Author's response to reviews

Title: DNA hypermethylation and decreased mRNA expression of MAL, PRIMA1, PTGDR and SFRP1 in colorectal adenoma and cancer

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Author's response to reviews: see over
Dear Dr. Dave Hoon,

Attached please find our manuscript “DNA hypermethylation and decreased mRNA expression of MAL, PRIMA1, PTGDR and SFRP1 in colorectal adenoma and cancer”, which we would like to resubmit to BMC Cancer after revision according to Referees’ comments and questions. Together with the revised manuscript, hereby I would like to provide a detailed point-by-point author’s response letter.

Authors’ response to Christos Kontos

Authors would like to thank the Referee for the aware and extensive review. Authors agree with the comments of the Referee and the manuscript has been modified according to the following answers.

1. The introduction is rather short. Moreover, important introductory information (e.g. on miRNAs) is missing. The authors should consider enriching appropriately the Introduction section.

The introduction section was extended with additional information about DNA methylation and miRNAs.

“Colorectal cancer (CRC) is regarded as one of the most frequent malignant tumours globally [1]. This heterogeneous disease can develop through at least three distinct molecular pathways by which genetic and/or epigenetic dysregulation influences gene expression and protein levels finally leading to colorectal adenoma and carcinoma formation [2, 3]. One of the epigenetic alterations that can contribute to CRC formation is the abnormal DNA hypermethylation of promoters, resulting in reduced or absent gene expression [4]. DNA hypermethylation occurs at regulatory sites e.g. promoter in a tissue- and cancer type-specific manner [5]. Besides genetic alterations, DNA hypermethylation of tumor suppressor genes is a frequently detected mechanism behind the inactivation of these genes leading to tumor initiation [6]. Although more and more genes are associated with various types of cancers, our knowledge of DNA methylation markers in CRC development remains incomplete.
Another key posttranscriptional epigenetic regulator of gene expression, miRNA, regulates the stability and translation process of mRNAs. The expression of miRNAs has been shown to differ in colorectal tumors compared to healthy colon tissue specimens and on the basis of several experimental results they play role in colorectal cancer formation. Up- and downregulation of certain miRNAs was identified along the adenoma-carcinoma sequence of CRC and evidence supports the role of miRNAs in CRC development and progression as these small non-coding RNAs affect proliferation and invasion [7].

The identification of genes affected by epigenetic changes can be achieved using whole genome gene expression analysis [8]. DNA methylation and miRNA expression alterations can both lead to a certain degree of dowregulation of mRNA expression and consequently of protein levels, which can be confirmed by immunohistochemistry.

In the present study, our aims were (1) to identify DNA methylation markers in left-sided CRC samples on the basis of whole genome gene expression analysis and (2) to analyse the methylation levels of these candidate marker along the colorectal adenoma-carcinoma sequence on colorectal adenoma and cancer samples. Furthermore, (3) our aim was to confirm the relationship between gene expression, DNA methylation status, miRNA expression and protein level of the analysed candidate markers.”

2. Was the current study performed in accordance with the ethical standards of the revised Declaration of Helsinki? If so, this should be mentioned in the Materials and Methods section.

The following sentence was added to the Materials and Methods section: „The study was performed according to the ethical standards of the revised version of Helsinki Declaration.”

3. The authors should discuss the role of miR-21 in more detail (end of the Discussion section). It is also advisable to add some more information about the epigenetic regulation of miR-21 in colorectal cancer.

The discussion part was extended with information about the role of miR-21 in cancer formation and the epigenetic regulation of miR-21.

„The expression level of miR-21 is elevated both in colorectal adenomas and cancers, and the degree of upregulation correlates with more advanced stages of CRC [7]. This small non-coding RNA could have a fundamental role in the progression of CRC, as elevated level of miR-21 was found to be predictive of poor survival [46], that may increase proliferation,
migration and invasion. In CRC cell lines with the EMT phenotype the expression of miR-21 oncomiR is regulated by AP-1 and ETS transcription factors and also by epigenetic factors. Activating histone modifications (H3K3me3, H3K914ac, H3K27ac), but no inactivating were detected on mir-21 promoter region [47]. These epigenetic mechanisms can affect the binding affinity of transcription factors to the miR-21 promoter regulating its expression level.”

4. The whole manuscript should be carefully checked for English language (grammatical and syntax) errors and these should be corrected, e.g. last sentence of the introduction “…we aimed to confirm…”.

The manuscript was proofread by a native English speaker and errors were corrected.

Author’s Response to Jiang Cao
Authors would like to thank the Referee for the aware and extensive review. Authors agree with the comments of the Referee and the manuscript has been modified according to the following answers.

1. The title may need to be revised: it indicates decreased expression of MAL, PRIMA1, PTGDR and SFRP1 in left-sided colorectal adenoma and cancer, but in the Abstract and the Results the authors focused more on COL1A2, SFRP2 and SOCS3. And, the authors didn’t provide any direct experimental evidence for that the upregulated miR-21 leads to the decreased expression of those genes in their work;

Agreeing with the opinion with the Referee, and taking the limitations of our miRNA experiments with the lack of functional analyses into consideration, the title and the abstract was modified as follows:

Title:

DNA hypermethylation and decreased mRNA expression of MAL, PRIMA1, PTGDR and SFRP1 in colorectal adenoma and cancer

Abstract, Conclusion

Conclusion: Hypermethylation of the selected markers (MAL, PRIMA1, PTGDR and SFRP1) can result in reduced gene expression and may contribute to the formation of colorectal cancer.

2. Figure 5, high resolution/good quality needed;

Figure 5 was replaced by images of better resolution.
3. Figure 7, more representative images are needed to show the decreased expression of the SFRP1 protein.

Figure 7 was modified to include more representative images along the adenoma-carcinoma sequence in order to illustrate the decreased SFRP1 protein levels in colorectal adenomas and carcinomas.

Revised version of Figure 7 (Figure 5 in the revised version of the manuscript):

4. Please describe more on the selection of SFRP1 for IHC but not other decreased proteins in their work (if they do have the IHC for other proteins, it would be better to show all).

In the present study protein level analysis was performed only for one candidate marker of our special interest. The reason for selecting SFRP1 was that our working group is currently focusing on the epigenetic regulation of SFRP1 and its expression in stromal cells of colorectal adenoma and cancer on both mRNA and protein levels. According to the results of
that parallel project, we hypothesized that myofibroblast-derived SFRP1 protein might be a paracrine inhibitor of epithelial proliferation in NAT areas and the loss of this signal may support tumor proliferation in CRC (Valcz G, Patai A V, Kalmar A, Peterfia B, Furi I, et al. (2014) Myofibroblast-Derived SFRP1 as Potential Inhibitor of Colorectal Carcinoma Field Effect. PLoS ONE 9(11): e106143. doi:10.1371/journal.pone.0106143). Although authors agree that protein level analysis of the other markers would provide more insight into the possible effect of DNA methylation, immunohistochemistry experiments for all the analyzed genes would have financial limitations.

Materials and methods section was modified in the revised manuscript:

„Among the analyzed 18 genes, only SFRP1 protein level was analyzed because of the special interest of our working group.”

5, **Confirmation of the finding in more clinical sampes are desired for a solid conclusion.**

In the present study we aimed to identify the candidate genes showing hypermethylation along colorectal adenoma-carcinoma progression. Currently, we had the opportunity to analyse gene expression data from a larger study including healthy colonic (n=49), colorectal adenoma (AD, n=49), and CRC (n=49) biopsy samples, furthermore, from laser capture microdissected epithelial and stromal cells from NAT (n=6) and CRC (n=6) clinical samples. Although for the methylation analysis we could involve a limited clinical sample set, besides biopsy and macrodissected samples we also included –time-consumingly collected– laser microdissected epithelial and stromal cells. Further validation studies are already planned with higher clinical sample number.

6, **Figure 4 legend, please indicate the case number of each group.**

According to the suggestion of another reviewer due to the complexity of Figure 4, DNA methylation data were summarized in Table 3. However, former Figure 4 was added to the revised manuscript as Additional figure 2, and the numbers of analyzed samples in each group were added to the figure legend.

7, **As HT-29 is not a suitable model to illustrate anything for the development/progression of CRC, and the results are not supporting the major findings the authors proposed, this part of work could be removed.**

We have selected the concentration of 5-Aza for demethylation treatment on the basis of literature data, and 72 hours of treatment with 5-Aza resulted in the gene expression changes presented in the manuscript. Although 5-Aza demethylated HT-29 cells did not show remarkable gene expression changes in case of the analyzed genes, these data can be helpful
for other working groups, as higher 5-aza-2’-deoxycytidine concentrations might result in more representative demethylation experiments.

According to the suggestion of the referee and taking the limitation of HT-29 cell line into consideration, more gene expression data sets were analyzed from GEO database containing 5-Aza treated colon cell lines other than HT-29: GSE14526 (3 µM 5-Aza treatment for 72 hours on HCT116 and SW480 cell lines); GSE32323 (0.5 µM 5-Aza treatment for 72 hours on colo32, HCT116, HT-29, RKO and SW480 cell lines.

The materials and methods of the revised manuscript contains the following part:

Expression of the selected gene set was also analyzed on gene expression datasets of human colorectal cell lines before and after DNA demethylation treatment with 5-Aza (GSE29060: 10 µM 5-Aza treatment for 72 hours on HT-29 cell line; GSE14526: 3 µM 5-Aza treatment for 72 hours on HCT116 and SW480 cell lines; GSE32323: 0.5 µM 5-Aza treatment for 72 hours on colo32, HCT116, HT-29, RKO and SW480 cell lines.

Data were represented on heatmaps together with the original HT-29 expression data (GSE29060) on Figure 2 in the revised version of the manuscript:

Gene expression of the selected marker set was analysed on data sets containing control and 5-Aza treated colon adenocarcinoma cell lines. According to GSE29060 data, in HT-29 adenocarcinoma cells after a demethylation treatment 4 transcripts showed a minimally decreased expression (TIMP1, FADS1, CYP27B and SULT1A1), while PTGS2 was found to be upregulated. HCT-116 cells showed higher re-expression of the selected genes, as PTGS2, THBS2 and TIMP1 also showed upregulation (1<lgFcontrol-treated) and TIMP1 was also upregulated in 5-Aza treated SW480 cells according to GSE14526. Among the 5 CRC cell lines of GSE32323 SULT1A1 in colo32 cells, PTGS2 in HCT-116 cells, ALDH1A3 and SOCS3 in HT-29 cells and ALDH1A3 and TIMP1 in SW480 cells showed remarkable upregulation after demethylation treatment (Figure 2, Additional table 3).
Demethylation treatment of colon adenocarcinoma cell lines led to varying degrees of upregulation of certain transcripts. In HT-29 cell line ALDH1A3 and SOCS3 was found to be upregulated by 0.5 µM 5-Aza. Interestingly, in HCT-116 cells PTGS2; and in SW480 cell line TIMP1 showed higher expression after 0.5 and 3 µM 5-Aza treatments, as well.

Author’s Response to Alexander Link

Authors would like to thank the Referee for the aware and extensive review. Authors agree with the comments of the Referee and the manuscript has been modified according to the following answers.

1. Methods part in the abstract are overloaded with information. The authors may wish to shorten it substantially providing the global overview of tissues and techniques.

According to the suggestion of the Referee, the original methods part of the abstract was shortened. The revised version of the modified abstract can be found below:

Methods: Whole genome expression profiling was performed on colonic biopsy samples (49 healthy normal, 49 colorectal adenoma (AD), 49 CRC); on laser capture microdissected (LCM) epithelial and stromal cells from 6 CRC-normal adjacent tissue (NAT) samples pairs, and on demethylated HT-29 human CRC cell line using HGU133 Plus 2.0 microarrays (Affymetrix). Methylation status of genes with gradually altering expression along the AD-CRC sequence was further analysed on 10-10 macrodissected and 5-5 LCM samples from healthy colon, from adenoma and from CRC biopsy samples using bisulfite-sequencing PCR (BS-PCR) followed by pyrosequencing. In silico miRNA prediction for the selected genes with miRWalk algorithm, miRNA expression was analysed on 3 CRC-NAT sample pairs
and 3 adenoma tissue samples using the Human Panel I + II (Exiqon). SFRP1 immunohistochemistry experiments were performed.

2. As authors point out, the study is based at least partly on the results that have been previously published. It may be helpful to include the clear statement in methods to what extent the data has been previously published (Ref. 6,7)? Why the selection gives a different set of genes?

In the present study we used gene expression data derived from previously published studies by our working group. Although we utilized the same raw data, our aims were now different from that in our previous publications. In our previous two papers our aims were:

1) to identify downregulated genes along the colorectal adenoma-carcinoma sequence and in parallel, identify genes that re-express in HT29 cells after 5’-aza-deoxycytidine treatment. Common hits were further analysed in validation experiments.

2, to identify transcript sets that can objectively discriminate between healthy colon, colorectal adenoma and carcinoma samples, and between high-grade dysplasia and early cancer cases.

In contrast to the above-mentioned goals, in the present study our aims were to identify genes differentially expressed genes along adenoma-carcinoma progression and that can potentially be regulated by DNA methylation. Furthermore, we aimed to validate their DNA methylation status in biopsy samples from healthy colon, colorectal adenoma and carcinoma samples, and to analyse on epithelial cells derived from those samples, as well.

The following statement was inserted in the Materials and Methods section:

„Although the bioinformatic analysis and the candidate selection was based on previously performed and published raw gene expression data of HGU133 Plus 2.0 microarrays, the aim of the present study was substantially different from the previously published studies’. We aimed to identify genes with gradually altering expression in adenoma and tumor samples that can be potentially regulated by DNA methylation.”

3. In both previous publications (6, 7) I was unable to find clinical information regarding the clinical samples which may provide valuable information from the clinical perspective.

The summary of clinical data of the previously published studies were added to the manuscript as Additional table 1. Furthermore, clinical data of the present study was also inserted as Additional table 2.
4. As the HT29 is a chromosomally instable (CIN) cell line with multiple chromosomal aberrations limiting DNA methylation analyses, do the authors expect different results in other CRC cell lines?

Encode RRBS data are available from Caco-2 and HCT-116 colon adenocarcinoma cell lines, that we could compare with our DNA methylation results (see pictures below, as examples). We could conclude, that Caco-2 and HCT-116 cell lines showed DNA methylation in the region we analyzed, as well.

SOCS3

COL1A2

SFRP2

However, taking the limitation of HT-29 cell line into consideration, more gene expression data sets were analyzed from GEO database containing 5-Aza treated colon cell lines other than HT-29: GSE14526 (3 µM 5-Aza treatment for 72 hours on HCT116 and SW480 cell lines); GSE32323 (0,5 µM 5-Aza treatment for 72 hours on colo32, HCT116, HT-29, RKO and SW480 cell lines.

The materials and methods of the revised manuscript contains the following part:

Expression of the selected gene set was also analyzed on gene expression datasets of human colorectal cell lines before and after DNA demethylation treatment with 5-Aza (GSE29060:
10 µM 5-Aza treatment for 72 hours on HT-29 cell line; GSE14526: 3 µM 5-Aza treatment for 72 hours on HCT116 and SW480 cell lines; GSE32323: 0,5 µM 5-Aza treatment for 72 hours on colo32, HCT116, HT-29, RKO and SW480 cell lines.

Data were represented on heatmaps together with the original HT-29 expression data (GSE29060) on Figure 2 in the revised version of the manuscript:

Gene expression of the selected marker set was analysed on data sets containing control and 5-Aza treated colon adenocarcinoma cell lines. According to GSE29060 data, in HT-29 adenocarcinoma cells after a demethylation treatment 4 transcripts showed a minimally decreased expression (TIMP1, FADS1, CYP27B and SULT1A1), while PGTS2 was found to be upregulated. HCT-116 cells showed higher re-expression of the selected genes, as PTGS2, THBS2 and TIMP1 also showed upregulation (1<lgFc_{control-treated}) and TIMP1 was also upregulated in 5-Aza treated SW480 cells according to GSE14526. Among the 5 CRC cell lines of GSE32323 SULT1A1 in colo32 cells, PTGS2 in HCT-116 cells, ALDH1A3 and SOCS3 in HT-29 cells and ALDH1A3 and TIMP1 in SW480 cells showed remarkable upregulation after demethylation treatment (Figure 2, Additional table 3).

The discussion of the revised manuscript contains the following part:

Demethylation treatment of colon adenocarcinoma cell lines led to varying degrees of upregulation of certain transcripts. In HT-29 cell line ALDH1A3 and SOCS3 was found to be upregulated by 0,5 µM 5-Aza. Interestingly, in HCT-116 cells PTGS2; and in SW480 cell line TIMP1 showed higher expression after 0,5 and 3 µM 5-Aza treatments, as well.
5. One of the shortcomings is the miRNA expression analyses. Unfortunately, the authors use only each 3 samples from each group to evaluate or validate results. The information regarding the used normalizer miR-423-5p may be helpful. The authors may wish to explain why they include the miRNA analyses. Did the authors confirm the array expression analyses for miR-21-3p, miR-181c or let-7i-3p using qPCR? Please correct the labeling in Figure 6?

The miR-423-5p is one of the 6 miRNAs represented on the Human Panel I + II qPCR plates recommended to be used as a normalizer by Exiqon. It was selected from the above-mentioned 6 miRNAs as a normalizer on the basis of its lowest standard deviation between all analysed samples.

The aim of the study was to identify candidates with altering expression along colorectal cancer formation and to investigate if DNA methylation could be an underlying regulatory mechanism. According to our results not all analysed genes showed alteration at the DNA level parallel with gene expression alterations. That was the reason why we investigated a posttranscriptional regulatory mechanism (miRNAs) that can influence gene expression. Although only a limited number of samples were analysed using miRNA assays, we aimed to include these results in order to have insight into another regulatory process, as well. However, agreeing with the opinion of the Referee, and taking the limitations of our miRNA experiments into consideration, the title of the manuscript was changed and miR-21 upregulation was excluded in order to have less emphasis on these results in the manuscript. Furthermore, with the lack of functional analyses, the discussion part containing miRNA results was modified suggesting the role of the selected miRNAs as potential regulators of the analyzed genes without remarkable DNA methylation alteration only hypothetically. However, as one of the Referees suggested to enrich more introductory information about miRNAs in the introduction part and more detailed discussion of miR-21 in the discussion part, these information were also included in the revised manuscript.

In the present study expression analysis of miRNAs was performed with miRCURY LNA Universal RT microRNA PCR that is a highly specific qPCR analysis using LNA optimized primers.

The label of Figure 6 (Figure 4 in the revised version of the manuscript) was modified.

6. In the present work the authors do not provide mechanistical or functional analyses therefore the current title is misleading. „DNA hypermethylation correlates with decreased mRNA expression...“. The study relates to 4 microRNA in very limited subset of subjects and may be not scientifically powered for the main message in title.

As mentioned above, agreeing with the opinion of the Referee, and taking the limitations of our miRNA experiments into consideration, the title of the manuscript was changed and miR-
21 upregulation was excluded in order to have less emphasis on these results in the manuscript. The title was modified accordingly:

DNA hypermethylation and decreased mRNA expression of *MAL, PRIMA1, PTGDR* and *SFRP1* in colorectal adenoma and cancer

7, Selection of 18 transcripts differs from previously selected targets from previous publication (7). The authors may wish to include the selection criteria (Table 1).

In our study we aimed to identify genes with gradually altering expression in adenoma and tumor samples that can be potentially regulated by DNA methylation. The transcripts were selected on the basis of Kendall (tau coefficient) rank correlation analysis.

The selection criteria was inserted in the legend of Table 1.: *Genes with gradually decreasing or increasing expression along the adenoma-carcinoma sequence with predictable CpG islands were selected on the basis of Kendall (tau coefficient) rank correlation analysis (−0.5 ≤ \( \tau \) ≤ 0.5).*

8, Expression of microRNA is shown on the Figure 6 with visually reduced expression.

Figure 6 (Figure 4 in the revised version of the manuscript) with miRNA expression data was modified. Raw Ct data were substracted from the maximal qPCR cycle number (45) in order to visually reverse qPCR results. Furthermore, these data were normalized with interplate calibrators and also with miR-423-5p Ct values.
Along colorectal adenoma-carcinoma progression, the following genes showed downregulation: BCL2, CDX1, ENTPD5, MAL, PRIMA1, PTGDR, SFRP1, and SULT1A1 while the following genes showed upregulation: ALDH1A3, COL1A2, CYP27B1, FADS1, PTGS2, SFRP2, SOCS3, SULF1, THBS2, and TIMP1.

10, Figure 1 and Figure 4 are too busy. Because of the great number of data sets, I would recommend to use a table (exp. N, Mean or median +/- SD and p-value) or alternatively heat-map.

According to the suggestion, Figure 1 and 4 was replaced with Table 2 and 3, respectively. However, as boxplot representation is a suitable was to illustrate gene expression alterations visually, former Figure 1 and 4 were added to the revised manuscript as Additional figure 1 and 2, respectively.

11, Could you please provide the information if any expression profiling data were validated by exp. PCR?

In the previously published studies data from HGU133 Plus2.0 experiments were validated with qPCR for gene sets selected in those publications on large independent sample sets [Galamb O et al. (2012) PLoS ONE 7(11); Spisák S et al. (2012) PLoS ONE 7(10); Galamb et al. (2008) Cancer Epidemiol Biomarkers Prev 2008;17(10)]. According to these results, a strong correlation could be found between array and qPCR data regarding tendency and extent of gene expression alterations. Although gene expression validation with qPCR would confirm the alteration of the selected transcripts, the present study did not include validation PCR experiments.

12, Description of statistical analyses is partly missing.

The statistical analysis was appended with additional information in the revised version of the manuscript, as follows:

The selection of candidate genes was based on expression data generated from 147 colonic biopsy specimens (from 49 normal, 49 adenoma, and 49 CRC patients), laser capture microdissected colonic epithelial cells (from 6 NAT, 6 adenomas, and 6 CRC), analyzed in a previous study by whole genome HGU133 Plus 2.0 microarrays (Affymetrix) [8, 9]. These
data files are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) at GSE series accession numbers GSE4183 (8 normal, 15 adenoma and 15 CRC), GSE10714 (3 normal, 5 adenoma and 7 CRC), GSE37364 (38 normal, 29 adenoma and 27 CRC) and GSE15960 (laser microdissected colonic epithelial cells from 6 normal, 6 adenoma and 6 CRC).

Although the bioinformatic analysis and the candidate selection was based on previously performed and published raw gene expression data of HGU133 Plus 2.0 microarrays, the aim of the present study was substantially different from the previously published studies’. We aimed to identify genes with gradually altering expression in adenoma and tumor samples that can be potentially regulated by DNA methylation. The data sets GSE4183, GSE10714, GSE 37364, and GSE15960 were analysed to identify genes potentially regulated by DNA methylation. Transcripts with gradually decreasing or increasing expression along the adenoma-carcinoma sequence were selected on the basis of Kendall (tau coefficient) rank correlation analysis (-0.5 ≤ tau coefficient ≤ 0.5). DNA methylation analysis was performed for genes with CpG island(s) on the basis of in silico prediction by the CpG Plot EMBOSS application (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html).

Expression of the selected gene set was also analyzed on gene expression datasets of human colorectal cell lines before and after DNA demethylation treatment with 5-Aza (GSE29060: 10 µM 5-Aza treatment for 72 hours on HT-29 cell line; GSE14526: 3 µM 5-Aza treatment for 72 hours on HCT116 and SW480 cell lines; GSE32323: 0.5 µM 5-Aza treatment for 72 hours on colo32, HCT116, HT-29, RKO and SW480 cell lines.

Student T-test and Benjamini-Hochberg methods were applied in order to determinate significance of gene expression and DNA methylation level comparisons (p < 0.05). For IgFc, abs (differences of average of intensity values) > 1 threshold was applied.

Looking forward to Your kind reply about the hopefully acceptance of our response to the Referees. We hope that the above modifications and enhancements make this manuscript acceptable.

Sincerely,

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