Reviewer's report

Title: Beyond KRAS mutation status: influence of KRAS copy number status and microRNAs on response to cetuximab in metastatic colorectal cancer patients

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Reviewer: Gillian Smith

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This manuscript addresses a topical issue of obvious clinical relevance – the identification of additional biomarkers of cetuximab response may significantly improve our ability to select the most appropriate patients for EGFR-targeted mAb therapy.

There are, however, a number of issues which must be addressed by the authors before this manuscript can be considered acceptable for publication.

Major Compulsory Revisions
1. Although K-Ras copy number variation has not previously been studied in relation to cetuximab response, we have developed quantitative Taqman-based gene copy number assays for assessment of Ras copy number which should be referenced (Smith et al, BJC 102, 693-703, 2010).
2. No data is shown to support the identification of chromosomal gains or losses – this must be provided.
3. Similarly, no information is provided about specificity of K-Ras gene amplification e.g. using gene specific assays, FISH etc. How do the authors know that their amplicon definitely contains the K-Ras gene?
4. The manuscript would be considerably strengthened by the inclusion of phenotypic data to demonstrated increased/decreased K-Ras expression and activity e.g. by Western blotting for K-Ras, B-Raf binding assays, assessment of phospho-ERK levels etc.
5. The authors appear not to have considered the possible consequences of different Ras mutations or different mutation burdens in their analysis, both of which could significantly influence therapeutic response.
6. Dukes’ stage or TNM staging information must be provided for all patients and included as co-variates in the analyses presented. This is particularly important as there is a significant difference in differentiation stage between the good and poor response groups, suggesting that outcome may be directly related to tumour pathology.
7. miRNA selection: It is of concern that there appears to be wide variability in the miRNAs identified using the different software programmes and of particular concern that these programmes failed to identify key miRNAs consistently reported (and functionally validated) in the literature. This point has not been recognised or addressed by the authors.
8. On what basis was RNU 6B selected as an endogenous reference gene? Data must be provided showing that the RNU-6B expression increases in direct proportion to input [cDNA] and that expression is invariant between paired normal and tumour samples.

9. Results, sections 2 and 3: what is the difference between a copy number gain and an amplification? How was Ras-specific aneuploidy assessed? How much variation was seen in the magnitude of copy number gains?

10. Why was the ##Ct method chosen? This assumes a single calibrator sample – presumably the matched normal tissue sample in each case – this should be more clearly defined. How were optimal baselines and thresholds determined?

11. No error calculations are provided for the Taqman data presented – compound errors combining variation in both target gene and reference gene must be provided and are essential to the interpretation of the data presented. Ideally, all reactions should have been performed in triplicate. All correlations and associated p-values must take these error calculations into account.

12. In light of the experimental errors associated with these experiments, it is usually not possible to accurately identify changes in gene expression #2-fold. Only one of the miRNAs studied therefore showed a relatively modest change in expression (assuming acceptable assessment of experimental error).

13. Discussion, the authors suggest that miRNA inhibition of K-Ras occurs only when K-Ras expression is “high” i.e. only in the presence of mutant Ras. They present no data to support this hypothesis i.e. that K-Ras expression is influenced by mutation status.

14. Can the “biomarkers” identified here be used to predict response in a blinded series of test samples? Presumably this analysis would be possible using the clinical material available to the authors?

Minor Essential Revisions

1. Introduction 2nd paragraph: “A point mutation” is incorrect – multiple point mutations in the K-Ras oncogene have been described.

2. “Ratio’s” should be “ratios” throughout.

3. Information should be provided on the number of cases where matched normal and tumour DNA were available for copy number analysis.

4. RNA integrity results should be presented.

5. Assays for mir-18a and mir-200c are commercially available from Applied Biosystems – it is not obvious why the authors think otherwise?

6. Table 2 is uninterpretable to a reader unfamiliar with Taqman qRT-PCR analysis and should be re-drawn describing fold changes in miRNA expression. Only 3 miRNAs (105, 143 and 217) show differences in gene expression which COULD be meaningful, assuming the appropriate error calculations were performed.

7. P-values should be provided for Figure 2.
Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

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