Author’s response to reviews

Title: Promoter methylation correlates with reduced NDRG2 expression in advance colon tumour

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Author’s response to reviews: see over
Dear Editors,

Thank you for reviewing our manuscript entitled “Promoter methylation correlates with reduced NDRG2 expression in advanced tumour colon stage” (MS: 4643427882078639). We have extensively revised the manuscript based on the criticism of the Reviewers.

Answers to Reviewer: Nagahide Matsubara

Major compulsory revisions:
Following suggestion of the reviewer we have explained in detail the text of figure 3 and we have added, as supplementary figure, the NDRG2 gene promoter region with the primers location of BSA and MSP assays.

As correctly noted, in the figure 3 there are three skipping CpG islands for NDRG2 gene that show unmethylation in normal tissue compared to tumour tissue. It’s true that usually consecutive methylation are observed in the promoter region but since these CpG islands show the same patterns in all normal tissues (both lymphocyte and colonocyte) as well as in all tumour tissues/cells (unmethylated and partial o full methylated, respectively) this strangely behaviour could probably suggest that these are important sites for correct gene function in normal cells.
The primers *NDRG2* for BSA and NDRG2_M for MSP assays overlap in the same region while the primers *NDRG2_M_2* are located to about 300bp at downstream from the first.

The difference in methylation status between BSA and MSP assay was probably related to number CpG islands insert in the MSPs primers. Indeed, in the primers sequence both *NDRG2_M* there are 4 CpG islands while in *NDRG2_M_2* there are 3 CpG sites. MSP is a qualitative test directly related to the number of CpG in the own sequence.

Minor Essential Revision:
The legend to Figure 2 was amended to better understanding and we have explain the meaning of 2-DCt.

Page 10 line 15: the NR3C2 gene was moved in correct group.
Page 11 line 17: hemimethylation was replaced with partial methylation.

**Reviewer:** Yutaka Kondo

**Major Compulsory revisions:**

1. We agree with the reviewer on confusing of our manuscript. In the revised manuscript we have removed the paragraphs on gene expression profile analysis both in Materials and Methods and in Results.

2. The sentence “Expression level of the post-treatment specimen compared to the pre-treatment specimen was calculated as a log-transformed ratio. A gene was classified as up-regulated by 5-Aza-CdR treatment when the log-transformed ratio was greater than 0.75 (equivalent to 1.65-fold up-regulation) in at least one treatment condition in one cell line. Genes with no change or very low expression levels in post treatment specimens were no further considered in the analysis.” was moved in statistical analysis. Moreover, since it was misleading we have changed it to better understanding.

3. We have added the information about the primer location of MSP and BSA in new Fig.3 and in supplementary of figure 3.

4. Fig.1: We have modified the text of the legend and change the number of genes with that correct: twenty-one genes. The expression profile values indicates the average of expression obtained by Comparative CT (or ΔΔCt) Method as reported in the text. The average cycle time (Ct) was calculated for each samples in three replicates, normalized to the average Ct of the reference genes (housekeeping) and compared to cDNA of normal tissue used as calibrator.
5. In fig.2 are shown the genes that are ri-expressed after treatment. At the point of non-treatment the genes are nothing silenced nor down-regulated but normalized. The values to which the reviewer refers correspond to error standard. In supplementary data the normalized values (N) are slipping in the error standard (SE) column. We have modified the column in supplementary table.

6. The 5-Aza-CdR was used to identify gene silencing in genome but confirmation of this was achieved after the identification of CpG islands methylated. Indeed, “epigenetic unmasking of methylated gene” is common used to discover new methylated genes and our results are consistent with the other studies (Hoque MO et al. Genome-wide promoter analysis uncovers portions of the cancer methylome. Cancer Res. 2008 Apr 15;68(8):2661-70; Yamashita K et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell. 2002 Dec;2(6):485-95). The best explanation for this mechanism in just few upregulated genes is most likely due to the fact that upstream genes might be ri-expressed by other transcriptional events.

We agree with the reviewer about the number of methylated genes in the CRC but our focus was only on 24 genes selected.

The sentence “these findings highlight the utility of combining genomic, epigenetic, and expression data to identify tumour biomarkers” was removed.

7. We have performed direct bisulfite-sequence. The sequence are added as supplementary figure 5A.
8. The data are expressed as the difference in methylation status between tumour and normal tissue.
9. Gel image of MSP are added in supplementary figure 5B.
10. We have revised the Discussion. The sentence with “reversible conversion….” was removed since the sentence was misleading.

Minor Essential Revision:

1. We have added the methods paragraph
2. The abbreviation are spelled out.

Finally, we would like to express our gratitude to the Reviewers for their remarks that have made our paper much more valuable. We hope that the extensive changes we made in the manuscript will be to your and Reviewers satisfaction, and now you will find our work worthy of publication.
Sincerely yours,

Piepoli Ada

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