**Author's response to reviews**

**Title:** APRIL is a novel clinical chemo-resistance biomarker in colorectal adenocarcinoma identified by gene expression profiling.

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**Author's response to reviews:** see over
18th September 2009

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Dear Dr Bucceri

MS: 1163450623266366
APRIL is a novel clinical chemo-resistance biomarker in colorectal adenocarcinoma identified by gene expression profiling.

Thank you for your recent correspondence inviting us to submit a revised version of this manuscript to BMC Cancer.

We have now addressed the issues raised by the two reviewers in the revised manuscript. On the following pages the comments of the reviewers are addressed on a point by point basis and reference to where changes have been made in the revised manuscript are included.

I hope that this meets with our approval. Please do not hesitate to contact myself if you have any queries or require further information.

I look forward to hearing from you

Yours sincerely

Dr Russell D Petty
(on behalf of all submitting authors)
APRIL is a novel clinical chemo-resistance biomarker in colorectal adenocarcinoma identified by gene expression profiling.


Reviewer: Andreas Teufel

Reviewer’s report:
The manuscript presented by Russell et al describes a novel biomarker, APRIL, overexpressed in 5fu/radiation treated colorectal carcinomas and was found to be predictive of outcome of adjuvant 5FU chemotherapy in patients with colorectal cancers. The manuscript offers a number of unclear points that need to be addressed before publication.

1) As the authors state the microarray experiments may only be seen as screening experiments since the ten samples were from 4 different experimental groups and even within the groups were from different tumor stages.
   a. It remains absolutely unclear though, as to why exactly APRIL was of highest interest among regulated genes? The authors describe a group of 17 cell death genes that are differentially regulated. What about the remaining 16?

Our response: APRIL was prioritised since over expression was also demonstrated in gene expression profiling microarray experiments in 5FU resistant colorectal cancer cell lines in our laboratory using the same bioinformatic analysis approach (this is stated in the manuscript). Among the sixteen remaining genes mentioned by the reviewer (see supplementary information 6, table S6), 3 genes were also identified in these 5FU resistant cell lines studies (details have been provided in additional supplementary information in the revised manuscript - in supplementary information_6, additional text and a new table has been added table S6.1), but APRIL was prioritised due to stronger evidence in the published literature of its involvement in carcinogenesis (although not specifically in colorectal cancer, where APRIL mRNA but not protein expression had been shown, therefore protein expression in colorectal cancer would also be of further interest as it would represent a novel finding). Furthermore, the data concerning the biological action of APRIL in promoting proliferation and inhibition of cell death would be relevant to and supportive of a potential mechanism for the hypothesised role in 5FU drug resistance and therefore it made ‘biological sense’. In addition, NFkB activation has been implicated downstream of APRIL and NFkB activation is implicated as a determinant of clinical response to 5FU containing chemoradiotherapy in rectal adenocarcinoma in our gene expression analysis and also in our 5FU resistance profiling studies on colorectal cancer cell lines.

The above reasons for prioritising/selecting APRIL are already included in the manuscript text (see page 11 paragraph2), and expanded upon and reiterated in the discussion section (see page 15, paragraph 3)- however in order to clarify, new text has been added on page 11 starting in the last four lines of the first paragraph starting ‘demonstrated 4 of the 17 genes…….’, and we have also added further supplementary information - within supplementary information_6, additional text and a new table has been added table S6.1Table S6.1
b. The authors cite several publications to “support further investigation of a putative functional role for APRIL in clinical 5 FU resistance”. Although these publications demonstrate an already established role of APRIL in cancer development, they don’t seem to deal with 5FU resistancy. Do the other 16 cell death genes not have any publications stating a role in cancer development?

**Our response**: Several of these genes have been implicated in carcinogenesis, or radiotherapy resistance, but none in 5FU resistance. Text has been added to the manuscript stating this (page 11, 7th line of first paragraph beginning ‘several of these’........) and further supplementary information added, outlining the known carcinogenic roles, roles in radiotherapy resistance- supplementary information _6- additional text added after table S6. Furthermore we wish to point out that this issue is also already addressed in the discussion section of the manuscript (page 15, paragraph 2)

2) The evaluation for a prognostic value for an adjuvant chemotherapeutic treatment in CRC was only investigated in stage III patients. It would be much more interesting to get a result in stage II patients as this may help to identify patients that would benefit from such a treatment in earlier stages not commonly treated with adjuvant chemotherapy.

**Our response**: While we agree that a predictive biomarker for benefit of adjuvant chemotherapy in stage II patient would be very useful, unfortunately none of the stage II patients in our series received adjuvant chemotherapy due to the limited and inconclusive benefit seen in unselected stage II patients in available clinical trials from the period that these patients were diagnosed and treated. (in line with current clinical practice at the time, as is stated). Therefore the numbers are insufficient to allow us to provide data on the role of APRIL for adjuvant 5FU benefit for stage II patients. In addition, for stage III patients, no more than a third of those treated adjuvantly with 5FU actually benefit from it in terms of increased cure(5 year survival) therefore it is very clinically relevant and interesting to have a biomarker for 5FU benefit in stage III patients where at present 5FU is given to the majority of such patients if medically fit to receive the treatment - we have made additions to the text in the discussion section of the revised manuscript to reflect these points-page 16 last, paragraph sentence starting with ‘Stage I and II patients did not receive........’) and also on page 16, first paragraph with the sentences starting ‘For example, currently adjuvant 5FU is used.......’ and ending with ‘............... and individualising clinical use of 5FU in this setting’.

Furthermore, adjuvant 5FU benefits only 2-3% of patients with stage II disease overall, and therefore the role of a biomarker for 5FU benefit is actually limited in this clinical setting, because combination adjuvant chemotherapy with additional agents is known to be more likely to be beneficial and is now used more often in this clinical setting. If APRIL had predictive value for any of these other combination agents e.g oxaliplatin, or biological agents in colorectal adenocarcinoma this would be more clinically useful, for stage II patients and also stage III- we have added text into the manuscript on this point as well (Page 16, second paragraph, added onto last sentence beginning ‘........ this would be potentially........’
3) The performance in comparison to established biomarkers is not discussed at all? Does APRIL perform better the previously identified markers?

**Our response:** A multivariate analysis has been performed demonstrating the HR identified for APRIL compares favourably to those reported for other previously identified putative 5FU predictive biomarkers in colorectal cancer (see table 2B) and text has been added to the manuscript stating this (page 15, first paragraph, third line text beginning ‘…… in which the Hazard ratio compares favourably ………’).

4) Since the marker is differentially expressed in stromal cells. Is the expression of APRIL in stromal cells associated with differential overall survival?

**Our response:** It is already shown in the data in the manuscript that APRIL stromal staining is associated with different overall survival- we have already stated this in the text on more than one occasion see results, discussion and conclusions and the potential mechanisms of this are also discussed. See also table 2B and figures 2 and 3.
Response to BMC cancer Reviewers Comments
MS: 1163450623266366
APRIL is a novel clinical chemo-resistance biomarker in colorectal adenocarcinoma identified by gene expression profiling.

Reviewer: Daniela Ellen Aust

Reviewer’s report:
Essential revisions
The conclusion of the authors that APRIL may be a predictive marker in 5FU-treated colorectal cancers seems a little far fetched since the authors write that APRIL is upregulated after CRT but do not indicate whether the expression of APRIL was also elevated in pre-treatment biopsies. In order to be of predictive value, APRIL expression has to be scored in pre-treatment biopsies and has predict the response to treatment. What the authors have shown so far is, that APRIL is a prognostic marker in stage III rectal cancers that were treated with RCT. The authors should state that. Maybe, strong APRIL expression in the resection specimens should prompt adjuvant treatment with other substances than 5FU.

Our Response: The reviewer raises an interesting point about predictive versus prognostic biomarkers. We believe in the context of this study that April is best described as a predictive biomarker as it appears to predict survival benefit in stage III colon cancer for those patients who received adjuvant (post-operative i.e. post resection) 5FU.

Many workers in the field accept and recognize the importance of the distinction between a predictive and prognostic biomarker as important both in terms of scientific investigation and clinical application – for example see the following authoritative reviews- Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. Nature Reviews Cancer 2005 Nov;5(11):845-856 or Wagner JA, Williams SA, Webster CJ. Biomarkers and surrogate end points for fit-for-purpose development and regulatory evaluation of new drugs. Clinical Pharmacology & Therapeutics 2007 Jan;81(1):104-107. Both types of biomarker should be assessable in biospecimens prior to any intervention (as carried out in this study) as stated by the reviewer, but a prognostic biomarker has impact upon therapy independent survival i.e. influences the natural history or ‘aggressiveness’ of the cancer in an individual patient and so correlates with survival in the absence of therapy. In contrast, a predictive biomarker has impact upon the probability of response and clinical benefit from a particular drug and so correlates with survival in patients treated with the drug. A ‘pure’ predictive biomarker (with no prognostic impact) has no impact upon therapy independent survival.

Bearing this in mind, the data presented in our manuscript shows the following as is stated in the manuscript text:

- mRNA expression of APRIL as measured by Affymetrix Plus2.0 Genechip Microarrays is upregulated in biopsies of rectal adenocarcinomas from patients taken after receiving preoperative treatment with concurrent 5FU and radiotherapy (chemoradiotherapy) as compared to biopsies taken prior to treatment, but no change in serial biopsies from patients before and
after radiotherapy alone (with no 5FU or any form of systemic chemotherapy). This is stated in the manuscript.

- It is not possible to comment on the relationship of APRIL mRNA in the tumours to response to Chemoradiotherapy due to the low numbers of patients analysed by the microarrays and hence low numbers of response groups. This is stated in the manuscript.

- APRIL mRNA measured by Affymetrix Plus2.0 Genechip is significantly upregulated in Colorectal cancer cell lines resistant to 5FU compared to parental ‘wild-type’ 5FU sensitive cell lines when the same bioinformatic analysis is used, as applied in the pre and post chemoradiotherapy biopsies from rectal adenocarcinoma patients. This is stated in the manuscript.

The Above data is presented as part of a hypothesis generating prospective biomarker discovery project - This is stated in the manuscript (introduction, results and also reiterated in the discussion) and recognised and acknowledged in comments by the other reviewer. The hypothesis generated - that APRIL has Predictive value for 5FU treated colorectal adenocarcinomas is then tested by measuring APRIL protein expression by immunohistochemistry in resected colorectal adenocarcinomas. This is detailed in the manuscript (results and discussion) and then as stated in the manuscript this analysis revealed:

- APRIL protein expression as assessed by immunohistochemistry in resection specimens correlates with survival in stage III colorectal cancer patients that receive adjuvant (post operative i.e. post resection) chemotherapy with 5FU, but NOT those Stage III patients that have not received adjuvant 5FU and there is no correlation with survival in Stage I and II patients who also did not receive adjuvant chemotherapy. This is stated in the manuscript.

As stated in the manuscript taken together this provides data to support APRIL as a predictive biomarker for 5FU in colorectal adenocarcinomas.

In particular the data from Stage III patients where APRIL is measured by immunohistochemistry supports a predictive as opposed to a prognostic impact because of the following:

- APRIL protein measured by immunohistochemistry is measured in resection specimens PRIOR to receiving adjuvant 5FU
- There is no correlation of APRIL protein expression with survival in stage III patients that have received no adjuvant chemotherapy. Therefore APRIL has no correlation with or impact upon therapy independent survival and accordingly is not prognostic
- There is a correlation between APRIL expression and survival in Stage III patients that have received adjuvant 5FU and the survival of such patients is also significantly different from patients who have not received adjuvant 5FU.

Therefore, we believe the data presented supports a role for APRIL as a Predictive Biomarker for 5FU benefit in stage III colorectal cancer patients rather than a prognostic marker. Furthermore, in the manuscript we specifically comment
that further independent large scale retrospective confirmation and prospective evaluation and further mechanistic evaluation is needed before concluding that APRIL is a clinically useful predictive biomarker.

The authors should give the number of tissue cores per case in the TMA that was used, so that the reader does not have to pull out the previous publications.

**Our response**: A sentence has been added to the methods section in the Immunohistochemistry subsection, detailing this information.