Author's response to reviews

Title: Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes

Authors:

George Pentheroudakis (gpenther@otenet.gr)
Vassiliki Kotoula (ykotoula@auth.gr)
Wendy De Roock (wendyderoock@gmail.com)
George Kouvatseas (G.Kouvatseas@heads.gr)
Pavlos Papakostas (oncologydepart@hippocratico.gr)
Thomas Makatsoris (maktom@yahoo.com)
Demetris Papamichael (demetris.papamichael@bococ.org.cy)
Ioannis Xanthakis (efipsarouli@yahoo.gr)
Joseph Sgouros (josephsgouros@yahoo.co.uk)
Despina Televantou (telev@hotmail.com)
Georgia Kafiri (hecogoff@otenet.gr)
Athanassios Tsamandas (hecogoff@otenet.gr)
Evangelia Razis (ERazis@hygeia.gr)
Eleni Galani (eleni_galani@yahoo.gr)
Dimitrios Bafaloukos (dimmp@otenet.gr)
Ioannis Efstratiou (hecogoff@otenet.gr)
Iliada Bompolaki (hecogoff@otenet.gr)
Dimitrios Pectasides (pectasid@otenet.gr)
Nicholas Pavlidis (npavlid@uoi.gr)
Sabine Tejpar (sabine.tejpar@uzleuven.be)
George Fountzilas (fountzil@auth.gr)

Version: 2 Date: 14 November 2012

Author's response to reviews: see over
REPLIES/ACTIONS TO REVIEWER REPORT

We’d like to thank the reviewers for their constructive comments which will help us improve our manuscript. The following actions were taken (red):

Editorial requests

-Competing interests - Please include a 'Competing interests' section between the Conclusions and Authors’ contributions. If there are none to declare, please write 'The authors declare that they have no competing interests'.
  Done.

-Authors' contributions - Please include an Authors' contributions section before the Acknowledgements and Reference list.
  For the Authors' contributions we suggest the following kind of format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.
  Done.

Reviewer’s report 1

Title: Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes

Version: 1 Date: 8 October 2012

Reviewer: Jeffrey Evans

Reviewer’s report:

Pentheroudakis and co-authors have submitted a manuscript with their results of a number of biomarkers in a retrospective study of patients treated with cetuximab. The authors are to be commended for their extensive studies and for identifying a significant number of patients and samples for their studies. However, this manuscript also has a number of weaknesses.

Major Revisions

1. The authors have performed a number of statistical analyses with significance values reported. However, it would have been helpful if they had stated in their methods what was their intended primary analysis and the proposed significant difference that they wished to observe, and estimated the sample size on that basis.

We thank the reviewer for the most helpful comment.

Prior to conducting any translational exploratory analysis with a prognostic objective, as the one presented in our retrospective study, we estimate the statistical power provided by the available sample size in order to avoid embarking into a futile project.

Based on the reviewer’s comment we have updated the methods section accordingly:
Power calculation
The major aim of our research proposal is the characterization of molecular biomarkers which would help to predict survival to cetuximab therapy. The study's power justification scheme was based upon the following assumptions: a) expected follow up of 60 months, based on 56 months accrual duration with 2 years additional follow-up period b) ratio of expressed to non-expressed samples of either 1:3, 1:1 or 3:1 and c) HR of 0.4-0.5. With this background information and risk assumptions the sample of 226 patients provided power from 91% to 99% at the 5% level of significance for performing a two sided log-rank test.

2. Patients were included who received cetuximab as 1st, 2nd, or 3rd line therapy. Outcome analysis, such as PFS or overall survival, will not be robust in such a heterogeneous group. Although the authors attempt to overcome this limitation by using objective response to cetuximab as indicator of efficacy, this may be influenced by the chemotherapy backbone to what the cetuximab was added (ie oxaliplatin - containing versus irinotecan - containing regimens). These limitations should be acknowledged.

Thank you. We acknowledge these limitations in the first para of the Discussion section: « Despite homogeneous management of all patients in standardised HeCOG recommended therapies, the retrospective nature of the translational research is an inherent limitation. Moreover, in the absence of a control arm with no treatment it is impossible to speculate on the prognostic (impact on outcome irrespective of therapy) or predictive (impact on benefit from cetuximab therapy) effect of the biomarkers studied. Finally, the administration of cetuximab across several lines of therapy combined with various chemotherapeutic agents constitutes an additional layer of heterogeneity, masking possible interactions and confounders. Moreover, survival analysis will not be robust and even tumor response may be influenced by the chemotherapy backbone to which cetuximab was added. Finally, it would be ideal for all of the tissue samples to have been collected prior to cetuximab therapy, as some of the biomolecules studied may have changed by the time patients received 1st, 2nd or 3rd line therapy»

3. The samples that were used were archival sample specimens ie taken at diagnosis. It is conceivable that some of the biomarkers studies may have changed by the time that patients received 1st, 2nd or 3rd line therapy. If the authors wish to study predictive markers then ideally they would use tissue collected prior to the intervention rather that archival material. I acknowledge the difficulties in doing this in retrospective studies. Nevertheless the authors should comment on these limitations to their study.
We agree, see above.

4. The main criticism of this manuscript is that the authors wish to develop predictive markers of cetuximab efficacy, for which there is no clear unmet need. However most of the data they present is on prognostic factors, which adds very little to the published literature and is of lesser impact. Furthermore, they have performed multiple analyses (and so, inevitably, some reach
statistical significance with a P value of <0.05) including on whether the tumour is in the left or right side of the colon. Much of this is of no impact and should be removed, concentrating instead on predictive markers. There are too many subgroup analyses on prognosis and the manuscript should be more concise.

We do not agree with the statement that there is no unmet need for cetuximab biomarkers. Currently we only possess negative biomarkers (KRAS mutation rules out cetuximab therapy) but sorely lack positive ones. Our potential findings of positive biomarkers (high EREG, high AREG in KRAS wild type pts) may help identify patients most likely to benefit from Cetuximab. As stated in our Discussion, since all patients received cetuximab, it is impossible to robustly dissect prognostic from predictive, however this does not rule out potential predictive utility of our markers. Only a confirmatory analysis in an independent cohort with both cetux-treated and non cetux-treated patients could answer this query. We agree on removing data on multiple analyses of several clinicopathologic factors.

5. The discussion is too long and repetitive. In addition they add that the absence of a control arm with no treatment makes it less robust to speculate on the prognostic or predictive effect of the biomarkers studied - which is a surprising comment as it undermines their own studies!
We agree and have shortened the Discussion considerably.

On the contrary, the comment on prognostic vs predictive is an absolutely necessary acknowledgement of limitation, essential for the correct interpretation of our data by the readers. As stated in our Discussion, since all patients received cetuximab, it is impossible to robustly dissect prognostic from predictive, however this does not rule out potential predictive utility of our biomarkers. Only a confirmatory analysis in an independent cohort with both cetux-treated and non cetux-treated patients could answer this query.

Reviewer's report
Title: Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes
Version: 1 Date: 27 October 2012
Reviewer: Murali Bashyam

Reviewer's report:
Pentheroudakis G et al., have studied “Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes”, a retrospective study among 226 colorectal cancer patients, who underwent cetuximab treatment (1st to 3rd line therapy). In this study authors have evaluated the efficacy of cetuximab treatment emphasizing the prognostic significance of biomarkers of EGFR dependent CRCs. Formalin fixed tumor biopsies were analyzed for mRNA expression of EGFR and its ligands (epiregulin-EREG and amphiregulin-AREG),transforming growth factor – alpha (TGFA) by real-time analysis and mutations were screened for KRAS, NRAS, BRAF, and PIK3CA. Authors observed BRAF and codon 12 KRAS mutations are associated with non-responsive CRCs to cetuximab treatment. It is observed that AREG and
EREG are having prognostic significance in cetuximab treated patients. However, AREG retains significance only in KRAS wt patients but EREG levels are shown to be significantly correlated both in KRAS wt and mutants. This study has revealed the possible prognostic significance of AREG/EREG mRNA expression levels in KRAS wild type and mutant patients of cetuximab treatment.

Major Compulsory Revisions:
1. The present work is depicting the efficiency of AREG and EREG as prognostic markers in CRC patients treated with cetuximab. Authors have mentioned in the discussion about EREG mRNA expression as independent of KRAS mutation due to HER4 receptor. Authors have not shown the functional significance of EREG-HER4 interaction in KRAS mutants with high EREG mRNA expression levels of cetuximab treated CRC patients.

   Our work aimed at identifying potential biomarkers of cetuximab benefit in colorectal carcinomas by applying qPCR studies of mRNA expression of EGFR ligands retrospectively. It would be outwith the scope of this paper to add preclinical data from cell line studies aimed at the functional significance of the EREG/HER4 interaction. Moreover, such studies have already been done and are cited in the Discussion (refs 28,29).

2. What could be the reason for lower EREG/AREG expression in KRAS mutant vs wild type tumors, especially with respect ot status of HER4? Is it only a selection phenomenon? Authors should discuss this observation in discussion section.

   Added in the Discussion: The correlation of low EREG/AREG expression with KRAS or BRAF mutated status could be due to the constitutive activation of the RAS/RAF/MAPK pathway which makes activation of the EGFR pathway redundant biologically. Alternatively, it could be due to a negative feedback loop linking MAPK axis activation with suppression of the EGFR pathway.

3. The authors should discuss the wide literature available on similar studies and highlight the unique and important contribution of this work.

   We think we fairly represented data from published literature in the field (refs 8-14, 16-30). Being more extensive would lengthen the manuscript excessively, especially in view of requests from the other reviewer to shorten the Discussion. The contribution of our work is emphasized in the last section as well as in other parts of the Discussion: «Additionally, we present data suggesting

   a) a differential impact of non-codon 12 KRAS mutations on outcome of cetuximab-treated Greek patients.

   b) a KRAS mutation-independent predictive significance of EREG mRNA expression. AREG and EREG are not biologically identical: AREG binds EGFR only, whereas EREG binds EGFR and HER4 and leads to a prolonged state of receptor activation [29]. Compared to AREG, tumour EREG mRNA expression was a stronger predictor of cetuximab benefit in KRAS wild type cases in three more series [13, 20, 27]. Consequently, we may speculate that even in the presence of KRAS mutations, cetuximab binding to EGFR prevents high levels of EREG from activating HER1/HER4 heterodimers and thus abrogates signalling pathways distinct from RAS/MAPK.»
c) a strong predictive value of AREG mRNA expression in KRAS wild type patients. In our cohort, patients on cetuximab with KRAS wild type, AREG-low CRC fared extremely poorly. In fact, the survival of these patients (median 15 months) was as poor as the survival of patients with KRAS mutant tumours (17-22 months). If this finding is confirmed in independent series, AREG expression in KRAS wild type cases would emerge as a robust biomarker of cetuximab efficacy. These findings warrant independent validation.

Minor Essential Revisions:
1. Cetuximab and panitumumab are already in clinical trials; previous work related to cetuximab role in EGFR mediated response in CRCs may be cited in the introduction.
2. Method section should be better organized. It is not as per BMC Cancer journal format.
   The Method section was organised in Subsections as requested.

3. Authors mentioned in methods that they followed manual tumor macro dissection in tumors with less than 50% tumor cells. They may mention about the criteria of tumor macro dissection and why low cellularity tumor samples were included.
   **No low-cellularity molecular samples were examined. The presentation of macrodissection issues has been appropriately modified in the revised manuscript.**

4. Typographical errors have to be taken care. Eg: On page 9, line #7 word ‘was’ is repeated.
   Done