Author's response to reviews

Title: Metallothionein 1G functions as a tumor suppressor in thyroid cancer through modulating the PI3K/Akt signaling pathway

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Author's response to reviews: see over
Dear Dr. Cree

This is a revision requested by BMC Cancer for this manuscript (MS: 7605478869294002) entitled “Metallothionein 1G functions as a tumor suppressor in thyroid cancer through modulating the PI3K/Akt signaling pathway”. Enclosed please find the revised version of the manuscript that we are re-submitting for your consideration for publication in BMC Cancer. We carefully studied the comments of the reviewers and thoroughly addressed them as detailed in the response letter and as underlined in the revised manuscript. We want to thank the reviewers for their positive comments and high interest in this work and for their suggestions which we found to be very useful in helping us revise the manuscript. We also rearranged the author order that Dr. Hongjun Lv should be a joint first author because he did most of the work to revise this manuscript. We hope that you are satisfied with our revision and will now find the manuscript acceptable for publication in BMC Cancer.

We want to thank you and the reviewers again for all of the efforts in helping improve this manuscript.

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Response to the reviewers

To reviewer 1:

We appreciate very much this expert reviewer’s careful and critical review and very helpful comments. The critiques of this reviewer are addressed as follows:

1. The reviewer commented that Figure 1 A-B and Figure 2 A-C were repeated from a previous article and suggested to remove them to additional file.

   Answer: Thanks for this reviewer’s suggestion! We have removed Figure 1A-B and Figure 2A-C to supplemental figures in the revised manuscript (Figures S1 and S2 in additional file).

2. The reviewer suggested that ideally primary thyroid cell lines obtained from patients should be used to perform our experiments. Alternatively, each experiment should be performed on two or more of the thyroid cancer cell lines we studied.

   Answer: This is a very good suggestion! We totally agree with this reviewer’s point. Unfortunately, it is not easy for us to obtain the thyroid tissues from patients to develop primary thyroid cell lines. However, we performed our experiments in FTC133, BCPAP and C643 cell lines, other than in K1 cell line. The growth-inhibitory effect of MT1G on K1 and FTC133 cells was better than C643 cells, which was related to their genetic background (Table S1 in additional file). K1 cells harbor the homozygous PIK3CA mutations and FTC133 is a PTEN-null cell line, indicating overactivation of PI3K/Akt signalling pathway in these two cells. Thus, we performed all the experiments in FTC133 and K1 cells as required by the reviewer. All data were presented in the revised manuscript and additional file. We hope our data can justify our conclusions.

Minor essential revisions:

1. The reviewer asked whether the stably transfected K1 cells used in this study were the stable pools, or individual clones.

   Answer: We used the stable pool, not individual clones. We apologize for the ambiguous description. It has already been modified in the revised manuscript.

2. Figure 3 (Figure 2 in the revised manuscript)
   a) State which thyroid cell lines were transiently transfected?
   b) Are similar results seen in the stable transfected vector or MT1G?
c) Add statistical analysis for cell cycle.
d) In text, sentence 5 of paragraph with title “MT1G induces cell cycle arrest and apoptosis of thyroid cancer cells” change the word “nuclear” to “nuclei”

**Answer:** a) K1 and FTC133 cells were transiently transfected in this study. This has been indicated in Figure 2 in the revised manuscript; b) As to the stable transfected cells, we only performed the cell cycle analysis in K1 cells. However, we failed to find any difference between MT1G and empty vector stably transfected cells; c) Done; d) It has already been modified in the revised manuscript. We apologize for the carelessness!

3. **Figure 4**
a) The reviewer asked which cancer cell lines were stably transfected in Figure 4.

**Answer:** K1 cells stably transfected with pEGFP-N1-MT1G or empty vector were used to perform Transwell migration and invasion analysis, whereas FTC133 were transiently transfected with pEGFP-N1-MT1G or empty vector to perform wound-healing assay. By the way, we failed to perform migration and invasion analysis in FTC133 cells using Transwell chambers. Thus, we have to perform the wound-healing assay for evaluating the effect of MT1G on cell migration. We hope that the reviewer agrees with us.

4. **Figure 5**
a) State which cancer cell lines were stably transfected?
b) Add data from another thyroid cancer cell line for experiments analyzing signaling pathway changes.
c) Fig. 5A: quantification of p-p70S6K is needed.
d) Fig. 5D: quantification of p-Rb is needed.
e) The reviewer suggested us to lighten all loading controls and quantify data.

**Answer:** a) K1 and FTC133 cells were stably transfected in the revised manuscript; b) The data for FTC133 cells have been added to explore signalling pathway changes in the revised manuscript; c) The expression of p-p70S6K in K1 and FTC133 cells-transfected MT1G or empty vector was quantified and no significant difference was found. Thus, we removed the corresponding data, also as suggested by the reviewer in the following question; d) Done; e) Thanks for the reviewer’s suggestion! Although the loading controls were over-exposed, expression of most of investigated proteins, such as p-Akt, Mdm2, p53, p21, Bak, Smac, and E-Cadherin, was remarkably changed after transfection of MT1G as compared to empty vector, particularly in K1 cells. Expression of p-Rb was only slightly decreased in MT1G-transfected cells compared with vector-transfected cells. Thus, we just quantified the levels of p-Rb using densitometry (Fig. 5 in the revised manuscript). We hope that the reviewer agrees with us.
5. Figure 6
a) Either a quantifiable western blot is needed, or remove the involvement of mTOR and pS6K in the model.

Answer: Thanks for the reviewer’s suggestion! We have already removed western blot data from Fig. 5 and the involvement of mTOR and pS6K in the schematic model (Fig. 6) in the revised manuscript.

6. Language edits
a) Discussion paragraph #1, sentence 4: change "no significant" to "not significant"
b) Discussion paragraph #3, sentence 1: change "evidences have" to "evidence has"
c) Discussion paragraph #4, sentence 9: change "evidences" to "evidence"

Answer: They have been modified in the revised manuscript. We apologize for the carelessness!

To reviewer 2:

We want to thank this reviewer for the positive comments on our work. The critiques of this reviewer are addressed as follows:

Discretionary Revisions:

1. The reviewer commented that the number of non-neoplastic lesions included in this study was small compared to the neoplastic cases.

Answer: We agree with the reviewer’s point! However, it is not easy for us to obtain the non-neoplastic tissues as compared to the neoplastic tissues. Although the number of normal tissues is small, we think that it should be fine for the statistic analysis. We hope that reviewer agrees with us.

To reviewer 3:

We appreciate very much this expert reviewer’s careful and critical review and very helpful comments. The critiques of this reviewer are addressed as follows:

1. The reviewer suggested that the origins and genetic characteristics of the thyroid cancer cell lines should be provided.
Answer: Thanks for this reviewer’s suggestion! We summarized the origins and genetic characteristics of thyroid cancer cell lines in Table S1 (additional file). According to the reviewer’s suggestion, we looked further into their phenotype-genotype relationships. K1 cells harbor the homozygous mutation in PIK3CA and FTC133 is a PTEN-null cell line, strongly indicating overactivation of PI3K/Akt signalling pathway in these two cells, thus growth-inhibitory effects of MT1G were more significant in these two cell lines as compared to other two cell lines, such as BCPAP and C643. We hope that the reviewer agrees with us.

2. The reviewer suggested that information regarding the transfection of other cell lines than K1 and FTC133 with MT1G and empty vector should be provided.

Answer: Thanks for reviewer’s suggestion! In this study, K1, FTC133, BCPAP and C643 cell lines were transfected with MT1G or empty vector. We chose these four cell lines because basal level of MT1G expression was relatively low. Importantly, MT1G expression could be dramatically induced by 5-Aza-dC and SAHA. Cell proliferation was analysed in all four cell lines, and the data were presented in Fig. S2B (see Additional file). As I mentioned in the above answer, we found that the growth-inhibitory effects of BCPAP and C643 was not as significant as that of FTC133 and K1. Thus, other experiments were only performed in K1 and FTC133 cells. We hope that the reviewer agrees with us.

Minor essential revisions:

3. The reviewer suggested that the terms of the current WHO classification for the list of follicular epithelial cell-derived thyroid tumors should be used.

Answer: Thanks for reviewer’s suggestion! We have already revised the classification of follicular epithelial cell-derived thyroid tumors in the revised manuscript.

4. The reviewer suggested that results of normal thyrocyte cell line HTori3 should be shown for comparison in Figure 1C lower part and D.

Answer: Thank you this reviewer’s suggestion! The data of HTori3 have already been added in the revised manuscript.

Discretionary Revisions:

1. The reviewer commented that no results of MT1G mRNA expression in other
carcinomas than PTC were given in the text or Figure 1A.

**Answer:** The number of other thyroid carcinomas, such as FTC (n =16), MTC (n =9) and ATC (n =9), was too small compared with PTC (n =178). We think the statistical data from these samples would not be reliable. Thus, we only focus on PTC samples in this study. We hope that the reviewer agrees with us.