Author's response to reviews

Title: Clinical and Biological Significance of STAT1 in Esophageal Squamous Cell Carcinoma

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Author's response to reviews: see over
Author's covering letter for initial submission

Title: Clinical and Biological Significance of STAT1 in Esophageal Squamous Cell Carcinoma

Authors:

Version: 1 Date: 14 July 2014

Comments: see over
Dear editors:

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Title: Clinical and Biological Significance of STAT1 in Esophageal Squamous Cell Carcinoma

We would like to thank the reviewers for their time and comments. In response to their suggestions and questions, we have made the following modifications to the manuscript and responses, detailed as follows:

Reviewer 1 comment:

1. The authors should be more careful when they state that “By subcellular fractionation, we also found that STAT1C transfection induced a dramatic decrease in the nuclear localization of NF-κB p65 or phospho-NF-κB p65 in both cell lines”. The blots presented in Figure 6b don’t show such a dramatic reduction with the exception of NF-κB p65 in the nucleus of EC109.

Response: We agree that our initial illustrations did not fully capture the biochemical changes we observed. We have taken new photos from the representative results and included them in Figure 6b. Densitometry data shows that the reduction in the nuclear p65 was around 80% after STAT1C expression.

2. In Figure 7a, the authors show a reduction in both STAT3 and phospho-STAT3 levels after STAT1C transfection in EC109 and EC1 cells. However, in the text they state that “Western blot studies showed that there was no appreciable change in the total expression level of STAT3”, probably referring to Figure 7c. This means that the results were not reproducible?

Response: Thank you for pointing out our mistake. The reduction of STAT3 upon STAT1C transfection is consistent. We have changed the manuscript and make it more clear (page 15, highlighted).

- Minor Essential Revisions

1. Table 2 should be formatted.

Response: Modified as suggested

2. The authors should revise the sentence “The expression of the phosphorylated/activated form of STAT1 (p-STAT1) in these cell lines was also
assessed in these 8 cell lines.” (lines 207-209).

**Response: Modified as suggested**

3. The authors should revise the sentence “One of the most findings of this study is that the absence of STAT1 expression in ESCC significantly correlates with a worse clinical outcome.”

**Response: Modified as suggested**

4. The authors should revise the sentence: “In parallel to our findings, we have identified two in-vitro studies in the literature that have may have implicated a role of STAT1 in the biology of ESCC [9, 33].”

**Response: Modified as suggested**

5. The authors should revise the sentence: “W attempted to delineate the mechanisms by which STAT1 induces apoptosis in ESCC.”

**Response: Modified as suggested**

- Discretionary Revisions

1. One of the STAT1-negative cell lines is a human immortalized esophageal epithelial cell line. How the authors see this finding?

**Response: This is an interesting observation, but we do not have a good explanation to account for this unique feature of SHEE cells.**

2. The authors could include, together with the blots, the quantification of the bands.

**Response: Modified as suggested**

3. Cell cycle analysis was not performed after STAT1 silencing? The authors should bring this information.

**Response: Data added as suggested (Figure 5f)**

4. In the in vitro model, it would be interesting to directly evaluate the apoptosis level in order to check whether the reduction/increase in cell number in STAT1C-transfected/siRNA-transfected cells was a direct consequence of alterations in cell proliferation or apoptosis.

**Response: Based on our overall data (Figure 4 and 5), the change in the subG1 fraction in response to STAT1C transfection or siRNA STAT1 was**
more dramatic and statistically significant than the change in the S phase. Thus, we speculate that regulation of apoptosis is likely to be more important than alterations in cell proliferation. This has been added to the DISCUSSION.

5. Since the authors found a correlation of STAT1 expression with depth of tumor invasion, wouldn’t it be interesting to check cell invasion after STAT1 overexpression and silencing in the in vitro model?

**Response:** STAT1C transfection significantly decreased cell invasion (Figure 3d).

Reviewer 2 comment:

1, Authors should provide the immunohistochemistry work on the matched normal esophageal epithelial tissue, which is of important in assessing the significance of STAT1 expression in esophageal carcinogenesis.

**Response:** As suggested, we have now included an illustration of the normal epithelia from the same case where the tumor cells were STAT1-weak (figure 1e and 1f).

2, Authors should describe in detail if they use the same antibody for immunohistochemistry and Western blot. Again, in Table 1, it should be addressed for relationship of the negative immunostaining cases and Western blot results.

**Response:** We have described the antibody in Methods and Materials. Table 1 has been modified as suggested. Of note, we randomly selected 57 cases (from the original 131 cases studied by Western blots) for IHC studies. It just so happened that none of the 57 cases were scored STAT1 negative based on the Western blot results.

3, It seem that the positive immunostaining rate is very high in this study (123 of 131, 93.8%), authors should explain the possible mechanism, and again, if only 8 of 131 cases with negative for immunostaining, is it logical to conclude “patients with STAT1-strong/weak tumors had a significantly longer survival compared to those with STAT1-negative tumors”?

**Response:** The STAT1 immunostaining rate is high only if we include STAT1-weak tumors in the calculation. In our cohort, 51% of the tumors are STAT1-weak or STAT1-negative. Based on in-vitro data, a low level of STAT1 is biologically significant. Regarding our conclusion the reviewer is referring, this is indeed an observation what we found (i.e. with
statistical significance), regardless of the small size of the STAT1-negative tumor group.

Once again, thank you very much for your comments and suggestions.

Sincerely Yours,

Ying Zhang