Title: A polymorphism of EGFR extracellular domain is associated with progression free-survival in metastatic colorectal cancer patients receiving cetuximab-based treatment

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Author's response to reviews: see over
Dear Editor in chief

Please, find enclosed a revised version of our manuscript now entitled “A polymorphism of EGFR extracellular domain is associated with progression free-survival in metastatic colorectal cancer patients receiving cetuximab-based treatment” (MS: 1860822335165339 previously “Genetic predictors of cetuximab-based treatment activity in metastatic colorectal cancer”) by Gonçalves et al. As you will see in the following pages, we have answered the essential questions raised by the reviewers and modified the manuscript as they suggested. Thus, we hope this improved version will meet approval for publication in “BMC Cancer”

Sincerely yours

Anthony Gonçalves, M.D Ph.D.
Response to Reviews

Reviewer 1: Alberto Bardelli

Major points

Point 1: “Small size of the patient cohort examined. The inclusion of additional patients will undoubtedly improve the statistical power of the study”.

We fully agree with this point and cohort enlargement for validation study is currently ongoing at our institution. This has been further emphasized in the revised manuscript, discussion section, page 17, line 1: “Importantly, EGFR genotyping on a larger cohort of cetuximab-treated patients, the accrual of which is currently ongoing, will be essential to confirm our findings.”

Point 2: “The lack of functional evidence that the R521K variant affects the binding of cetuximab to the EGFR or somehow influences the signaling activity of this receptor and its partners. Experiments on CRC cell lines (genotyped for the presence of the R521K variant) could help addressing this issue”

We also agree with this comment and have initiated functional studies including an investigation into the correlation between genotype and CRC cell cetuximab sensitivity and comparative phenotypic studies in specifically engineered cell lines expressing wild type or R521K variant EGFR. This was also added in the discussion section, page 16, line22 : However, such hypotheses remain to be proven: we have initiated specific functional studies evaluating the correlation between cetuximab sensitivity and the EGFR exon 13
genotype in various CRC cell lines and in cellular models expressing wild type EGFR or the EGFR R521K variant.

However, even though experimentally addressing these 2 points is still ongoing, we believe that it should not preclude early publication of our data. This is also the opinion of reviewer 1 in his final recommendation.

**Minor revision**

**Point 1:** “The title is very generic, as the novelty is related to the data on the R521K variant this should be reflected in the title.”

We have changed the title of the paper, which is now: “A polymorphism of EGFR extracellular domain is associated with progression free-survival in metastatic colorectal cancer patients receiving cetuximab-based treatment”

**Point 2:** “Table 3 is mainly a meta-analysis of previous work (from other groups) and would be more appropriately included in a review article. Main conclusion could be listed in the text.”

Table 3 has been deleted and the main conclusion has been listed in the revised text: discussion section, page 14, line 24: “Moreover, pooling all published studies evaluating this putative association further suggests that KRAS mutations strongly negatively affect the probability of objective response to cetuximab treatment. In these studies, the response rate in KRAS mutated tumors was 9 out of 115 (7.8%, CI95%: 3.6-14.3%) versus 81 out of 192 (42%, CI95%:35.1-49.5%) in wild type tumors (p=3.5 \(10^{-10}\)).”
Point 3: “Sample used for genetic analysis were all from primary tumors of the liver.
met? This is important as it could affect the genetic analysis.”

The origin of samples subjected to genetic analyses has been listed in the revised text: results section, page 10, line 21:

“Of the 32 genotyped tumors, 19 were metastases and 13 were primary tumors. In six patients, paired primary and metastatic tumors were available; no discordance was observed between the genotypes.”

Point 4: “It seems implicit that the genetic analysis was performed on samples obtained from tumors that were not previously exposed to cetuximab. This should be stated more clearly in the text.”

This has been clearly stated in the revised text: methods section, page 6, line 18: “We retrospectively assessed 32 patients with EGFR-positive mCRC treated with cetuximab-irinotecan combination at the Institut PAOLI-CALMETTES, Marseille, France between March 2004 and July 2005, evaluable for tumor response and with available pre-treatment frozen and/or formalin-fixed and paraffin-embedded tumor tissues (from primary and/or metastatic tumor tissue).
Reviewer 2: Stuart Schwartz

Major Points

Point 1: “Overall the manuscript should be reviewed by a native English speaker and the manuscript should be edited.”

The manuscript has been reviewed by a native English speaker and has been edited.

Point 2: The manners in which the results are presented in the abstract are somewhat confusing and should be rewritten to be clearer. How many responders are there? How many patients have a partial response? How many non-responders are there?

The results section in the abstract has been rewritten to clearly present the response data, abstract section, page 3, line 13:

“Nine patients experienced an objective response (partial response) and 23 were considered as nonresponders (12 with stable disease and 11 with progressive disease).”

Point 3: Again within the text it is difficult to follow the different treatment regimens; this needs to be made clearer.”

This comment probably refers to the results subsection “Clinical features of KRAS-mutated benefiting from cetuximab based-treatment”, where various treatment regimens were listed. To make clearer the treatment regimen acronyms used in the text we have added a clear definition of all regimens listed (results section: page 11, line 7)

In addition, treatment regimen acronyms are clearly defined in table 2 legend.

Point 4: When talking about the number of gene copies of EGFR, does this refer to the
*absolute number of copies of EGFR or of the ratio of EGFR/7 centromere. What if there are three copies of each (EGFR and the centromere). How is this classified.*

And **Minor point 3:** The description of the EGFR amplification appears to be written differently in the methods than the results. This needs to be consistent. What is an increased EGFR copy?

The gene copy number refers to the absolute number of copies of EGFR, whatever the ratio of EGFR/7centromere. However, only a ratio of EGFR/7centromere > 2 or a number of at least 15 gene copies in at least 10% of cells, whatever the ratio, defines an authentic amplification. An authentic amplification as well as a high polysomy (at least four gene copies in more than 10% but less than 40% of cells, ratio ≤ 2) had a significant increase in EGFR copy number and were considered FISH positive.

The definitions we used to describe the FISH patterns which are associated with a landmark reference paper were clearly stated in the methods section and were the same throughout the entire manuscript. To make it clearer, we have added that trisomy and low polysomy were considered as nonsignificant increases in EGFR copy number: Methods section: page 8, line 9: “FISH patterns were defined as described in [17]: Briefly, the samples were grouped as follows: normal disomy, two gene copies in more than 90% of cells; trisomy, three gene copies in more than 10% of cells and ratio gene/chromosomes < 2; low polysomy, at least four gene copies in more than 10% but fewer than 40% of cells and ratio gene/chromosomes < 2; high polysomy, at least four gene copies in more than 40% cells, ratio gene/chromosomes < 2; and gene amplification, ratio gene/chromosome more than two or 15 gene copies in at least 10% of cells. Trisomy and low polysomy were not considered as increases in EGFR copy number. Tumors showing high polysomy and/or
gene amplification were considered to be FISH positive and as significant increases in EGFR copy number.”

Therefore, to answer specifically the question of reviewer 1: if there are three copies of each EGFR and the centromere, this is a trisomy, as defined above and this is a nonsignificant increase in EGFR copy number.

In the results section, the terms are consistent with this definition, page 10, line 2: “No authentic regional amplification was observed. An increased EGFR copy number was noted in 12 patients, but was considered as significant in only 2 patients, corresponding to high polysomy (patients 1 and 3, Figure 1).” In addition, the discussion part entitled EGFR copy number is entirely dedicated to discuss the difference between an increase in copy number and actual amplification and also to confirm that these events are rather rare in CRC. However, we fully agree with reviewer 1 that it is confusing to use alternatively the terms of EGFR amplification (method and result subtitles) and EGFR copy number (discussion subtitle). Thus, in the revised version, we have decided to use the term of EGFR copy number uniquely for the section subtitles.

Point 5: In the results the authors say that there are only 2 responding patients with KRAS mutations; but 3 are given in the next section. Why is this section included if most of the patients with KRAS mutations didn’t respond?

1/ We do not agree with the first part of this remark. In the section entitled “Clinical features of KRAS-mutated benefiting from cetuximab based-treatment” the described medical records include only two responding patients along with one nonresponding patient but with a long-lasting stable disease. This was clearly stated in 2 places of this paragraph in the initial version “Medical records of KRAS-mutated patients with objective response or long-lasting disease stabilization were reviewed.” and page12, line 11: “Finally, a third patient (pt 21)
with a KRAS mutation achieved long-lasting stable disease (8 months), under cetuximab-irinotecan treatment.” It is again clearly stated in the introductive sentence of this paragraph in the revised version, page 11, line 7: “To better examine the clinical relevance of response or long-lasting stable disease obtained by cetuximab-irinotecan combination in 3 patients with KRAS-mutated tumors, their medical records were reviewed.” Thus, there is no discordance at all.

2/ Why is this section included if most of the patients with KRAS mutations did not respond?

Convergent data are demonstrating that KRAS mutated tumors have a low probability of response to cetuximab. Thus, some physicians are now proposing to exclude those patients from cetuximab-based treatment. This section has been included to clearly show that, although rare, it is possible to obtain a clinically relevant benefit with cetuximab in some patients with KRAS mutated tumors. This is one of the critical messages of this paper. To make it clearer we have added at the beginning of this section, the following sentence: page 11, line 7: “To better examine the clinical relevance of response or long-lasting stable disease obtained by cetuximab-irinotecan combination in 3 patients with KRAS-mutated tumors, their medical records were reviewed.”

Point 6: In many cancers (e.g. lung cancer) mutations in the EGFR tyrosine kinase domain (exons 18, 19 and 21) are studied, however in this study exons 6 to 14 are studied. Why the differences between the exons studied.

It is widely accepted and documented that these mutations usually found in exons 18-21 are absent in CRC, whereas no data are available about exons coding for the extracellular domain of EGFR, the binding site of cetuximab (exons 6-14), thus justifying our approach. This was explained in the introduction section: page 5, line 22: “Somatic mutations of EGFR tyrosine kinase domain are associated with exquisite sensitivity to EGFR-tyrosine kinase inhibitors.
erlotinib and gefitinib in non-small cell lung cancer (NSCLC) [1-3], but such mutations are rare or absent in CRC [4, 5].” And in the discussion section: page 15, line 12: “Activating mutations of the intracellular kinase domain of EGFR have been associated with human malignancies and/or responsiveness to small molecule EGFR tyrosine kinase inhibitors[4-6]. These mutations are rare or absent in mCRC, and are thus unlikely to explain the reported antitumor activity of cetuximab in this population. Nevertheless, little is known about the extracellular region of EGFR which represents the binding site of cetuximab.”

In addition, we have also sequenced exons 18-21 coding for tyrosine kinase domain of EGFR in these patients and found, as a large number of other groups, no mutations.

Minor points

Point 1: With the FISH studies for EGFR amplification; it needs to be stated that paraffin sections are being used and what their thickness is.

These 2 points have been added in the text: methods section, page 7, line 16: “Formalin-fixed paraffin-embedded (FFPE) tissue sections (5 µm) were placed in pretreatment solution for 60 min at 80ºC, and digested with pepsin solution for 15 min at 37°C.

Point 2: In Table 1 define what is meant by partial response, object response and stable disease. All of this terminology must be kept consistent throughout the entire manuscript.

Criteria for response evaluation were better defined in the methods section: page 7, line 6: …every 3 months, until disease progression. WHO criteria of response were used. Briefly, the sum of products of target lesions was calculated and response was determined as follows: complete response (CR), disappearance of all target lesions without any residual lesion; partial response (PR), 50% or more decrease in target lesions;
progressive disease (PD), 25% or more increase in the size of measurable lesions or appearance of new lesions; stable disease (SD), neither PR or PD criteria are met.

and this definition was referenced in table 1. The same terminology was used throughout the entire manuscript.

Point 3: See major points

Point 4: “How often is the R521K mutation detected in the general population? Explain why this is a variant (polymorphism).”

These data have been added to the discussion section: page 16, line 1: “This substitution, considered as a polymorphism (rs11543848 in SNPdb, heterozygosity of 0.41), may be relatively conservative, as both Arg and Lys are positively charged amino acids with similar side chains. It is also found in DNA from normal human lymphocytes [25] obtained from individuals without malignant diseases with a frequency of about 20% (homozygous variant) to 50% (heterozygous variant) in the general population [26].”

Point 4: “I do not think that the data present allows for as strong of conclusions as given by the authors.”

The conclusions have been modulated by adding the following sentence: discussion section: page 17, line 8: “However, these data from a small-sized patient population are still preliminary. Thus, validation of these results on larger cohorts and prospective studies are imperatively needed”. In addition, the following sentence has been removed from the discussion section: “Importantly, the study population, although of a limited sample size, was mostly representative of the current routine use of this compound, conferring to our findings a significant level of clinical relevance.”