Author's response to reviews

Title: Epigenetic inactivation of the NORE1A gene correlates with malignant progression of colorectal tumors

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Author's response to reviews: see over
Dear Prof. Ian Cree and Natasha Mellins-Cohen,

Re: BMC Cancer : MS# 5940755764177257

MS Title: Epigenetic inactivation of NORE1A correlates with malignant progression of colorectal tumors

Thank you very much for your e-mail dated 2nd September, 2010. We agree that the manuscript is much improved after incorporation of the reviewers’ comments, and have amended the manuscript accordingly. We listed our responses point by points as followed.

Neither this submitted paper nor any similar paper has been or will be submitted to or published in any other primary scientific journal while under consideration by *BMC Cancer*. All of the authors are aware of and agree to the content of the paper and their being listed as authors on the paper. This manuscript does not contain any information conveyed either by personal communication or release of unpublished experimental data.

**Reviewer #1**

We would like to thank the reviewer for the constructive and useful comments.

1. Epigenetic inactivation and its reactivation by 5-aza-dc has been already reported in SW48 colon cancer cell line (Ref. 15). More importantly, Akino et al. have also reported NORE1A methylation in colon cancer cell lines and colorectal cancer tissues (*Gastroenterology* 2005;129:156–169). The latter paper is not cited. These results should be described and discussed in detail in the manuscript.

Reply: Thanks for this kind indication. According to the reviewer’s indication, we cited the paper (listed as ref #29) in this revised manuscript. Although this published study showed that RASSF5 (NORE1) expression is down-regulated in several colorectal cancer cell lines by promoter hypermethylation, expression status of NORE1A and NORE1B in primary carcinomas and its association with tumor progression have not been reported. In this revised manuscript, we demonstrated expression status of both NORE1A and NORE1B in cell lines and primary tumors (Figs 1A-1D), its association with tumor stage and grade (Figs 2A-2C), effects of its expression on tumor cell growth (Figs 5A-I).

2. Epigenetic inactivation of RASSF1A and RASSF2 has been reported in colorectal cancer. Because only NORE1A was analyzed in this study, the relationship between NORE1A inactivation and inactivation of other RASSF family is unclear. This should be clarified.
Reply: According to the reviewer’s indication, we newly included NORE1B data in this revision. As described in Results and Discussion sections, we found that NORE1B expression is frequently down-regulated in cancer cell lines and primary tumors (Figs 1-3). Interestingly, however, all of cancer specimens harboring NORE1B reduction also showed low NORE1A level, but none of tumors showed NORE1B-specific reduction, indicating that NORE1A is more commonly down-regulated in colorectal cancers (Fig 2B). As the reviewer indicated, several members of the RASSF family including RASSF1A have been identified. Previous studies show that each member of the family has distinct and tissue-specific roles. We think that comparison of expression status of these RASSF members in the absence of functional evidences would not provide valuable information and be out of scope in this study. More detailed characterization of other RASSF members need to be performed in separate studies.

3. It would be interesting to analyze the relationship between NORE1A inactivation and KRAS status.

Reply: We determined the mutational status of K-Ras in 80 primary carcinoma tissues examined in this study using DNA-SSCP and sequencing analysis. As summarized in Fig 2D, we found no association between expression status of NORE1A or NORE1B and the mutational status of K-Ras in tumor specimens we tested. The result was described in the Results and Discussion sections in this revision.

4. Abnormal reduction of NORE1A was correlated with advanced stage. In the abstract, the authors conclude that epigenetic inactivation of NORE1A might be implicated in the malignant progression of colorectal tumors. However, in the last paragraph of discussion, the authors conclude that epigenetic inactivation of NORE1A might be implicated in the development and/or early progression of colorectal tumors. This needs to be better clarified.

Reply: Our study shows that abnormal reduction of NORE1 (both NORE1A and NORE1B) is more frequently in tumors of advanced stages and high grades compared to tumors of early stages and low grades, indicating that NORE1 reduction is associated with malignant tumor progression. However, the observation that a substantial fraction of early stage and low grade tumors also shows NORE1 reduction suggests that NORE1 abnormality might be implicated in early progression. Considering that epigenetic gene silencing is a relatively early event during tumorigenesis processes, our finding suggests that epigenetic inactivation of NORE1 might be implicated in the development and/or early progression of colorectal tumors. We agree that
more detailed and large scale study is required to define the role of NORE1 inactivation in colorectal tumorigenesis, and in this context, our comment is a general suggestion based on the limited results.

5. The authors arbitrarily defined a value less than a half of normal mean (0.64) as abnormally low level. The criterion for the reduction of NORE1A expression in 80 matched tissue sets needs to be described.

Reply: When we compare expression levels of NORE1A and NORE1B between cancerous and noncancerous tissues of the same patients, we regarded more than 40% difference in expression level as low expression. This is also an arbitrary criterion we have utilized for several translational studies considering tumor tissues contain at least 10% contamination of normal cells such as infiltrating lymphocytes. We describe this in the text. We think that there can be no absolute criteria for determination of abnormal expression levels of mRNA or protein.

6. Regarding the reactivation experiments shown in Figure 3c, did the authors confirm significant demethylation in these cell lines treated with 5-aza-dC? Are there any reasonable reasons why the authors did experiments at a single concentration of 5-aza-dC and a single point after treatment? It would be interesting to analyze the effects of trichostain A (TSA) treatment and those of the combination of 5-aza-dC and TSA.

Reply: We did not show the data in the original manuscript, but we actually obtained a dose-associated induction results from a preliminary study. We newly included two representative examples in this revision (Fig. 3C). We also tested effect of TSA and found that combined treatment with TSA leads to higher induction of NORE1 compared to single treatment (Fig. 3C). The main purpose of 5-aza-dC in this study is to test whether NORE1 reduction in tumors is associated with DNA hypermethylation. Because the involvement of DNA methylation is suggested from 5-aza-dC assay, we next performed bisulfite DNA sequencing analysis of the NORE1 promoter to verify aberrant promoter hypermethylation. We believe that our findings of the reactivation of NORE1 by 5-aza-dC and a clear correlation of mRNA expression status with CpG methylation content are good enough to conclude that DNA hypermethylation is associated with abnormal down-regulation of NORE1 expression in tumors.

7. Correlation of NORE1A methylation with inactivation of expression in clinical samples should be presented at protein levels as well by using western blotting and/or immunohistochemistry.
Reply: We examined protein expression status of NORE1A in 10 cancer cell lines by immunoblot assay using NORE1A-specific antibody, and found that NORE1A protein levels reflect well the mRNA expression levels in these cells. The immunoblot result was presented in Fig 1B in this revision. We have a separate research plan for immunohistochemical study of NORE1A and NORE1B in clinical specimens. We hope to present the data in near future.

Discretionary Revision

1. Functional studies such as growth, apoptosis, and colony formation assay would strengthen the conclusion.

Reply: According to the reviewer’s comment, we constructed expression vectors encoding wild-type (WT) NORE1A and WT-NORE1B and siRNAs against NORE1A and NORE1B, and performed cell number counting, apoptosis, and colony formation assays. As we presented in Figs 5A-5I in this revision, using two cancer cell lines (Caco-2 and HCT116), we observed that both NORE1A and NORE1B exert growth suppression effects (inhibition of cellular growth and colony formation and induction of apoptosis). We newly described these results in Results and Discussion.

Reviewer #2

We would like to thank the reviewer for the constructive and useful comments.

1. NORE1 isoforms: NORE1 encodes two major transcripts (NORE1A and NORE1B), which are derived from alternate splicing and different promoter usage. Macheiner et al. reported that NORE1B was epigenetically silenced in human hepatocellular carcinoma. On the other hands, Hessen et al. reported that NORE1A was inactivated in several human cancers. In this manuscript, the authors demonstrated both NORE1A and NORE1B expression were commonly decreased in colorectal cancer cell lines. The authors should consider which transcript is responsible for malignant progression of colorectal tumors. Otherwise, it is hardly to state that only NORE1A gene correlates with malignant progression of colorectal tumors. In addition, the authors should elucidate whether epigenetic inactivation inhibited the NORE1B expression as well.

Reply: Thanks for this helpful comments. According to the reviewer’s indication, we performed
expression study of NORE1B in cancer cell lines and primary tumors. As described in Results and Discussion sections, we found that NORE1B expression is frequently down-regulated in cancer cell lines and primary tumors (Figs 1A-2C). Interestingly, however, all of cancer specimens harboring NORE1B reduction also showed low NORE1A level, but none of tumors showed NORE1B-specific reduction, indicating that NORE1A is more commonly down-regulated in colorectal cancers (Fig 2B). Likely NORE1A, abnormal reduction of NORE1 is more frequently in tumors of advanced stages and high grades compared to tumors of early stages and low grades (Fig 2C), indicating that NORE1B reduction is also associated with malignant tumor progression. We also observed that NORE1B mRNA expression was re-activated in low expressor cells following treatment with 5-aza-dC (Figs 3C and 3D). In addition, we carried out functional studies to examine effects of NORE1A and NORE1B on tumor cell growth. For these study, we constructed expression vectors encoding wild-type (WT) NORE1A and WT-NORE1B and siRNAs against NORE1A and NORE1B, and performed cell number counting, apoptosis, and colony formation assays. As we presented in Figs 5A-5I, using two cancer cell lines (Caco-2 and HCT116), we observed that both NORE1A and NORE1B exert growth suppression effects (inhibition of cellular growth and colony formation and induction of apoptosis). We newly described these results in Results and Discussion.

2. In abstract, the authors describe as follows; Our data indicate that epigenetic inactivation of NORE1A due to promoter hypermethylation is a frequent event in colorectal tumorigenesis and might be implicated in the malignant progression of colorectal tumors. However, the author did not show any direct evidence such that inactivation of NORE1A was associated with colorectal tumorigenesis. Is NORE1A implicated with the initiation of colorectal tumors or progression? I am interested whether epigenetic inactivation of the NORE1A gene is associated with poor prognosis in each stage as well.

Reply: Our study shows that abnormal reduction of NORE1 (both NORE1A and NORE1B) is more frequently in tumors of advanced stages and high grades compared to tumors of early stages and low grades, indicating that NORE1 reduction is associated with malignant tumor progression. However, the observation that a substantial fraction of early stage and low grade tumors also shows NORE1 reduction suggests that NORE1 abnormality might be implicated in early progression. Considering that epigenetic gene silencing is a relatively early event during tumorigenesis processes, our finding suggests that epigenetic inactivation of NORE1 might be implicated in the development and/or early progression of colorectal tumors. We agree that more detailed and large scale study is required to define the role of NORE1 inactivation in colorectal tumorigenesis, and in this context, our comment is a general suggestion based on the
limited results. Tumor tissues examined in this study include many specimens obtained from recently diagnosed cancer patients, and we have a separate plan for clinical study including an immunohistochemical analysis of NORE1A and NORE1B in clinical specimens whose histopathologic characteristics and prognosis information of the patients, including survival status, are available. We hope to present the data in near future.

3. The authors demonstrated that NORE1A promoter was completely or partially methylated in 6 of 10 (60%) colorectal cell lines while Hessen L. et al. reported NORE1A promoter was methylated in 1 of 6 colorectal cell lines. What do the authors explain this discrepancy?

Reply: Hessen L. et al examined 6 colorectal cancer cell lines and found promoter methylation from one (SW48) of the cell lines. The six cell lines they examined include HCT116 and DLD1, which were also included in our study. In both studies, these two cell lines were identified to express detectable levels of NORE1A mRNA and have no aberrant promoter hypermethylation. Therefore, the results from these cell lines are consistent in both studies. Using a COBRA assay, Akino et al (Gastroenterolgy 129:156-169, 2005) showed that 10 colorectal cell lines exhibit variable levels of promoter methylation depending on the regions analyzed. They showed 4 of 10 cell lines have no or low NORE1 mRNA expression. Among these 10 cell lines, 4 cell lines (Caco-2, HCT116, DLD-1, and Colo320) were also examined in our study and showed the identical results. Considering these, we think that there is no discrepancy in real data among reports. We predict that if a large scale study is performed, approximately 30-60% of colorectal cancer cells might display aberrant promoter hypermethylation.

Minor essential revisions

1. Fig 3B does not show all of the low expressors and SNU-C4 (Page 10 Line 6). Fig 3C is collect.

Reply: Thanks for this kind comment. According to the reviewer’s indication, we replaced Figures and presented new results from all 7 cell lines (Fig 3D). We also included a dose-associated effect of 5-aza-dC and combined treatment with TSA (Fig 3C).

2. A grammatical mistake was found as follows; NORE1A expression correlates with tightly with loss of p21 (Page 4 Line 7).

Reply: Thanks again for this kind comment. We have corrected this sentence.
Reviewer #3
We would like to thank the reviewer for the constructive and useful comments.

This paper reports the epigenetic inactivation of NORE1A, a member of the RASSF family, in colorectal cancer. Authors have analyzed gene expression and the DNA methylation status of NORE1A promoter CpG island in a series of 80 primary colorectal carcinomas and 10 cell lines. Downregulation of NORE1A is more frequent in advanced stages of the disease. It is concluded that epigenetic inactivation of NORE1A is a frequent event in colorectal cancer and that it might be implicated in the progression of the disease. Experimental analysis is well performed and presented. The conclusions are consistent with the data, but show no novelty. The design of the study is very modest. Specifically, NORE1A epigenetic activation should be accompanied by the analysis and study of possible associations with other genetic and epigenetic alterations (i.e. K-ras and p53 mutations, microsatellite instability, RASSF1A epigenetic silencing, etc) and additional clinical data (i.e. outcome, location).

Reply: Thanks for this helpful indications. In this revision, we newly included expression and functional studies of NORE1B. Our revision is summarized as followed (1-5).

(1) As described in Results and Discussion sections, we found that NORE1B expression is frequently down-regulated in cancer cell lines and primary tumors (Figs 1-3). Interestingly, however, all of cancer specimens harboring NORE1B reduction also showed low NORE1A level, but none of tumors showed NORE1B-specific reduction, indicating that NORE1A is more commonly down-regulated in colorectal cancers (Fig 2B).

(2) We determined the mutational status of K-Ras in 80 primary carcinoma tissues examined in this study using DNA-SSCP and sequencing analysis. As summarized in Fig 2D, we found no association between expression status of NORE1A or NORE1B and the mutational status of K-Ras in tumor specimens we tested. The result was described in the Results and Discussion sections in this revision. Although we did not evaluate the possible role of NORE1 in p53 signaling, our finding of the significant growth suppression of mutant p53-carrying Caco-2 cells by NORE1A or NORE1B indicates that both NORE1A and NORE1B have p53-independent growth arrest and apoptosis-enhancing functions.

(3) We carried out functional study to define effects of NORE1A and NORE1B expression on tumor cell growth. For these assays, we constructed expression vectors encoding wild-type
WT) NORE1A and WT-NORE1B and siRNAs against NORE1A and NORE1B, and performed cell number counting, apoptosis, and colony formation assays. As we presented in Figs 5A-5I in this revision, using two cancer cell lines (Caco-2 and HCT116), we observed that both NORE1A and NORE1B exert growth suppression effects (inhibition of cellular growth and colony formation and induction of apoptosis). We newly described these results in Results and Discussion.

(4) Our study shows that abnormal reduction of NORE1 (both NORE1A and NORE1B) is more frequently in tumors of advanced stages and high grades compared to tumors of early stages and low grades, indicating that NORE1 reduction is associated with malignant tumor progression. However, the observation that a substantial fraction of early stage and low grade tumors also shows NORE1 reduction suggests that NORE1 abnormality might be implicated in early progression. Considering that epigenetic gene silencing is a relatively early event during tumorigenesis processes, our finding suggests that epigenetic inactivation of NORE1 might be implicated in the development and/or early progression of colorectal tumors. We agree that more detailed and large scale study is required to define the role of NORE1 inactivation in colorectal tumorigenesis, and in this context, our comment is a general suggestion based on the limited results.

(5) Tumor tissues examined in this study include many specimens obtained from recently diagnosed cancer patients, and we have a separate plan for clinical study including an immunohistochemical analysis of NORE1A and NORE1B in clinical specimens whose histopathologic characteristics and prognosis information of the patients, including survival status, are available. We hope to present the data in near future.

Minor issues: The classification of tumors into low and high “expressors” is not justified and has no biological meaning. Taking into account that authors have quantified the expression levels using densitometry, it would be more appropriate to use statistical parameters (mean, SD) to compare the different groups of tumors as it has been done for normal-tumor comparisons.

Reply: We agree to the reviewer opinion that the classification of tumors into low and high “expressors” is not justified and has no biological meaning. For simple and convenient discrimination, we have used this arbitrary criterion for several years, which is based on a half of normal mean as described in the text. We think that there can be no absolute criteria for determination of abnormal expression levels of mRNA or protein. According to the reviewer’s
indication, we newly presented expression data (NORE1/GAPDH) from some matched tissue sets (Fig. 2B). When we compare expression levels of NORE1A and NORE1B between cancerous and noncancerous tissues of the same patients, we regarded more than 40% difference in expression level as low expression. This is also an arbitrary criterion we have utilized for several translational studies considering tumor tissues contain at least 10% contamination of normal cells such as infiltrating lymphocytes. We understand your consideration on this issue and appreciate this indication.