Author's response to reviews

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

Authors:

Leonie J.M. Mekenkamp (l.mekenkamp@pathol.umcn.nl)
Jolien Tol (j.tol@aig.umcn.nl)
Jeroen R. Dijkstra (j.dijkstra@pathol.umcn.nl)
Inge de Krijger (ingedekrijger@gmail.com)
Elisa Vink-Borger (e.borger@pathol.umcn.nl)
Steven Teerenstra (s.teerenstra@ebh.umcn.nl)
Eveline Kamping (e.kamping@antrg.umcn.nl)
Eugene Verwiel (e.verwiel@antrg.umcn.nl)
Miriam Koopman (m.koopman-6@umcutrecht.nl)
Gerrit A. Meijer (ga.meijer@vumc.nl)
Han J.M. van Krieken (j.vankrieken@pathol.umcn.nl)
Roland Kuiper (r.kuiper@antrg.umcn.nl)
Cornelis J.A. Punt (c.punt@onco.umcn.nl)
Iris D. Nagtegaal (i.nagtegaal@pathol.umcn.nl)
Shannon van Vliet (s.vanVliet@pathol.umcn.nl)

Version: 2 Date: 7 March 2012

Author's response to reviews: see over
Dear Sir, Madame,

Please find enclosed our revised manuscript entitled "Beyond KRAS mutation status: influence of KRAS copy number status and microRNAs on clinical outcome to cetuximab in metastatic colorectal cancer patients" by Mekenkamp et al., which we hereby re submit for publication in BMC Cancer.

We thank the referees for the helpful and valid comments on our manuscript. The manuscript has been revised taking into consideration the comments from the reviewer, as shown below. In the manuscript the changes are marked in red.

We hope that the revised paper is acceptable for publication in BMC Cancer.

Remaining with best regards,

Dr. Iris Nagtegaal
Reviewer 1:

Major revisions:

1. Although KRAS 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).

We agree with the reviewer that the prevalence of KRAS amplifications in colorectal cancer is previously mentioned in their article. We added the next sentence in the introduction of our paper:

“However, little is known about the prevalence and effect of CNA of the KRAS locus on chromosome 12p12.1. By using a TaqMan-based KRAS copy number assay, KRAS amplifications were observed in approximately 2% of the 106 investigated colorectal primary tumours [26]. In CRC cell lines, gains of the KRAS locus were shown to be associated with an eleven-fold increase in RAS-GTPase activity, which is comparable with the twelve-fold increase caused by a codon 12 or 13 mutation[27].”

2. No data is shown to support the identification of chromosomal gains or losses – this must be provided.

We agree with the reviewer that it is illustrative to support the identification of our chromosomal aberrations with plots. We added additional plots of 2 patients with chromosomal loss and amplification at the 12p12.1 locus established by array CGH, and the heatmap of all patients with CNA of the KRAS locus in Additional Figure 2. We have added this Additional Figure in the first and second paragraph of our result section about 12p12.1 copy number changes in good an poor responders.

3. Similarly, no information is provided about specificity of KRAS gene amplification e.g. using gene specific assays, FISH etc. How do the authors know that their amplicon definitely contains the KRAS gene?

We used high-resolution (720k) array CGHs and our amplicon widely covered the KRAS gene. Therefore we are convinced that our amplicon contained the KRAS gene. However, we agree with the reviewer that validation can fortify our results and we have chosen to perform MLPA. This technique confirmed the (high level) gains we detected by array CGH. The losses were more difficult to interpret with MLPA and we confirmed only one KRAS loss. The other 4 deletions detected by array CGH appeared to be present subclonal, below the detection threshold of MLPA. The deletions we observed with array CGH consisted of a high number of array probes (median of 800 probes). Therefore we are convinced that our KRAS deletions observed by array CGH are real.

We completed our result section about 12p12.1 copy number changes with the MLPA data. In addition, we describe our MLPA data also in the legend of Additional Figure 2.

4. The manuscript would be considerably strengthened by the inclusion of phenotypic data to demonstrated increased/decreased KRAS expression and activity e.g. by Western blotting for KRAS, BRAF binding assays, assessment of phospho-ERK levels etc.

We agree with the reviewer that phenotypic data could strengthen our data. However, we used patients who participated in a large multi-center randomized clinical trial, and only paraffin-embedded tissue was provided. The assays above mentioned are really interesting but only informative in fresh frozen tissues. Moreover, these functional assays are performed before and the phenotypic data of KRAS amplifications and several miRNAs are established (Ref 10, Soh et
al). Therefore, we performed translation research and focused on the correlation with therapeutic outcome, which is not published before.

5. **The authors appear not to have considered the possible consequences of different KRAS mutations or different mutation burdens in their analysis, both of which could significantly influence therapeutic response.**

   We agree with the reviewer that there are additional KRAS mutations described at codons 19, 22, 61, 117, 146, 164 and 173. We address in the discussion that we only tested the most common KRAS mutations in codon 12 and 13. The new KRAS mutations are rare and the correlation with therapeutic outcome is missing. Our paper is a translational study focusing on new additional biomarkers and in the current clinical practice, these rare mutations are not used yet.

6. **Dukes’ stage or TNM staging information must be provided for all patients and included as covariates 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.**

   We agree with the reviewer that the TNM staging is an important prognostic indicator and we have added the T and N stage in the table. All the patients had metastatic disease, therefore we did not include the M stage. The T and N stage were equally distributed between both groups, therefore we did not include these variables in the subsequent analyses. Differentiation grade was the most significant variable that was skewed distributed between both groups, and we can only include one variable in our analyses (due to the small sample size).

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 recognized or addressed by the authors.**

   We agree with the reviewer that there is a large inconsistency in the prediction of miRNA targets. This is recognized in the literature and probably due to the different strategies used by each target prediction algorithms. Sethupathy et al. (Nature Methods 2006) reported the performances of five individual programs (TargetScan, DIANA-microT, miRanda, TargetScanS, and PicTar) and of various combinations of these programs. They showed that the intersection of all programs achieved the highest specificity but the lowest sensitivity. On the other hand, the union of all programs achieved the highest sensitivity but the lowest specificity. They recommended that the intersection of PicTar and TargetScanS predictions could achieve both high sensitivity and high specificity.

   We hope that this comment cleared the confusion. We have chosen not to comment on this in our article because it is well described in the literature.

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.**

   We selected RNU6B as an endogenous reference gene because this gene was widely used in the previously published ‘microRNA’ qRT-PCR literature. Thereafter, we used NormFinder and GeNorm to establish if the use of this reference gene was justified. In GeNorm our M value of the RNU6B controls were below 0.5, which means that the can be used as a reference gene. In NormFinder, the intragroup and intergroup validation was acceptable. By using these programmes we justify that the expression of RNU6B is stable enough and can be used as a reference gene for our miRNA quantification.

   We added the next sentence to our results section:
“MiR-205 expression was undetectable in both tumour and normal mucosa, therefore 17 miRNAs were included in our final analysis. By using NormFinder[40] and GeNorm[41], the use of RNU6B as a reference gene was justified.”

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?

By using array CGH, copy number gain is defined as 3 copies and an amplification means that there are more than 3 copies. We had one patient with a multi-copy amplification (at least two extra copies) and one patient with a duplication (one extra copy). RAS-specific aneuploidy was assessed by MLPA and array CGH, where the amplicon widely covered the KRAS gene.

10. Why was the ΔΔCt 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?

The ddCt method was indeed chosen since we used paired (tumor vs normal tissue) samples. This is described in the section ‘statistics’ of the material and methods. Optimal thresholds were determined by averaging the software generated thresholds per gene of interest (GOI) from the individual runs. When applying these averaged threshold it was manually checked if they were in the exponential part of the amplification curve. We have chosen for this option even though we are aware of the fact that an arbitrary threshold can be selected for each GOI as long as this is situated within the exponential part of the amplification curve and the efficiency of the GOI and reference gene (RG) is comparable. The baseline was set equally for each individual run, the precise baseline settings were determined by comparing all settings and selecting the minimal settings.

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.

We apologize for the typographical error we made underneath table 2. The SD mentioned in the table is not the standard deviation but the standard error of the mean ddCt of each of the miRNAs. This standard error combines both the within-patient error in the target gene and reference gene measurements (which is reduced by the double duplicate measurement) and also accounts for the between-patient variation in the ddCt. Indeed, to calculate the ddCt, the average over the measurements is taken in both the target and reference gene, which decreases the within-patient error in both the target gene and the reference gene measurement. Also the between patient variation is reduced by the square root of 32 by taking the average over 32 patients. Thus, the standard error of the mean ddCt combines both the within-patient error in the target gene and reference gene measurements and also accounts for the between-patient variation in the ddCt. Since all further analyses were based on the 32 ddCt values from the patients, the within patient error in the target and reference gene are accounted in all correlations and p-values.

The RT reaction was performed in duplicate and the qRT-PCR was also performed in duplicate. This resulted in quadruplicates of which the mean was used for the further analyses. In some cases we determined the ddCt from triplicates instead of quadruplicates due to the fact that one of the measurements clearly deviated from the other three.

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).
We agree with the reviewer that the differences we have found are still in the range of experimental errors. This is largely due to the small sample size of our cohort. However, we frequently mention that this study is only hypothesis generating and should be performed in a larger sample size to really draw any conclusion.

13. Discussion, the authors suggest that miRNA inhibition of KRAS occurs only when KRAS expression is “high” i.e. only in the presence of mutant RAS. They present no data to support this hypothesis i.e. that KRAS expression is influenced by mutation status.
We agree with the reviewer that we do not have functional assays to support our hypothesis. However, we want to explain our findings and it is established in the literature that de RAS GTPase activity is influenced by the KRAS mutation status in CRC cell lines (Soh et al., PLOS One 2009) as we state in the Introduction section.

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?
We agree with the reviewer that the data presented in our manuscript are hypothesis-generating and should therefore be confirmed, like all other biomarkers in independent series. The samples included in the current analysis were selected based on the most extremes in progression-free survival in cetuximab-treated patients in the CAIRO2 trial. Therefore, any other selection from the same series would yield an inferior discriminatory power.

Minor Essential Revisions

15. Introduction 2nd paragraph: “A point mutation” is incorrect – multiple point mutations in the K-Ras oncogene have been described.
We agree with the reviewer and changed this sentence in the text, although the presence of multiple point mutations in KRAS in a single tumour is uncommon. Mostly, the effect a KRAS is caused by one single point mutation (of which many different example are possible.:
“Point mutations in the KRAS oncogene leads to a significantly increased RAS-GTPase activity, ultimately resulting in the stimulation of cell proliferation and the inhibition of apoptosis via the RAS/MAPK pathway[6].”

16. “Ratio’s” should be “ratios” throughout.
We changed this.

17. Information should be provided on the number of cases where matched normal and tumour DNA were available for copy number analysis.
In 8 out of 34 patients we were not able to match tumour with normal DNA. In these cases we used comparable cases (public and private databases) to correct for germline copy number changes. We added the number of samples in the manuscript.

18. RNA integrity results should be presented.
RNA integrity was assessed by means of spectrometry (using the Nanodrop). We are aware of the fact that this technique is not capable of retrieving all preferentially known information. However as we have also addressed in a recent manuscript (Dijkstra JR, Mekenkamp LJ, et al. J Cell Mol Med 2011) there is, to this current date, no technique available which does give us all the preferred information. We have therefore chosen for spectrometry besides the fact that this is a rather cost-effective method.

19. Assays for mir-18a and mir-200c are commercially available from Applied Biosystems – it is not obvious why the authors think otherwise?
At the time we planned and performed the experiments, the two Taman assays were not commercially available. Because we performed the RT reaction in duplicate and the qPCR in duplicate, we had to make a selection in the number of miRNAs. Therefore, we have chosen not to design primers for these two miRNAs.

20. Table 2 is uninterruptable to a reader unfamiliar with Taqman qRT-PCR analysis and should be redrawn 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.

As mentioned above, the appropriate error calculations were performed. The fold changes are described in the relative quotient, and this calculation is clarified in the section ‘statistical analysis’ of the material and methods.

21. P-values should be provided for Figure 2.

We agree and added the p-values in Figure 2.

Reviewer 2:

Major compulsory revisions:

1. Unfortunately, despite the sound rationale, the study suffers from a lack of patient numbers and the authors are aware of this and do acknowledge it to an extent. While some interesting statistical trends and potentially significant results are observed, the paper does not provide any robust evidence of the influence of these markers. With some of the subgroups analyzed possessing as few as 6 patients (Kras mutant; good responders), this is not surprising. With such low patient numbers, the influence of other variables is extremely difficult to account for - although the authors did attempt to control for those that were possible. However, considering this, with appropriate acknowledgement as a pilot study and the limitations that exist with regard to the patient numbers and statistical power available, with some modifications the paper would be acceptable for publication. The authors do provide sound biological rationale and supporting references for the observed effects. The discussion is well written (if a little lengthy) but gives the distinct impression of a successful study with positive data and sound observations which may be overstating the strength of the data. I would request that the second last paragraph of the discussion expand upon the limitations of the study in more detail in a constructive manner to allow the reader to interpret the results in the context of the limitations.

We adjusted the second last paragraph according to the reviewer’s suggestions.

2. One of my concerns with the manuscript is the authors use of the term ‘response to cetuximab’ in the manuscript title when the measure of clinical outcome used in the study was progression-free survival on cetuximab treatment. The methods state that the 34 patients were selected based on the extremes of PFS. The authors should perhaps reconsider the use of this term as a good progression-free survival is not always synonymous with a ‘good response’ and can be a measure of a cytostatic or disease stabilization scenario. The term ‘response’ in the context of clinical trials is well defined (usually by RECIST) and the authors are not comparing the influence of copy number or miRNA expression to tumour response. In the abstract the authors use the term ‘clinical outcome’ in their background - I recommend this. This also applies to other areas of the manuscript where the term ‘good responders’ and ‘poor responders’ are used.

We agree with the reviewer that the term good response is not synonymous with a good progression free survival. In the title and abstract we changed the term response into PFS. We also address this item at the end of the first paragraph (Patients) of the Methods section, but for reasons of legibility brief the initial terminology is kept.

We added the next sentence:
Throughout the article the terms good and poor responders are used, which does not apply to response according to RECIST, but to the patients with the longest and shortest PFS on cetuximab-based treatment.

3. What was the rationale behind selecting PFS vs response rate? This should be mentioned. Incidentally, the correlation between response rate and PFS in the 17 patients selected in each category might be useful to know - particularly in the first results section and Table 1 where the PFS range for each group is specified. If there is a valid reason why PFS is more appropriate than response rate for this analysis, it should be mentioned.

The primary endpoint of the phase III clinical trial upon which the current study is based was progression-free survival. Especially with respect to targeted agents the correlation between response according to RECIST and (progression-free) survival appears to be suboptimal. Lastly, the response rate in the clinical trial was 50% in both study groups, and did not correlate with PFS, and therefore the number of patients potentially eligible for this pilot study is large, and the choice of response rate as endpoint would impair the selection of patients with the best outcome (PFS) on this treatment. In the group of patients with good outcome the response rate was 88% (15/17 patients) whereas in the group of poor responders only 29% (5/17 patients) had an objective response according to RECIST.

We added the next sentences:

Throughout the article the terms good and poor responders are used, which does not apply to response according to RECIST, but to the patients with the longest and shortest PFS on cetuximab-based treatment. This outcome parameter was chosen for the current study because it is the best reflection of the clinical trial upon which this analysis is based. Next, especially with respect to targeted agents PFS appears to be superior of response rate in terms of clinical outcome.

4. In the introduction the authors do not adequately delineate the differences between KRAS mutations. Specifically, the recent observation that patients with the G13D KRAS mutation may actually benefit from an EGFR monoclonal antibody vs mutations in codon 12. This is particularly important since in their 34 patients, only 1 patient has a codon 13 mutation. This also applies to the discussion where the authors discuss the nature of certain KRAS mutations including mention of codon 61 mutations - the data regarding codon 13 mentioned much later in the discussion should be brought forward. De Roock et al. JAMA. 2010 Oct 27;304(16):1812-20.

Since only one patient in our series had a codon 13 mutated tumour we do not think that the distinct clinical behavior of codon 13 mutations in KRAS will influence the results presented in our study. We added the requested citation to the Introduction section, and also comment on this in the discussion.

5. Another point regarding the introduction - some mention of additionally tested yet unconfirmed predictive markers that have been analyzed with regard to response to anti-EGFR antibodies are mentioned in the discussion (PI3K, ligands, SNPs etc) but might benefit from being brought forward to the introduction to help support the rationale for the study...i.e. support the need for continued efforts to identify biomarkers to these therapies. This would help as the discussion is bordering on lengthy with some repetition.

To address this comment we moved the paragraph on other potential markers for cetuximab from the Discussion to the Introduction section.

6. In the discussion the authors do bring up the interesting results of the CAIRO2 trial whereby the cohort receiving chemotherapy, bevacizumab and cetuximab had decreased PFS compared to chemotherapy plus bevacizumab. An explanation as to why this limitation may not apply to this retrospective subset analysis is provided, but is not satisfactory and the potential interaction between the two biologics and the potential impact on this study should be expanded upon briefly.

The text has been changed according to the reviewer’s suggestion.
“The patients used in this study were derived from a clinical trial, and the outcome will also be influenced by the effect of the other used agents, and not cetuximab exclusively. The phase III CAIRO2 trial showed that cetuximab plus chemotherapy and bevacizumab resulted in a significantly decreased median PFS compared to patients treated with chemotherapy and bevacizumab alone. This suggests at least that the detrimental effect of cetuximab was not present in the good responders and that these patients even may have benefitted from this treatment. The explanation of this detrimental outcome is unclear[43]. Excessive toxicity in the cetuximab group does not appear to be cause of these results. Negative interaction between the antibodies or between antibodies and chemotherapy might have influenced the outcome although preclinical observations supporting this hypothesis are not yet available. The interpretation of the current analysis is complicated by the detrimental outcome of the trial. Whether this outcome also affects the PFS in the good responders remains unclear.”

Minor essential revisions

7. In the methods, the authors used a different patient population to derive their KRAS gene copy number data. Although they provide a reference, they refer to it as ‘our previous Phase III study’. Since this population of patients contribute directly to the data in the manuscript, they should provide some brief details on this population including the nature of any therapy received and informed consent etc…simply for consistency and to inform the reader.

The Methods section has been changed:

“The patients included in this study participated in the CAIRO2 trial (CKTO 2005-02; ClinTrials.gov NCT00208546) of the Dutch Colorectal Cancer Group (DCCG)[17]. In this multicenter phase III trial, 755 mCRC patients were randomized between first-line treatment with capecitabine (1250 mg/m2), oxaliplatin (130 mg/m2), and bevacizumab (7.5 mg/kg), or the same schedule with the addition of weekly cetuximab (250 mg/m2, after initial 400 mg/m2). Translational research on tumour tissue was part of the informed consent procedure. The primary end point of the study was progression free survival (PFS), and secondary end points were overall survival, response rate, and toxicity. The median PFS in patients treated with cetuximab was 9.4 months (95% CI 8.4-10.5 months), which was significantly shorter than the PFS of patients treated in the group without cetuximab (median PFS 10.7 months, 95% CI 9.7-12.3 months, p=0.01). Patients in the cetuximab-group with a KRAS mutated tumour had a significantly decreased median PFS compared to patients with a KRAS wild-type tumour (8.1 versus 10.5 months, respectively, p=0.04).”

Minor discretionary revisions

8. In the final sentences of the introduction the authors state that there is currently no data available on the clinical relevance of miRNAs involved in KRAS activity, but I am aware of at least one study that utilized a cetuximab-treated clinical cohort and analyzed the influence of polymorphisms in a LET-7 miRNA binding site and response to cetuximab in KRAS wild-type patients. Although it is indirect evidence by analyzing SNPs vs directly measuring miRNA expression, it supports the concept nonetheless and perhaps warrants a mention. Zhang et al. Ann Oncol. 2011 Jan;22(1):104-9.

We agree with the reviewer that there is an association reported about the LCS6 T/G variant (rs61764370) and response in wild-type codons 12 and 13 KRAS metastatic colorectal cancer patients treated with cetuximab monotherapy (Zhang et al. Ann Oncol 2011). The authors reported that patients with wt KRAS and with LCS6 T/T genotype had worse objective response rate compared with patients with T/G or G/G genotype (G-allele). Moreover, it was shown that patients with G-allele had also a longer progression-free survival and overall survival. In 2010, Ruzzo et al. published on the association between the LCS6 SNP and survival outcomes in patients with metastatic colorectal cancer treated with cetuximab and irinotecan (Ruzzo et al. J
Pharmacogenomics 2010). Surprisingly, they found that patients with wild type \textit{KRAS} and wild type \textit{BRAF} and with LCS6 G-allele showed worse OS and PFS than T/T genotype carriers (confirmed in the multivariate model including the \textit{KRAS} status). Apparently, these two papers are conflicting and the association between the LCS6 variant and response to cetuximab is controversial and uncertain.

As mentioned by the reviewer, SNPs and directly measuring miRNA expression is quite different. Due to the fact that the predictive value of the SNP (LCS6) is controversial we have chosen not to comment on this in our article.

\textbf{Reviewer 3}

The manuscript by Mekenkamp et al. evaluated \textit{KRAS} CNA and miRNAs in correlation to clinical outcome in mCRC patients treated with cetuximab in combination with chemotherapy and bevacizumab from the CAIRO2 Trial. Even though such biomarkers are novel and of interest for prediction of response/resistance to cetuximab in mCRC, the setting that Authors chose for this retrospective analysis is not appropriate to this aim. Patients indeed received cetuximab in combination with chemotherapy and bevacizumab, so that a good clinical outcome can be due to the other components of the regimen associated to cetuximab. Conversely, an unfavorable outcome can be due to the detrimental effect of combining the two monoclonal antibodies, as it was reported by the same Authors in the NEJM paper, and not to lack of efficacy of cetuximab. Generally speaking, in order to drive meaningful conclusions about any association between a given biomarker and a therapeutic effect one should be confident that such therapeutic effect is attributable only to the candidate drug basing on the proposed rationale (ideally with a control arm). In the case of mCRC treated with anti-EGFR this can be achieved in the chemorefractory setting as in the papers by Karapetis et al. (cetuximab) or Amado et al. (panitumumab). Moreover, it should be noted that the cohort analyzed is really small and any association with proposed biomarkers should be taken with extreme caution (for example more solid data about association with miRNA from blood and outcome to cetuximab in an appropriate setting and in a much larger cohort were recently reported at the ASCO Meeting [Abst. 3532]).