG1 (pleomorphic adenoma gene 1) measurement. PLAG1 is a proto-oncogene whose overexpression is a crucial oncogenic event in salivary gland pleomorphic adenomas. Our data indicates that the majority of patients - 15 (68.18%) and 22 (100 %) had a positive response to HPV 16 and PLAG 1, respectively. These findings may explain that despite of high expression of proapoptotic miRNAs in the salivary gland tissue adjacent to the tumor, proliferation and possible malignization still occur in this area. This information is added to the manuscript in the "Discussion" section.
CHANGES IN THE TEXT 12. Lines 295-306.

COMMENT 13. Conclusions – Odds ratio should be used for reporting 10 times
REPLY 13. This sentence has been revised.
CHANGES IN THE TEXT 13. Lines 355-359

Reviewer B

REPLY. Thank You very much for careful revisions of our manuscript and useful suggestions.

Throughout the manuscript
COMMENT 1 - The correct name of the miRNAs is miR-34a and miR-29a and not miRNA-34a and miRNA-29a.
REPLY 1 – These changes are dome in the text.
CHANGES IN THE TEXT 1 – All together 72 changes were performed through the text.

COMMENT 2 - Extensive grammatical English editing needed.

REPLY 2 – The text of the manuscript has been revised. Certificate of the proof reading service is added to the submission.

CHANGES IN THE TEXT 2 – throughout the text.

Introduction

COMMENT 3 - In the first two paragraphs there are a numerous percentages and proportions that are difficult to follow.

REPLY 3 – This paragraph was revised and rewrited.

CHANGES IN THE TEXT 3 – lines 57-75.

COMMENT 4 - miRNAs are not only pro- and anti-apoptotic, but might also be tumor-suppressors and oncogenes; pro-fibrotic and anti-fibrotic; pro-angiogenic and anti-angiogenic., etc.

REPLY 4 – That is correct. It has been changed to “Among microRNAs with different functions some of them have strongly pronounced proapoptotic and antiapoptotic functions.”

CHANGES IN THE TEXT 4 – Lines 85-89

COMMENT 5 - miR-29a is from miR-29 family that are mainly fibrosis-related miRNAs (have anti-fibrotic function and are down-regulated in fibrosis states).

REPLY 5 – Taking into consideration that miRNAs usually have more than one target and function it is possible that one molecule has fibrosis-, apoptosis-related properties, etc. Apoptotic assay was not performed in this study but according to the literature data miR-29a has demonstrated proapoptotic abilities. These literature sources are cited in the text (References 15, 16, 17).

CHANGES IN THE TEXT 5 – lines 91-97.

COMMENT 6 - Why apoptosis- related miRNAs? And why only two of them and why these two? No explanation on background for the decision.

REPLY 6 – Choose of these miRNAs was based on the literature data. The aim of the study was mainly to determine their localization in the perytumor zone in order to

explaine border functions of this zone and to elucidate the necessity of surgical removal of it. The role of these miRNAs as a predictive markers (especially blood level of them) that is not linked with their proapoptotic functions was also a goal of present study. These goals confirm the necessity of a surgical removal of the border zone despite the high expression of proapoptotic microRNAs and possible barrier functions of apoptosis. Etiological role of these microRNAs level was not studied in this work. This explanation is added to the “Introduction” section.
CHANGES IN THE TEXT 6 – Lines 92, 111.

COMMENT 7 - Why apoptosis in pleomorphic adenomas?
REPLY 7 – This is a question of a general pathology. In any type of tumor mechanisms of apoptosis deviation and escape are activated. This fundamental question has its practical realization due to high expression of proapoptotic factors in perytumor zone leads some researchers to the conclusion that this zone should be saved during surgery. This formulation is added to the “Introduction” section.
CHANGES IN THE TEXT 7 – Lines 99-111.

Materials and methods
COMMENT 8 - Sub-titles should be included to separate methods used.
REPLY 8 – This revision is done now.
CHANGES IN THE TEXT 8 – Line 113, 123, 157, 175.

COMMENT 9 - Was all RNA isolated using phenol-extraction, tissue and blood?
REPLY 9 - Yes
CHANGES IN THE TEXT 9 – N/A

COMMENT 10 - Was efficiency determined for qPCR?
REPLY 10 – Yes, we have performed analysis of each sample three times.
CHANGES IN THE TEXT 10 – N/A

COMMENT 11 - p16 IHC is not equeal to HPV16 infection!
REPLY 11- Exactly! It was a mistake in the text. P16 corresponds to any type of HPV infection. This is revised in manuscript including tables now.

CHANGES IN THE TEXT 11 – Lines 292-298, 446.

COMMENT 12 - LMP1 IHC is not equal to EBV infection!

REPLY 12 - Epstein–Barr virus latent membrane protein 1 (LMP1) is an Epstein–Barr virus protein that regulates its own expression. It is used for latent EBV infection confirmation (for example, DOI 10.5812/ijp.2359). We have also added to the manuscript results of additional evaluation of the PLAG1 marker (Figure 1B).

CHANGES IN THE TEXT 12 – Lines 144, 147, 73, 193, 298-313, 446, 471.

Results

COMMENT 13 - No systematic overview and description, comparison of the results. Very difficult to follow.

REPLY 13 – The descriprion of the results has been changed in the manuscript now.

CHANGES IN THE TEXT 13 – Changes through the “Results” section.

COMMENT 14 - Too many tables, might be combined.

REPLY 14 – Table 4 and 6 were combined. Also all the figures were combined in one.

CHANGES IN THE TEXT 14 – pages 19-21.

COMMENT 15 - It is not clear, what is the main text and what is the Figure/Table legend.

REPLY 15 - Figure and Tables legends are present in the appropriate sections now and text is revised.

CHANGES IN THE TEXT 15 - pages 8-10.

COMMENT 16 - What are the numbers for blood in Table 7? Is fold change? How was that calculated?

REPLY 16 – These are relative units of miR/U6. These changes are added to the table. Now it is Table 6.

CHANGES IN THE TEXT 16 – line 459.

Discussion

COMMENT 17 - Modern genetic area, modern science and similar are not scientific

language.

REPLY 17 – We are agreed. These sentences are changed.

CHANGES IN THE TEXT 17 – Lines 43, 272.

COMMENT 18 - Third paragraph: please, specify the conventional units.

REPLY 18 – These are relative units miR/ small nuclear RNA U6. Changes are added to the text.

CHANGES IN THE TEXT 18 – Lines 168-170.

COMMENT 19 - Fourth paragraph: this is not genetic level, but it is transcriptional level.

REPLY 19 – This revision is changed in the manuscript.

CHANGES IN THE TEXT 19 – line 288.

COMMENT 20 - Since p53 is mentioned, the discussion should also include a little bit more about correlation between p53 and miR-34a. And p16.

REPLY 20 – This information is added to the manuscript.

CHANGES IN THE TEXT 20 – Lines 293-304, 333-341.

Conclusion

COMMENT 21 - Where are the ROC and AUC curves to be certain that these two miRNAs might have diagnostic potential?

REPLY 21 – Our primary task was to estimate changes in the levels of expression of two miRNAs in 4 groups of tissues including blood. We demonstrate that this difference exists. The task about prognosis that needs logistic regression, higher amount of observations, etc. was not among our goals. ROC analysis is used for binary classification only and not for 4 groups of comparison. Still we mention in the discussion that blood level of miRNAs of interest cannot be used as a prognostic marker.

CHANGES IN THE TEXT 21 – N/A

COMMENT 22 - However, I do not believe that these two miRNAs might have promising diagnostic utility due to their wide spectrum of role.

REPLY 22 - Whenever there was a sufficiently high level of miRNA-34a and miRNA-29a expressions in the tissues of the large salivary glands pleomorphic adenomas compared with the intact salivary gland tissue evaluated as 10:1, these molecules can be used as a genetic markers for the verification (identification) of this type of tumor. This was changed in the Conclusion 1. Blood, showed levels of expression of miRNA-34a and miRNA-29a lower than normal (intact gland), and this was not statistically significant; therefore, it cannot be a diagnostic criterion for the genetic verification of pleomorphic adenomas at the preoperative stage.

CHANGES IN THE TEXT 22 – Lines 304-313 and 347-351.