Author's response to reviews

Title: 14-3-3epsilon contributes to tumor suppression in laryngeal carcinoma by affecting apoptosis and invasion

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Author's response to reviews: see over
Dear Editors,

Thanks for your kind consideration. I have revised my manuscript (m/s) word by word according to reviewers’ opinions.

**Reviewer:** Krzysztof (Chris) Szyfter

1. Why the authors use the term 14-3-3-epsilon instead of the existing (they call it “official”) tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein?

   **Answer:**
   Because the term 14-3-3-epsilon has been used comprehensively in almost all the articles which have been published with respect to 14-3-3-epsilon, in our m/s, we use 14-3-3-epsilon instead of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein.

2. There is a variety of terms for tumor-free margin, histological normal margin, surgical margin etc. The authors use instead the own term paired adjacent normal laryngeal tissue (PANL). Do we need so many terms for the same entity?

   **Answer:**
   In the revised m/s, the term “paired adjacent normal laryngeal tissue (PANL)” has been changed to “clear surgical margin tissues”.

3. The section Methods should provide more information about cell line derived from laryngeal cancer (generalia, TNM, grading, treatment).
Hep-2 cell line derived from the metastatic epidermoid carcinoma of the larynx, the donor patient was a male aged 57 years. The relevant information has been added to the section Cell culture and Transient Transfection on Page 8, Lines 11-12, with the literature reference: “TOOLAN HW: Transplantable human neoplasms maintained in cortisone-treated laboratory animals: H.S. No. 1; H.Ep. No. 1; H.Ep. No. 2; H.Ep. No. 3; and H. Emb.Rh. No. 1. Cancer Res 1954, 14(9): 660-6.”, but, TNM, grading, treatment of the laryngeal cancer can not be found in any literature reference.

4. There are no literature references in the section Methods. Are all the methods established or modified in own laboratory? If YES at least the ones used for quantization should contain more information.

Answer:
According to the demand, all the literature references have been added in the section Methods in the revised m/s.

5. Results, 1st paragraph should refer to the Table 2 not 1.

Answer:
Table 1 has been changed to Table 2 on line 12 of Page 11 in the revised m/s.

6. Results. Comparisons done between different stages of tumor are not readable, at least at mine copy. Information concerning staging is missing.
Answer:

In the revised m/s, the comparisons done between different stages of tumor have been revised on Page 12, Lines 2-5, as “There was no difference in mRNA levels with respect to patients clinical stages (Table 4). However, the protein expression level of 14-3-3-epsilon in stage III or IV tumors was significantly lower than that in any stage I or II tumors (P<0.001, Table 4)”.

The information concerning staging was shown in Table 1 in our original M/S and we have added a sentence “The information of the patients was shown in Table 1.” on Page 5, Line 1 from bottom to describe it.

7. Fig. 1. I suggest to be more specific for ordinates by using estimation or (better) determination instead of analysis.

Answer:

According to the demand, the “analysis” has been changed to “Determination” in Fig. 1 in the revised m/s.

8. Editing of the manuscript is horrible. A number of typing errors, missing spaces and regularly added spaces between words and commas appear.

Answer:

In the revised m/s, all the editing errors like missing spaces have been corrected.

**Reviewer:** Demitrios Vynios
1. The m/s contains a lot of grammar errors and needs word by word correction.

Answer:

Our revised manuscript has been word by word corrected carefully.

2. “Background, Page 4, Line 2”, they use the number 5,00,000 that was written in ref. 2.

Answer:

In our revised manuscript, the number “5,00,000” on “Background, Page 3, Line 2”, has been changed to “half-million”. We also give a criticism in the last sentence of the same paragraph.

3. “Background, Page 4, Lines 3-5”, they incorporate the sentence written in ref. 3, however the occurrence rate is exactly opposite for men and women to that written (see the web pages of the British Cancer Society and the Finnish Cancer Society, or a Basic Otolaryngology edition). To my opinion, these points are extremely unfair and a possible reason for rejection of the paper independently to the quality of work done.

Answer:

Frankly, the sentence “Larynx squamous cell carcinoma (LSCC) accounts for the vast majority of SCCHN, with an occurrence rate of 0.4% in men and 2.2% in women[3].” on “Background, Page 4, Lines 3-5” is the original one. We also think it is unfair. Therefore, the reference 3 has been deleted in our revised manuscript.
4. Page 7, Line 10: The composition of the “protein extracting fluid” and the volume to weight ratio must be given.

Answer:

The composition of the “ protein extracting fluid” are RIPA Lysis Buffer (50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and PMSF. And the volume to weight ratio is 20 mg/250 µl.

Both the composition and the volume to weight ratio have been shown in our revised manuscript on “Western blot, Page 6, Lines 2-4”.

5. Page 12, second para: “The analyse result of 14-3-3epsilon gene expression levels with respect to the clinical characteristics (age, sex and the clinical stages) showed no differences in both protein and mRNA levels of 14-3-3epsilon regarding to patients’ age and sex, and in mRNA level to clinical stages (datas not shown)”. (A sentence with many grammar errors): The authors should include the results of RT-PCR in table IV (mRNA levels according to clinical stage).

Answer:

In our revised manuscript ‘Page 11, third para’, the sentence has been corrected as: “We assessed the 14-3-3epsilon gene expression levels with respect to the clinical characteristics (age, sex and the clinical stages). No differences were identified in protein and mRNA levels of 14-3-3epsilon with respect to patients ages and sex (data not shown). There was no difference in mRNA levels with
respect to patients clinical stages (Table 4). However, the protein expression level of 14-3-3-epsilon in stage III or IV tumors was significantly lower than that in any stage I or II tumors (P<0.001, Table 4). " And the statistics analysis results of relationship between mRNA levels and clinical stages have also been shown in table IV.

6. Page 15, line 2 from bottom: "Our results implies that something wrong probably happens in G2 DNA damage checkpoint and there maybe molecular mechanism of 14-3-3-epsilon on S phase arrest of laryngeal cancer cells." It is not a clear sentence.

Answer:

The sentence has been changed to “However, our study showed that the growth of Hep-2 cells overexpressing 14-3-3-epsilon was inhibited and these cells were only halted in S phase, which indicates that the low proliferation of Hep-2 cells transfected by 14-3-3-epsilon-GFP originates partly from S phase arrest. The molecular mechanism on which the arrest of Hep-2 cells in S phase affected by 14-3-3-epsilon will be an interesting target in our further study. ” on Page 15, Lines 10-15 in our revised manuscript.

7. Page 16, second and third paragraphs: The results of the study indicated that 14-3-3-epsilon affected cell apoptosis, and thus, as the authors mentioned, it might be responsible for the development of cancer. However, the protein levels were
constant in PANL and stages I and II samples, and decreased in stages III and IV indicating that the protein has a distinct role in cancer invasion. So, the authors must clearly discuss their observations.

Answer:

Due to that the observations in the original manuscript on “Page 16, second and third paragraphs” were not discussed clearly, the discussion related to these parts has been clarified in the revised m/s on “Page 15, third paragraph and Page 16, second paragraph” as: “Some studies show that 14-3-3epsilon, an inhibitor of apoptosis protein, prevents apoptosis progression by inhibiting the activities of pro-apoptotic proteins such as Bad and Bax [23-26]. But in the present study, our results from both apoptosis and cell cycle assays showed that the apoptotic cells in 14-3-3epsilon-GFP group increased, which indicates 14-3-3epsilon has the ability in promoting apoptosis. We speculate that the increased apoptosis in Hep-2 cells transfected by 14-3-3epsilon can also lead to reduction of cells. Meanwhile, the down-regulation of 14-3-3epsilon gene detected in LSCC in the study plays a role in the development of LSCC probably by inhibiting apoptosis.

Tumor invasion and metastasis are hallmarks of malignant tumors. Cancer cell metastasis to distant organs is the major cause of death in almost all kinds of cancer patients. Metastasis is a multi-step process, and the initial step is considered to be the invasion of surrounding tissue by cancer cells. Inhibition of the invasion and metastasis of tumor cells could be a new pathway in the treatment of patients with cancer [27-28]. Tak et al. found that 14-3-3epsilon inhibits cell
migration in HeLa cells by interacting with MAPK-activated protein kinase 5 (MK5) [29]. Our present study showed that 14-3-3-epsilon displayed a lower expression in the metastatic lymph nodes than that in cancer tissues and 14-3-3-epsilon protein levels were significantly lower in stage III or IV than those in stage I or II, which implies that 14-3-3-epsilon might inhibit the metastasis of LSCC. And our transwell result also supports this conclusion. The results from apoptosis, cell cycle and cell viability assays combined with those mentioned above in the study implies that lower expresion of 14-3-3-epsilon which results in decreased apoptosis and high proliferation could contribut to invasion and aggression of LSCC. 

8. Page 5, Lines 7-8: Other six mammalian isoforms (#, #, #, #, #, # and #) have also been identified. The isoform “#” should be removed from the parentheses.

Answer:
On Page 4, fourth para in our revised manuscript, the sentence “14-3-3-epsilon is one of mammalian 14-3-3 protein family members which contain a few regions of diversity and have been proposed to interact with more than 200 proteins [9]” has replace the original sentence “14-3-3-epsilon is a member of mammalian 14-3-3 protein family. Other six mammalian isoforms(#, #, #, #, # and #)have also been identified, which contain a few regions of diversity, but have been claimed to interact with more than 200 proteins.” In the new sentence, we also add a literature reference as reference 9.
9. Page 5, Line 17: “are” instead of “were”.

Answer:
In our original manuscript, Page 5, Lines 16-17, the sentence “But the mechanism and partly contradictory biological functions which 14-3-3epsilon exerts in many tumors were not well elucidated.” has been revised as “However, the exact function and regulatory mechanism of 14-3-3epsilon in carcinogenesis are not clear.” in the revised manuscript on Page 5, Lines 4-5.

10. Page 6, Line 4 from bottom: “an internal” instead of “a internal”.

Answer:
The “a internal ” has been revised as “an internal” in our revised manuscript, Page 6, Line 7.

11. Page 7, Line 8: “were” instead of “was”.

Answer:
The word “was” has been revised as “were” in our revised manuscript, Page 6, Line 5 from bottom.

12. Page 10 Line 5 from bottom: “were” instead of “was”.

Answer:
The word “was” has been revised as “were” in our revised manuscript, Page 10, Line 8.
13. Page 11 Line 3 from bottom: The sentence “The result showed significantly lower in LSCC tissues than those in PANL tissues” is grammatically uncorrect.

Answer:

The sentence has been revised as “The result showed that 14-3-3-epsilon mRNA expression level in LSCC tissues was significantly lower than that in clear surgical margin tissues.” in our revised manuscript, Page 11, Lines 10-12.

14. Page 12, Line 1: The sentence “The 14-3-3-epsilon protein expressed level were significantly lower in LSCC tissues than those in PANL tissues” is grammatically uncorrect.

Answer:

The sentence has been revised as “The 14-3-3-epsilon protein expression level in LSCC tissues was significantly lower than that in clear surgical margin tissues” in our revised manuscript, Page 11, Lines 6-8 from bottom.

15. Page 12, Line 6: what is the meaning of “analyse”?

Answer:

The “analyse” is a spelling mistake, and in our revised manuscript, Page 11, paragraph 3, the whole paragraph has been re-edited as “We assessed the 14-3-3-epsilon gene expression levels with respect to the clinical characteristics (age, sex and the clinical stages). No differences were identified in protein levels
of 14-3-3epsilon with respect to patients ages and sex (data not shown). There was no difference in mRNA levels with respect to patients ages, sex and clinical stages (Table 4). However, the protein expression level of 14-3-3epsilon in stage III or IV tumors was significantly lower than those in any stage I or II tumors (P<0.001, Table 4)."

Answer:
The word “datas” has been revised as “data” in our revised manuscript, Page12, Line 1.

17. Page 13 Line 3: “a” instead of “an”.
Answer:
The word “an” has been revised as “a”, in our revised manuscript, Page 12, Line 4 from bottom.

Answer:
In our revised manuscript, Page 14, Line 11, the word “produced” has been changed to “regulates”.

19. Page 14 Line 3 from bottom: The sentence “The abnormal expression of
14-3-3-epsilon are found in only a few kinds of cancers” is grammatically uncorrect.

Answer:
The sentence has been revised as “The abnormal expression of 14-3-3epsilon has been found in several types of cancers.” in our revised manuscript, Page 14, Lines 11-12 from bottom.

20. Page 16 Line 6: what is the meaning of “analyse”?

Answer:
The word “analyse” is a spelling mistake, and in our revised manuscript, Page 15, the third paragraph has been extensively revised as: “Some studies show that 14-3-3epsilon, an inhibitor of apoptosis protein, prevents apoptosis progression by inhibiting the activities of pro-apototic proteins such as Bad and Bax [23-26]. But in the present study, our results from both apoptosis and cell cycle assays showed that the apoptotic cells in 14-3-3epsilon-GFP group increased, which indicates 14-3-3epsilon has the ability in promoting apoptosis. We speculate that the increased apoptosis in Hep-2 cells transfected by 14-3-3epsilon can also lead to reduction of cells. Meanwhile, the down-regulation of 14-3-3epsilon gene detected in LSCC in the study plays a role in the development of LSCC probably by inhibiting apoptosis.”

Reviewer: Gino Marioni
1. The relations between 14-3-3-epsilon levels and the essential prognostic parameters (at least recurrence rate, disease-free survival, disease-specific survival) should necessarily be investigated in the considered cases of laryngeal SCC.

Answer:

All the patients who donated specimens received operation from 2008-2009 and they are all disease-free survivors at presence.

2. The potential clinical applications of the achieved results should be described in the Discussion section.

Answer:

The potential clinical application has been supplemented in our revised manuscript, Page 16, paragraph 3, as “According to the achieved results in the present study, 14-3-3-epsilon gene could be a useful parameter in diagnosis of LSCC. It could also be used as a molecular marker to decide the clinical stages. Meanwhile, 14-3-3-epsilon protein may be a potential target of a new drug, which is able to control the initiation and progression of LSCC effectively.”

By the way, some words and sentences including the title which were not clear or native in our original manuscript have been revised and marked as red color in the corresponding places of our revised manuscript.

Looking forward to your kind reply.
With my best regards

Sincerely yours

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