Author's response to reviews

Title: LATS2 is De-methylated and Overexpressed in Nasopharyngeal Carcinoma and Predicts Poor Prognosis of the Patients

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Author's response to reviews: see over
Dear Editor in Chief:

We are appreciate you for your kindly help for arrangement of reviewing this manuscript. The revision of this manuscript has been edited by a native English speaker from England recommended by the agency of International Science Editing. The primary version of this manuscript with track change labeled was sent to you by email independently to conceive you.

We also would like to thank all reviewers for their timely review for our manuscript. We are pleased to know that our study is of general interest for the readers of BMC Cancer. We have carefully evaluated the reviewers’ critical comments and thoughtful suggestions, responded to their suggestions point-by-point, and revised the manuscript accordingly. All changes made to the text are in red so that they may be easily identified. With regard to the reviewers’ comments and suggestions, we wish to reply as follows:

Answer to Reviewer (Hiroshi Nojima)

1) In breast cancer and leukemia, it was reported that not only Lats2 but also Lats1 was down regulated in tumor cells but not in paired normal cells due to hyper-methylation of LATS2 and LATS1 promoter regions. This is at least partly because Lats2 and Lats1 cooperatively downregulate the cell growth (see Hippo pathway). In this manuscript, the authors claim the reverse phenomena that Lats2 is overexpressed in tumor cells and LATS2 promoter region is demethylated. If true, this finding is potentially very important. Then, the authors should ask to what extent Lats2 and Lats1 cooperate in NPC cells. For example, the authors should examine if Lats1 expressions are also upregulated and/or LATS1 promoter region is also demethylated in NPC cells and tissues.

LATS1 and LATS2 are members of the warts/lats subfamily. LATS2 is most homologous to but distinct from LATS1. LATS2 is expressed in many human tissues, as is LATS1, but it appears to have a preference for certain organs. For example, LATS2 is scarcely expressed in brain, liver, and kidney, while LATS1 is expressed at considerable levels in these organs (Tao et al., 1998). As to their biological roles, one possibility is that LATS2 and LATS1 are involved in the same cellular events but in a different or reverse fashion, as has been reported for the members of the bcl-2 family. Another and more likely possibility is that they are mutually complementary and either of them can play a predominant role depending on the type of tissue (Hori et al., 2000). We thank the reviewer for this useful suggestion, and we hope to be able to
detect LATS1 expression in NPC cells and tissues in future studies.


2) In terms of Lats2 function in cancer cells, the authors should not ignore the contribution of the Hippo pathway to the regulation of tumor growth. It is important to examine, for example, the phosphorylation levels of the Lats2 target protein such as YAP, using Phospho-YAP (Ser127) antibody, which is one of the key players of the Hippo pathway.

We thank the reviewer for this insightful comment. Our data suggested that LATS2 stimulated cell growth in NPC cells, but the precise mechanism for this stimulation was not clear. LATS2 may have regulated cell growth by dysregulation of the Hippo pathway or another pathway. This is an interesting point and deserves further study.

3) The authors should also examine if Lats2-Mdm2-p53 pathway is activated or inactivated (see Aylon, et al, Gene Dev. 2006). Western blot analysis (WB) of p53 and related proteins may provide valuable information.

The 5-8F cell line is a subtype of SUNE1. In SUNE1, a mutation has been detected in the exon 8 of the p53 gene (Chen J, et al., 1994). A heterozygous mutation of G → C at codon 280 of p53 was also detected in the CNE2 cell line (Sun Y, et al., 1992). The mutant p53 gene product has been postulated to override wild-type function by a dominant negative mechanism, as indicated by direct inactivation of the wild-type protein by mutant p53 protein through oligomer formation, or by conformational adoption of the mutant form [2]. We therefore did not examine the LATS2-MDM2-p53 pathway.


4) Fig.1: the conclusion of this paper largely depends on the results presented in Fig.1C. However, no data are presented that confirm the authenticity of the immuno stained signals as Lats2 itself. The authors should perform peptide competition in immunohistochemistry and siRNA mediated LATS2 knockdown in the cell lines and confirm the disappearance of these signals in order to show that these antibody signals are not derived from background or nonspecific recognition of other proteins.
This query is posed because Lats2 localizes in the nucleus only during M phase, but these cells are mainly at the interphase. It seems to me that these immunostained images are not from Lats2 proteins.

To ensure the specificity of the primary antibodies, consecutive sections were incubated either in LATS2 primary antibody or with a non-immunized goat IgG antibody. No immunostaining was detected with the control IgG antibody (Supporting Fig.1). Transfection with LATS2 siRNA1 caused a dose dependent decrease in LATS2 protein levels 72 h post-transfection (Fig. 4C). These results show that the antibody signals are not derived from background or nonspecific recognition of other proteins.

Mitotic figures are rare in NPC, which makes it difficult to determine when cells are at M phase or at the interphase. Localization of LATS2 in the nucleus was also shown in human prostate tissue (Powzaniuk M, et al., 2004).


5) Fig3: the authors should perform RT-PCR and/or WB using the same sample as was used in Fig.3, to show the direct relationship between transcription and methylation (Fig.1 Aversus Fig.3).

We have detected the methylation status of LATS2 in NPC cell lines (Fig.3 in the revised manuscript). LATS2 mRNA and protein expression were detected by RT-PCR and WB (Fig.1.)

6) Fig5: the authors should examine if phenotypes of LATS2-knockdown (S phase arrest and apoptosis) can be rescued by overexpression of siRNA resistant Lats2-refractory construct.

We thank the reviewer for this suggestion, but we do not consider it possible to complete the suggested experiment in one month.

7) Fig5A: the authors should examine if overexpression of exogenous Lats2 in NP69 cells (with low expression level of Lats2) increase its growth rate in comparison with vector alone expression.

As shown in Fig.6 in the revised manuscript, the overexpression of LATS2 in NP69 stimulates cell growth.

8) Fig5A: successful knockdown of siRNA1 should be confirmed by Western analysis for every sample used in Fig.5A and the results should be presented below the line graph.

As shown in Fig.5A in the revised manuscript, 5-8F and CNE2 cell lines were transfected with LATS2 siRNA1 (75 nM) or control siRNA (75 nM). Cell lysates were generated at 72 h post-transfection, followed by immoblot analysis to determine LATS2 expression. GAPDH was used as the loading control.
Fig5B: apoptosis should be examined and confirmed with other methods. The presented data alone cannot be concluded that the cell deaths are due to apoptosis (Fig.5B). Flow cytometry has been used previously for the detection of apoptosis (Wang YY and Guo X). Cells negatively stained by both PI and annexin V are considered as live cells; annexin V positive staining is considered as indicative of the early apoptotic cells, and both PI and annexin V positive staining indicates cells that are primarily in the late stages of apoptosis.


Fig5C: S-phase cell cycle arrest should be examined and confirmed with other methods. FACS data alone cannot be concluded that the cell deaths are due to S-phase arrest (Fig.5C). It is suggested to perform Western analysis using Cdk and S phase related proteins.

We thank the reviewer for this suggestion; we have rewritten the sentence as follows: LATS2 silencing induces S-phase cell cycle increase in 5-8F and CNE2 cells.

Minor comments:
1) The references in the background section should be updated. At least, more detailed comments on the Hippo pathway are required in the Introduction and Discussion sections.
   We have introduced the Hippo pathway in the Introduction of the revised manuscript.

2) Size marker should be presented for Lats2 WB of Fig.1B, Fig.4A and Fig.4C. In Fig1B, an bans at lane for C666 is truncated.
   Size markers have been included in the LATS2 WB of Fig.1B, Fig.4A, Fig.4C and Fig.5A in the revised manuscript. And Fig.1B was also revised according to the original Figure.

   We changed “siNRA1” to “siRNA1” in the revised manuscript.

4) Font size should be increased in the X- and Y-axis of Fig.5B.
   The correction is shown in Fig.5C in the revised manuscript.

5) “DNA content” and “Cell number” are missing in the X-axis and Y-axis of the 5-8F siRNA1 panel, respectively (Fig.5C).
   The correction is shown in Fig.5D in the revised manuscript.
11) English is not good. The authors should ask a native speaker (scientist) to edit English of the whole text
The manuscript has been edited by a native English speaker.

Answer to Reviewer (Miho Ohsugi):
Minor Essential Revisions
1) Authors should spell out the abbreviation on its first occurrence but NPC is used without spelling out in “Abstract” section.
We changed “NPC” into “nasopharyngeal carcinoma” in the “Abstract” section.

2) Figure1D; Authors declare that nospecific LATS2 staining was observed in paired normal nasopharyngeal epithelial cells (page 12 and Figure1D legend). However, there are small but significant numbers of signal-positive cells in the section shown in Figure1D. Authors should rewrite the sentences or describe the evidence that these signals are non-specific.
We rewrote the sentences as “LATS2 was expressed weakly in normal nasopharyngeal epithelium.”

3) Page 13; Figures should be referred to in the text in consecutively order.
Fig.2C, Fig.2D, Fig.2E and Fig.2F were referred to in the text in consecutive order.

4) Pages 14 and 18; Although data in Figure 3 strongly suggests the increased de-methylation frequency of LATS2 promoter region in NPC tumor tissues, the result of methylation-specific PCR only is insufficient evidence to conclude that LATS2 is overexpressed due to de-methylation of the promoter region.
We thank the reviewer for this suggestion. The sentence “the overexpression of LATS2 may be associated with de-methylation of its promoter” was deleted from the conclusion.

5) Figure5; Concentration of transfected siRNA in each experiment should be described.
The concentration of transfected siRNA in each experiment was described in the Results and in the Figure legend sections.

6) Authors used scrambled siRNA named NC as a negative control in siRNA transfection experiments. I suggest authors to use an easily comprehensible term such as scrambled siRNA or control siRNA instead of NC in the text in the result section and figure legends.
We changed “siRNA1” and “NC” into “LATS2 siRNA1” and “Control siRNA” in the text in the results section and in the figure legends.