Author’s response to reviews

Title: EGFR modulates complement activation in head and neck squamous cell carcinoma

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Author’s response to reviews:

Editor Comments:

We would like to thank the editor for his feedback and for considering our article for publication, below is our response to the editor’s comments.

”Please address the following editorial concerns

1) Did you measure the expression of EGFR at the protein in all cells lines. Although RNA did not change, is it expected given the different sensitivities of the cell lines.”

• Yes, we measured the expression of EGFR on the protein level using an immunoassay that we are currently working to publish and found no significant difference on the protein level.

• In a study that examines response to Iresssa in non-small cell lung cancer, significant correlation between EGFR gene copy number and gefitinib sensitivity was found. EGFR protein was necessary but not sufficient for predicting sensitivity (1). It is worth noting that EGFR status do not always correlate with treatment success in general, several mutations in the EGFR signaling pathways (Raf, Mek, Erk) can be responsible for resistance (2, 3).

“2) Can you use the TCGA to examine EGFR expression in other cancers.”

This is a very good point. However, TP53 status but not EGFR data are available from those cell lines on the International Agency for Research on Cancer (IARC). Further data on the cell lines can be found on IARC with the following links,
http://p53.iarc.fr/CellLines.aspx Searching for (LU-HNxSCC4, LU-HNxSCC5, LU-HNxSCC7, LU-HNxSCC8)

Or at

https://scicrunch.org/resources/Cell%20Lines/source/SCR_013869-1/page/5/search?q=*&l=&facet%5B%5D=Disease:Head%20and%20neck%20squamous%20cell%20carcinoma&sort=asc&column=Disease&sort=asc

“3) Line 101 "but was in some instances beneficial for the cancer cells." Please be more specific.”
• This was changed to (Background, page 5, line 100-101):
  ” Complement activation did not cause cell lysis and rather increased ERK phosphorylation for one of the cell lines tested.”

“4) Does the understanding of the complement system add value to EGFR as a diagnostic tool.”
• Further studies can investigate the feasibility of using complement activation fragments in the cancer microenvironment like the terminal complement complex (TCC), to examine the response to EGFR inhibition before and after treatment.

Response to reviewers

We would like to thank the reviewers for the very constructive and useful feedback to our manuscript

Specific points:

Anantha Lakshmi Marisetty (Reviewer 1):
• “The authors have put in great efforts to finding the relation between EGFR signaling and modulation of immune response specifically the complement system. The experimental plan and designs are very simple and clear with appropriate controls, cell lines and controls used.”

• “I have a question for the authors, the authors claim that growth is inhibited but the viability is not affected on the cells, did the authors perform a cell cycle analysis to see what stage of the cell cycle the cells were arrested?”

Response:
When we did cell counts for the Iressa sensitivity assays, the device that we used (LUNA™ Automated Cell Counter (Logo Biosystems)) provides viability - along with cell numbers - using trypan blue staining (Materials and methods, page 8, line 139-146), the viability did not fall below 90% for controls and Iressa treated cells (Results, page 17, Line 297-300). We did not perform a cell cycle analysis, but EGFR inhibitors and specifically Iressa usually work by delaying cell cycle progression with arrest in the G1 phase (1, 4).

• “Did the cells look happy when the growth is inhibited or did they look sick?”

Response:

We examined cell morphology using light microscopy at every step following any manipulation of those cell lines. We can confirm that treatment with EGFR inhibitors did not induce cellular changes that we could observe. But we did notice a change in morphology following confluence of a monolayer and changing the DMEM medium to KGM serum free medium in both EGFR inhibitor-treated and non-treated cells. We added information on the morphology of the cells in the manuscript (Results, page 17, Line 300-302) and added a figure (supplementary figure3) to the supplementary material.

• “Could they passages for a longer time or due to growth inhibition cannot be passaged or stopped growing?”

Response:

Although growth was slowed down with Iressa, it did not stop even with the highest concentration of Iressa used (10µM). By comparing cell numbers at seeding and after 48 hours of Iressa, we observe an increase in cell number in all cell lines tested, this increase was larger in resistant cell lines in comparison to sensitive ones, and of course growth in non Iressa treated cells was the highest.

Cells could be passaged after EGFR inhibitors, this was demonstrated when making Cetuximab resistant cell lines (HN4-cet and HN5-cet, line 126), which were serially passaged in increasing Cetuximab concentrations. The cells we use for the 48h Iressa experiments are used only for the experiment and are discarded.

Rahul Jangid, Ph.D (Reviewer 2):

• “Presented study by Anas H. A. Abu-Humaidan et al., aimed to study the role of EGFR in modulating the complement activation in head and neck squamous cell carcinoma. Authors show that human HNSCC cell lines activate the complement system when incubated with human serum and this same activation was increased in the cell lines sensitive to EGFR inhibition. They used pharmacology drug Iressa and validated their results. They also used sensitive cell lines which made resistance to EGFR inhibitors and
showed that they displayed complement activation and a decrease in complement regulatory proteins even in absence of EGFR inhibitors.

Experiments were designed and this study show some novel things regarding EGFR and complement activation the cell head and neck squamous cell carcinoma. Authors have analyzed and interpreted their results well in light of known literature. They have also discussed their results with the recent finding and also pointed out the future needs for this field. I was delighted to read the manuscript”

References:


