Reviewer’s report

Title: Displayed correlation between gene expression profiles and submicroscopic alterations in response to cetuximab, gefitinib and EGF in human colon cancer cell lines

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Reviewer: Ashwin Dihal

Reviewer’s report:

The manuscript entitled “Displayed correlation between gene expression profiles and submicroscopic alterations in response to cetuximab, gefitinib and EGF in human colon cancer cell lines” by Solmi et al. describes the effect of the EGFR inhibitors cetuximab and gefitinib, and of EGF as an EGFR ligand in the colon cancer cell lines HT-29 and Caco-2.

The authors have described a number of changes upon treatment, including morphology of microvilli, cellular EGFR-localization, but a lack of an effect on the cell cycle.

The study is well-performed and a major plus is the fact that a wide field of techniques is applied, ranging from microarrays to scanning electron microscopy. However, there are some aspects that need to be considered by the authors, as described below.

Major Compulsory Revisions:

1. The EGFR expression data as found by immunohistochemistry are not found among the significantly affected genes found by micro arrays. This raises the main question whether the data analysis is performed with proper criteria.

2. The observations with respect to cellular changes are descriptive, e.g. “a small number of microvilli”. As the authors mention an effect on cell differentiation, a better objective method to quantify cellular differentiation is measurement of the alkaline phosphatase activity, that is expressed exclusively in the microvilli, and a generally accepted differentiation marker for HT-29 and Caco-2.

Minor Essential Revisions:

1. Gefitinib is not specific for EGFR, in contrast to cetuximab. Briefly discuss this difference in specificity in relation with the data.

2. Describe the confluency grade of the cell lines at the moment of exposure. This is of importance as post-confluent Caco-2 cells start to differentiate and develop microvilli in contrast to HT-29 cells.

3. Section “scanning electron microscopy”: focus the results on the most important observations relevant to the main goal and outcome of this research,
instead of describing (almost) all figures.

4. Pathway analysis: in the discussion on page 17 the authors state "Several involved pathways refer to these cellular element, EGF treatment pathways:â##â##â##â##â##. In the results section however, not a single pathway is highlighted/explained, since on page 13 the authors only refer to table 4. In the results section, make a link between the pathway analysis and these list of genes on page 17 (that would be more appropriate in the results section) to better understand the microarray results.

5. In the conclusion the authors link their results with Enteropathogenic E. Coli and H. pylori. As the main goal of this study was to analyze the effect of EGFR-directed therapeutics in colon cancer, this is an incorrect conclusion. This can only be mentioned in the discussion.

6. Page 4: mention a reference for the low and high expression levels for the 2 colon cancer cell lines.

7. Avoid descriptions as "an effect on differentiationâ##", for example (but not only) on page 5. Clearly mention whether this effect is a stimulation of inhibition.

8. Change the description "â##â##EGF 10nMâ##" into 10 nM EGF. The same applies for all the other conditions.

9. Page 5, last sentence above the Methods section: "â##â## profiling with respect to EGF and gefitinibâ##". Clarify if this is EGF alone and gefitinib alone, or the combination of EGF and gefitinib.

10. Section "â##cell linesâ##": as it is described now it seems that there are 2 variants of the culture medium: one containing penicillin and 10% FCS and the other streptomycin with 20% FCS. Is this an error? Culture medium is frequently enriched with a combination of penicillin and streptomycin. Please check whether in both cases it is U/mL or U/mL for penicillin and ug/uL for streptomycin.

11. Section "â##Cell-cycle analysisâ##": details regarding cell culture are already described in the section "â##Cell-viability assayâ##". Therefore, in the section "â##cell-cycle analysisâ## you can refer to the "â##Cell-viability assayâ## section for culturing conditions. The same (partially) holds for the sections, including "â##Scanning electron microscopyâ##"

12. Cell viability:

a. in the discussion (p.17) the authors describe: "â##and this could also suggest an apoptotic effect as confirmed by the pathway displayedâ##. As no pathway is described in the results section, it is unclear what pathway is mentioned here. Please explain this pathway in the results section and also mention its name in the discussion.

b. The low EGFR expression the Caco-2 cell line is associated with a response to different treatments. A somewhat mechanistic explanation/speculation based on previous articles - in addition to the authorâ##'s hypothesis regarding an apoptotic effect - would be more appropriate. Can this difference also be caused by the different genders these tumors were derived from?

13. Figure 1:
a. Instead of describing the significances in the legend, visualize these data in the graph by incorporating *, ** and *** above the bars for P<0.05, P<0.01 and P<0.001. Describe only once that comparisons are made relative to the NT group.

b. When comparing the untreated HT-29 cells with Gb+E, the latter shows a limited SD, suggesting that there would be a statistical significance. Please double check whether this is the case.

14. The figure numbers are described somewhat unusual, e.g. Fig. 3A, a. Do the authors mean Fig. 3a?

15. Table 2: untreated HT-29 have an intermediate staining in both the cytoplasm and the membrane. In the case of E, Cx5+E, Cx10+E, Gb+E treatment, the membrane staining was negative, which is a reduction relative to the control situation, whereas the cytoplasmic staining stayed intermediate, equal to the control cells. Does this mean that there is no internalization of EGFR (from the membrane to the cytoplasm), but only degradation occurs in the membrane? An explanation would be helpful.

Discretionary Revision:
As technical aspects related to miroarrays might have caused the discrepancy between EGFR expression in the array data set and EGFR expression found by immunohistochemistry, the authors may want to consider to sustain their EGFR immuno data by looking for EGFR downstream targets that show supporting direction of fold changes in the microarray data set.

**What next?:** Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**
I declare that I have no competing interests