Author's response to reviews

Title: Promoter methylation correlates with reduced ndrg2 expression in advanced colon tumour stage

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Version: 5 Date: 10 October 2008

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

“First, they performed a gene expression profile assay on both cancer and normal colonic tissues by DNA microarray to choose up- and down-regulated genes. If they really focus on the TSGs, only down-regulated genes have to be further examined”.

We agree with the reviewer; now we have modified our manuscript taking into account only TSGs.

“Expression analysis by qPCR or rt-PCR seemed well correlated with the microarray data, however, it is difficult to understand why some of the genes were analyzed in tissue samples and others were analyzed on cell lines to match the result. No gene was analyzed by both methods. It would be better that all genes have to be analyzed on tissue samples compared with the normal counter part.”
In the revised manuscript the paragraph on the cells was deleted together with figure 2 and validation of microarray data was carried out only on tumour tissue samples compared with the normal counterpart using the same method (qPCR) for all genes analysed.

“By the treatment with demethylating agent, 6 genes showed elevation of their mRNA expression. It is interesting that 3 of the constitutively over-expressed genes confirmed by microarray were upregulated by treatment with the demethylating agent. How could it be explained?”

Our primary interest was to evaluate whether some silenced genes had an increased expression after treatment with demethylating agent. A secondary aim was to search for a possible down-regulation of constitutively over-expressed genes due to either a possible hypermethylation of other regulatory genes or depletion of DNMT1 levels. For this reason, we originally included in our study CSE1l, HSPH1 and SOX9 genes which showed overexpression in our microarray assay. Surprisingly, after 5-Aza treatment, the expression of these genes, as evaluated by qPCR, further increased. Since we were unable to find a clear explanation of this finding, we decided to exclude these genes from the final analysis. We are going to perform additional experimental work on these genes in order to confirm these preliminary data in other cell lines.

“Only remaining candidate TSG was the NDRG2 whose expression is possibly controlled by promoter methylation. However, throughout the steps (array analysis, expression analysis or demethylation analysis) to identify NDRG2, it is important that well-known TSGs whose expression is controlled by promoter hypermethylation, such as MGMT or p16, should be assayed in the same experiments as its control.”

Indeed we used p16 gene as positive control in the demethylating assay. The result of p16 expression was added in the figure 2.

“Also, the characteristics of NDRG2 in comparison with the well-known (for example classical CIMP and new CIMP (by Laird et al.)) genes should be evaluated. We would like to know the importance of the NDRG2 in comparison with other important epigenetically controlled genes such as CIMP”.

We did not perform this kind of analysis because we were more interested in searching for new TSGs than in sorting our cancer samples according to CIMP status. Clearly, the next step will be to correlate the NDRG2 methylation status with CIMP phenotype.
“There are so many spelling mistakes especially the name of the genes in the text and figures. HSPH1 vs HPS1, NRDG2 vs NDRG2, HPDG1 vs HPGD etc.”

We have corrected all the spelling mistakes.

“ Figure 4, what is (N), (T), (K)?”

(N) is normal tissue, (T) and (K) stand for tumour (T) or cancer (K) tissue. We have added the meanings in the legend and modified the Figure.

“Table 5, frequency of the promoter methylation of MLH1 is 20% in all colorectal cancers. Also the frequency in descending colon (means distal colon including descending and sigmoid(?)) is 14%, and it is hard to believe because MSI-H cancer, which has promoter methylation of MLH1 unless Lynch syndrome, is commonly identified in proximal (cucum, ascending and transverse) colon. Methylation of MLH1 matched to the MSI-H?”

MSI is shown in approximately 10%-15% of sporadic CRC cases without history of predisposing family history of cancer. (ref. Thibodeau S.N. et a., Science 1993, 260:816; Aaltonen L.A. et al., Science 1993, 260:812). Our data are in agreement with these prevalence rates. In fact, we found a MSI-H prevalence of 13% (4/30) in all our CRC samples. When MSI-H status was matched with MLH1 methylation, we found that 3 out of 4 MSI-H samples (1 proximal and 2 distal colon samples) had also MLH1 promoter methylation. Accordingly, we checked carefully table 5 and found that percentage values were wrong. Indeed, the prevalence of MLH1 methylation is 22% (2/9) in the proximal colon and 19% (4/21) in the distal colon. Tumour location was significantly associated neither with MSI-H status nor with MLH1 methylation but generalization of these data is not possible for the low number of samples studied.

Reviewer: Yutaka Kondo

“This manuscript might need a lot of revision. The content of the Abstract is very misleading and seems different from the contents of the main text. It is hard to follow the manuscript because of the lack of whole data sets, appropriate controls, and standard error bars (how reliable are these data?). In addition, I cannot read some of the letters (e.g. Figure 3). I feel that I cannot comment on this manuscript until it has been carefully revised by the authors.”
The manuscript has been completely revised according to the careful suggestions of the other referee. The abstract has been modified by eliminating the discrepancies with the main text. The data sets have been added in a supplementary part of the manuscript. Control data have been also included. Standard errors bars have been added in the figure. Figure 3 has been re-drawn.

Now, we hope that the reviewer will be able to comment on our paper.

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,

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