Author's response to reviews

Title: Presence of activating KRAS mutations correlates significantly with expression of tumour suppressor genes DCN and TPM1 in colorectal cancer

Authors:

Vid Mlakar (vid.mlakar@mf.uni-lj.si)
Gašper Berginc (gasper.berginc@mf.uni-lj.si)
Zdravko Štor (zdravko.stor@mf.uni-lj.si)
Miran Rems (miran.rems@sb-je.si)
Damjan Glavac (damjan.glavac@mf.uni-lj.si)
Metka Volavsek (metka.volavsek@mf.uni-lj.si)

Version: 2 Date: 18 May 2009

Author's response to reviews: see over
Dear Editor-in-chief Professor Melissa Norton, MD

Please find enclosed our revised manuscript entitled:

**Presence of activating KRAS mutations correlates significantly with expression of tumour suppressor genes DCN and TPM1 in colorectal cancer**

Vid Mlakar, Gašper Berginc, Metka Volavšek, Zdravko Štor, Miran Rems, Damjan Glavac

The following corrections to the manuscript were made based on reviewers comments.

Reviewer: Mailo Frattini

**Major Compulsory Revisions**

- The paper is based on microarray and Real-Time PCR methods, and to be sure about the obtained results, a careful selection of tumour blocks is mandatory. However, this information is completely absent in the manuscript. Therefore, the Authors must describe how they selected the tumour blocks for RNA extraction.

We added the description of tumour blocks selection for RNA extraction. In this study we only included samples that showed 50% of tumour cells or more (16 samples). Other samples for which we could not confirm 50% or higher tumour cell percentage were excluded from analysis and therefore not described.

Adjacent to samples stored in RNA later tissue sections were obtained for histological evaluation. Only samples including within the range 60 to 90% of invasive tumour cells (as evaluated by expert pathologist) were accepted for further analysis. Corresponding normal tissue samples contained no cancer cells as evaluated by pathologist. Tumour stage and nodal status were determined for each patient (Table 1).

**Minor Essential Revisions**

- Antibodies against the protein encoded by DCN gene for IHC analysis are available: the authors must investigate DCN by immunohistochemical techniques and then match these results with those obtained through the methodologies outlined in the present manuscript.

It is author’s opinion that such analysis would be helpful. However, due to the short time given for reply and extensive amount of work we were not able to perform these experiments. On the other hand, studies using IHC for decorin protein expression were performed. They clearly support our
results and are clearly showing that DCN protein is lost during the development of tumours. The authors are providing the reference for latest of these studies.

Interestingly, although many tumour suppressor effects have been associated with decorin, no precise quantification by real time PCR has been performed. However, decorin protein under-expression in CRC has been clearly established using immunohistochemistry [31].

- DCN encodes for a protein that acts through EGFR. The Authors did not investigate the EGFR deregulation, but analyzed the association with K-Ras, a member of an EGFR downstream pathway that is altered in an independent manner with respect to EGFR. Therefore the Authors must investigate the EGFR deregulation, ideally by FISH as IHC is not a reliable method for the evaluation of EGFR deregulation.

Unfortunately the authors were not able to perform these experiments in a three week period given for reply. To discuss the matter we can say that mutations in KRAS activate the pathway independently of EGFR status; therefore making the EGFR state irrelevant for subsequent effects of KRAS activity on level of transcription. Of note is also that EGFR is overexpressed in more than 80% of all CRC cases and that EGFR is rarely mutated (<1%) in colorectal cancer [19]. Thus, it is unlikely that EGFR status could influence our results. As far as the effects of decorin protein on the functional EGFR protein and function of other pathways much studies have been done and the authors cited all major studies, showing the importance and effects of decorin deregulation.

- Finally, for the association with K-Ras alterations, the Authors must discuss this datum in the context of the model proposed by Vogelstein for the development of colorectal cancer.

The authors included the discussion of association of DCN with K-Ras alterations.

In the Fearon and Vogelstein model, activating KRAS mutations are one of the first genomic events leading to the development of CRC. This in turn suggests that underexpression of the DCN gene might be early consequence of acquired activating KRAS mutations [32]. Moreover, as reported previously, overexpression of decorin also plays a role in TGF-alpha by inhibiting its synthesis and bioactivity, thus entering another important CRC development pathway as a tumour suppressor gene [30]. The importance of decorin downregulation for the development of CRC is further emphasized by its downregulation despite the absence of activating KRAS mutations.

Reviewer: Donia Macartney-Coxson

Major criticisms

1. Abstract/Discussion/Conclusion

- The authors should be careful not to overstate the significance of their gene expression data. For instance in the abstract "We are the first to report under expression of three important tumour suppressor genes...." Under expression of TPM1, DCN, and SLC26A3 mRNA has been previously reported in CRC in independent micro-array analyses (as per authors table 2). It would be more accurate to say that their observations provide further weight/evidence for decreased mRNA expression of TPM1, DCN, SLC26A3 and CALM3 in colorectal cancer.

We checked the manuscript for above mentioned statements and corrected them in two occasions as suggested.

Our study provides further evidence of decreased mRNA expression of three important tumour suppressor genes in cases of CRC, thus implicating them in the development of this type of cancer.
In conclusion, we detected significant under-expression of genes *SLC26A3*, *TPM1*, *DCN*, and *CALM3* in CRC, providing further evidence of their decreased mRNA expression and thus implicating them in the development of this type of cancer.

- With regard to the real-time PCR data the authors have carried out this analysis on the same samples which they used for the micro-array and as such have validated their micro-array data rather than independently validating the observation of differential expression. Independent validation would need to be carried out in additional samples not used for the micro-array. Real-time PCR is indeed a robust and more economic way to do this in a larger number of samples.

Unfortunately, the authors could not obtain more samples, since the study was limited in number of patients and only samples with higher tumour cell percentage were included in study. However, despite this limitation, the authors do not see how the microarray data or microarray experiment could influence the outcome of real time PCR results since both experiments were done independently. We did not use aRNA or any part of the microarray protocol in real time PCR reactions and vice versa. Therefore real time PCR results could not be biased by microarrays and are therefore independently describing the amount of motioned genes.

2. Real-time PCR.
   a). The ABI assay-on-demand which the authors have selected for DCN spans a SNP (single nucleotide polymorphism) in the primer or probe sequence (see ABI assay information). If any of the 16 samples contain this SNP it is likely to significantly affect the efficiency of the PCR reaction and therefore the gene expression value observed. Therefore, the genotype of the 16 samples needs to be verified. If any individual has the SNP the analysis will have to be repeated with another assay. From the variability in expression observed in Figure 2 I would guess that this might well be the case.

   Authors were aware of this fact when ordering the assay for DCN. However when looking at the PubMed database for SNP present in DCN assay the frequency of the polymorphism in Caucasian (116), 2 Asian (2 x 88) and Sub-Saharan African (116) population was 0.000. All four studies combined together contained 408 chromosomal counts of individuals of different ethnicity. Authors have performed additional sequencing for presence of V64L (rs17018909) polymorphism and report no presence of minor allele in 16 corresponding normal tissues.

Methods

All 16 corresponding normal tissues were also sequenced for V64L (C>A, rs17018909) polymorphism of the DCN gene, which might have an effect on performance of Hs00266491_m1 Assay-on-Demand™ (Applied Biosystems).

Results Real time PCR

We also report that we did not detect V64L polymorphism in 16 corresponding normal tissues, so no adverse effects on performance of real-time PCR was expected, as suggested by the manufacturer.

b). The real-time PCR methods section states that VIPR1 was measured in tumour samples and their adjacent normal tissue. No primer/probe sequence or Assay-on-Demand is referenced. Was VIPR1 used as a control? The authors used GAPDH as an endogenous control. They need to clearly state whether GAPDH showed significant variation in expression between tissues and samples.
We removed the VIPR1 section as it is not relevant for this article. GAPDH was used as endogenous control. No significant variation of GAPDH between normal and tumour tissue was observed. We corrected the manuscript as follows.

Methods Real time PCR
Expression of the SLC26A3, DCN, CALM3 and TPM1 genes in tumour samples relative to their normal adjacent tissues was measured using quantitative real time PCR based on the TaqMan® fluorescence methodology. … and Pre-Developed TaqMan Assay Reagents Human GAPDH (20x) mRNA (Applied Biosystems) were used as the endogenous control gene.

Data analysis
The paired t test was also used to check the difference in GAPDH expression between corresponding normal tissue and tumour samples.

Results
We used GAPDH as endogenous control for normalization. No statistically significant difference in expression of GAPDH between corresponding normal tissue and tumour samples was detected (paired t test p=0.113).

3. Graphs should clearly state that mean data are shown on the Y-axis (Figures 1 and 2). Error bars should show Standard error of the mean, rather than standard deviation, as statistical inference is being made. (Altman DG & Bland, J.M. Standard deviations and standard errors. British Medical Journal 2005, 331: 903).

The graphs were changed as suggested. Authors used standard error of the mean. See also 5. Figure 1 where additional suggestions regarding presentation of results are given.

4. Table 2:
This is an important table which summarises the micro-array results, and places them in the context of other publically available micro-array data. As such the columns showing “Down”, “Up” and “No Change” would be more informative if they contained a ratio of change in expression equivalent to the authors observed value (“Log2 ratio”). The appropriate data could be referenced in superscript e.g. -1.1616. The table could also be simplified by removing Location and RefSeq GI (as this will be available in their publically submitted data). The “Name” and “Function” columns would more accurately be labelled “Gene Symbol” and “Gene Name”. For ease of comprehension the “other methods” column could be renamed along the lines of “independent validation”.

The authors have changed the table as suggested. We changed the Log2 ratio in fold change, removed Location and RefSeq GI, and changed “Name”, “Function and “other methods” for “Gene Symbol”, “Gene Name” and “independent validation”.

5. Figure 1.
The strength of this study is the 16 matched normal/tumour samples. By presenting the normal and tumour gene expression data as independent groups this reduces the power of the study. The dCt data for each set of paired samples could be presented, or alternatively the fold change. Real-time PCR analyses fold change could be calculated for each of the 16 pairs, and the mean fold change compared to the null hypothesis of no fold change. Using the power of the matched pairs will increase the statistical power of the analyses. Admittedly, this would make the variation between individuals more obvious, but if anything this adds strengths to the authors argument that there are a number of pathways (including ones yet to be identified) to CRC development. This would also neatly summarise the real-time PCR results section into a figure rather than text.
The authors have made corrections as suggested. For each sample, expression of all four genes is presented as average \( \Delta \Delta C_T \) of three replicates. The presentation is made in the way that negative bar represents under-expression of gene in tumour and positive bar represents over-expression of gene in tumour.

6. With regard to the KRAS activating mutations did the authors investigate the normal DNA for those with a mutation in order to differentiate between germline or somatic mutation? Authors report that no KRAS mutations were found in corresponding normal tissue of 16 samples. This result is presented along with table 1 where sample histology and presence of KRAS mutations in tumours were presented and also in results under Mutation detection section.

Methods

**KRAS mutation detection and rs17018909 detection**

The first exon of the KRAS gene of each tumour sample and corresponding normal tissue was amplified with PCR as previously described by Konig and co. [11].

Table 1 and Results Mutation detection

No activating KRAS mutations were found in corresponding normal tissue.

Minor revisions

1. Abstract:
   a) “despite the identification of the major genes” delete “the”.
   Corrected
   b) “using real-time PCR, we also searched for chromosomal abnormalities…” Edit sentence to make it clear that real-time was used for mRNA expression, and copy number variation analyses, and that potential correlations between gene expression and KRAS mutation were investigated.
   Corrected

Real time PCR was used for mRNA expression as well as to search for chromosomal abnormalities within candidate genes. The correlation between the expression obtained by real time PCR and the presence of the KRAS mutation was investigated.

2. In the methods section define dC. For instance, test gene expression was normalised to GAPDH (dC). Corrected

\[
\Delta \Delta C_T (\Delta C_T = \Delta C_{T_{normal}} - \Delta C_{T_{tumour}}; \Delta C_T = \Delta C_{T_{xx}} - \Delta C_{T_{GAPDH}}) \text{ method, unless stated otherwise.}
\]

3. The results section states that 31 differentially expressed genes were identified. Table 2 shows 30, and the discussion mentions 30.
   Corrected

4. The authors should clearly state in the results why they looked for KRAS activations in codons 12 and 13 alone. In addition, it would be useful to highlight why they initially used SSCA, and its utility for codon 12 and 13 mutations, rather than directly sequencing the samples.

Authors added the explanation on activating KRAS mutations and SSCA method used. SSCA was used as a screening method. It is able to distinguish between all possible mutations of codon 12 and 13. The method is sensitive as it is able to detect mutant allele in as low as 20% of all alleles in sample (in-house data on optimization of SSCA for KRAS codon 12 and 13 mutations (Department of Molecular Genetics, Institute of Pathology)).

In order to detect mutations, we used the SSCA method for preliminary testing and automatic sequencing for subsequent confirmation of different patterns. We searched for mutations in codon
12 and 13, since these mutations represent 96 – 99% of all mutations detected in CRC. Mutations in other positions, such as 66 and 146, account for around 1-4% of all mutations, although their clinical relevance in CRC is unclear [19]. We found activating mutations in codon 12 and 13 in 8 out of 16 samples (Table 1). No activating KRAS mutations were found in corresponding normal tissue.

5. Discussion
The background on each gene of interest is quite long and could be edited. It could more briefly highlight agreement/disagreement with the literature and discuss why down-regulation might be pertinent to cancer development, and possible alternate pathways (as per the initial aim).

The authors were trying to reach the compromise as other reviewer suggested adding additional discussion.

Discretionary Comments
1. The Table 2 legend says that “fold change” is given as Log2 (tumour/normal). The authors may wish to change the values to an actual transposed fold change as this is intuitively easier for the reader to understand. For instance, Log2 ratio of -0.50 would be -1.4 fold change. The table legend also refers to NSCLC rather than CRC.

Corrected as suggested.

2. It would be useful to know why the authors selected the particular previous micro-array data to compare their results to. Do they feel these are representative of the publically available data?

Authors used the described studies as they were available on GEO (PubMed) and are therefore subject of MIAME standards.

Results on differentially expressed genes were compared to 5 major expression studies available from the PubMed GEO project and evidence of independent validation of gene expression data was checked using the PubMed database.

We sincerely hope that we have made all necessary corrections and that you will find now our paper ready for publishing in BMC Cancer.

Kind regards,

*Correspondence to:
Damjan Glavac, Ph.D.
Associate Professor of Human Genetics
Department of Molecular Genetics, Institute of Pathology, Korytkova 2,
Faculty of Medicine, 1000 Ljubljana, SLOVENIA
E-mail: damjan.glavac@mf.uni-lj.si
Tel: 386 1 543 7180
FAX: 386 1 543 7181