Author's response to reviews

Title: Deregulation of microRNAs Let-7a and miR-21 mediate aberrant STAT3 signaling during Human Papillomavirus-induced Cervical Carcinogenesis: Role of E6 Oncoprotein

Authors:

Gauri Shishodia (gaurishishodia@gmail.com)
Gaurav Verma (gverma.gaurav@gmail.com)
Yogesh Srivastava (yansh111@gmail.com)
Bhudev C. Das (bcdas48@hotmail.com)
Alok C, Bharti (bhartiac@icmr.org.in)

Version: 4
Date: 21 October 2014

Author's response to reviews: see over
Deregulation of microRNAs Let-7a and miR-21 mediate aberrant STAT3 signaling during Human Papillomavirus-induced Cervical Carcinogenesis: Role of E6 Oncoprotein. (Shishodia et al.)

Point-wise Response to the reviewers’ comments

Reviewer 1 (File no. 1713873601142306_comment)

Major comments:
Q1. Authors showed accumulation of PTEN after silencing of miR-21 in Figure 3 and claim that: “It is likely that STAT3-induced miR-21 forms an important part of positive feedback loop in cervical cancer cells that keeps various apoptosis-inducing death regulators including PTEN, under control and miR-21 inhibition alleviates PTEN suppression leading to abrogated STAT3 signaling.” This claim should be justified if they could demonstrate the status of STAT3 under this same experimental scenario. This experiment need to be included.

Response: We are thankful to the reviewer for the suggestion. The level of STAT3 in the stated experimental condition is now included in Figure 3E.

Q2. Authors demonstrated that silencing of HPV16 E6 results in a specific up-regulation of Let-7a and abrogation of miR-21 level (Figure 5). The author should demonstrate the status of STAT3 and PTEN under this condition?

Response: The level of STAT3 and PTEN in the stated experimental condition is now included in Figure 5E.

Q3. Fold change expression generally calculated as ratio against the loading control. From Figure 3D, it is clear that miR-21 inhibitor treatment resulted in PTEN accumulation at 10 nM of dose but a further increase in inhibitor dose did not enhance PTEN level. But in Figure 3E, the bar graph showed dose-dependent increase in PTEN expression which is not justified with Figure 3D. The author should recalculate the fold change.

Response: We appreciate reviewer's concern. As indicated in the revised manuscript, the panel shown in Figure 3D is a representative of 3 independent experimental sets whereas Figure 3E depicts cumulative data of thethree experiments, and hence these panels are not exactly the same. Nevertheless, we re-examined the fold changes calculations but did not find any difference.

Minor points
Q1. The authors should use either h or hr throughout MS.
Response: Needful has been done, ‘hr’ has been replaced with ‘h’ throughout the manuscript.

Q2. The pSTAT3 (Y705) of Figure 2H is not displayed. Therefore, this figure cannot be assessed by the reviewer. Need clear figure.

Response: Needful has been done.
Reviewer 2 (File no. 3244759901426941_comment)

Main comments:

1. Introduction is too long and unfocused.
   **Response:** As suggested, we have edited the text to make it more focused.

2. How specific are curcumin and stattic as STAT3 inhibitors?
   **Response:** Present investigation utilized 2 different inhibitors that block STAT3 activation. Among these, curcumin, a strong but non-specific inhibitor of STAT3 phosphotyrosination at Y705 that control STAT3 dimerization, nuclear translocation and subsequent DNA-binding and transactivation; has been shown to manifest its effect through blocking upstream STAT3 signaling [24, 25]. On the contrary, stattic electively inhibits the function of the STAT3 SH2 domain regardless of the STAT3 activation state in vitro and selectively inhibits activation, dimerization, and nuclear translocation of STAT3 [26].

   This information has been appropriately added in the manuscript on page no. 10 line no. 2-8.

3. Is there any evidence that miR-21 and Let-7a interact with STAT3?
   **Response:** Till date there is no report that indicates that miR-21 or Let-7a directly interact with STAT3. However, an upstream enhancer containing two highly conserved STAT3 binding sites control the gene encoding miR-21 (Loeffler 2007). Similarly, STAT3 3'UTR possess a strong putative Let-7a binding site (Wang 2010).

   This information/lacunais highlighted in the Introduction section of the revised manuscript.

4. Is it possible that decrease in STAT3 expression and miR-21 (Fig. 1C & 1D) due to loss of cell viability (Fig.1B). This needs to be ruled out. And; 5. Same logic as #4 may also apply to curcumin.

   **Response:** Upon completion of the indicated time period, cultures were terminated by washing the cells with ice cold PBS, prior to isolation of the RNA and proteins. Since SiHa cells are adherent in nature and dying cells get rounded and loosely attached, the majority of left over cells that were isolated constituted only the live cells. Further, the assays were controlled with beta-actin and U6 RNA in each run to avoid bias due to dead cells.

   This information is now included in the revised manuscript in Methodology section, page no. 7.

6. Figure 1A, 2A, 3A are redundant with panel B in the all figures shown.
   **Response:** As suggested by the reviewer, redundant panel in Figure 1, 2, and 3 have been deleted in the revised manuscript.

7. Authors presented no evidence that they are dealing with active STAT3 as stated in discussion.
   **Response:** As claimed by the manufacturer, and later established by us using STAT3-specific electrophoretic mobility shift assay (Shukla et al 2010), the anti-STAT3 used in the present study specifically detects the active form of STAT3.

   This information is now included in the revised manuscript in Methodology section on page no. 6.

8. Discussion is too long and unfocused.
   **Response:** As suggested, we have edited the text to make it more focused.