Author's response to reviews

Title: Temporal Evolution in Caveolin 1 Methylation Levels During human Esophageal Carcinogenesis

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Dear Editor:

We greatly appreciate the reviewers’ insightful suggestions and are encouraged by their strong interest in our manuscript. Please find below our point-by-point responses to their comments:

Reviewer's report:

Reviewers: 1

In this manuscript entitled "Temporal Evolution in Caveolin 1 Methylation Levels During human Esophageal Carcinogenesis" the authors investigated whether and at which neoplastic stage promoter hypermethylation of CAV1 is involved in human esophageal carcinogenesis. The authors found CAV1 methylation is significantly higher in Barrett’s metaplasia (BE), low-grade and high-grade dysplasia occurring in BE, esophageal adenocarcinomas (EAC), esophageal squamous cell carcinomas (ESCC) than in normal esophagus. They also observed that normalized methylation values (NMVs) of CAV1 in EAC and ESCC are significantly higher than in corresponding NE. Based on these findings, they concluded that CAV1 promoter hypermethylation is a frequent event in human esophageal carcinomas and is associated with early neoplastic progression in Barrett’s esophagus.

In overall, the manuscript is well organized and contains potentially interesting findings.

We are very pleased that this reviewer appreciated the value of our cohort study and its clinical ramifications.

However, there are some concerns.

1. Although this study contains well-performed statistical and comparative analysis of CAV1 methylation, the manuscript lacks the novelty considering that several previous studies reported CAV1 methylation status in subtypes of esophageal cancer.

2. The CAV1 promoter includes several CpG-rich regions that display differential methylation patterns, depending on tumor type or stage. In this context, authors need to provide more detailed information and analysis results for the promoter region (CpG sites) examined in this study.

We thank the reviewer for this very astute suggestion. Our group previously demonstrated that CAV1 promoter hypermethylation in this CpG-rich region correlates with silencing of this gene in colon cancer (see ref. 27, below and in our mss.). Furthermore, in the current study, our data revealed reversal of methylation and restoration of CAV1 expression induced in EAC cells (OE33) by 5-Aza-dC treatment (Figure 4). These results further support our conclusion that DNA hypermethylation of this promoter region silences CAV1.

Ref.27.

3. Considering the possible differential effects of the CpG-rich regions on gene transcription as mentioned above, authors need to validate that the promoter methylation status reflects CAV1 protein expression status in tumor tissues.

We, too, would have liked to perform these experiments, but unfortunately, due to the severely limited size of these small biopsy samples, there was no tissue remaining with which to perform these protein studies.

4. Authors found no significant association between CAV1 promoter hypermethylation and pathological characteristics of tumors and patient survival. In this context, authors need to describe clinical relevance of their findings.

In this study, we did not find a significant association between CAV1 promoter hypermethylation and pathological characteristics of tumors or patient survival. However, our results demonstrate that CAV1 promoter hypermethylation occurs frequently in both human EAC and ESCC (Table 1). CAV1 NMVs in T were significantly higher than those in corresponding NE (p < 0.01, Student’s paired t-test) in 41 cases with corresponding NE and T (Figure 2). Moreover, hypermethylation of the CAV1 promoter was significantly more frequent in premalignant lesions, such as BE and D, as well as in EAC, than in NE (Table 1). These results suggested that hypermethylation of CAV1 may represent an early epigenetic event in these patients. Therefore, the following sentence has now been added in the Results and discussion section(p.12): “These results suggest that hypermethylation of CAV1 may represent an early epigenetic event in these subjects, that the frequency of this epigenetic event increases during esophageal
carcinogenesis, and that this event is highly prevalent in human esophageal cancers.”

5. Several previous studies demonstrated that expression of CAV1 and/or CAV1 is elevated in esophageal squamous cell carcinoma compared to corresponding normal tissues and its elevation is associated with malignant progression and poor survival of the patients (Cancer 2002: 94:929-33; Oncol Rep. 200718:601-9). Therefore, authors need to discuss in more detail on this discrepancy.

We thank this reviewer for pointing out this important issue. Indeed, Kentaro et al. (Cancer 2002: 94:929-33) and Takuya et al. (Oncol. Rep. 200718:601-9) investigated the relationship between ESCC clinicopathological factors ESCC and the expression of CAV1. Although both studies demonstrated that overexpression of CAV1 is associated with clinicopathological factors, the two studies disagreed with each other. Specifically, Kentaro et al. showed that CAV1 immunostaining correlated with N classification (p=0.023), but not with T factor (p=0.593) or lymphatic invasion (p=0.476). In contrast, Takuya et al. revealed that CAV1 immunostaining showed a significant positive correlation with T factor (p=0.0468) and lymphatic invasion (p=0.0378), but not with N factor (p=0.3007).Kentaro and Takuya concluded that overexpression of CAV1 is associated with a worse prognosis in ESCC. However, by multivariate analysis, Kentaro found that CAV1 was not an independent prognostic factor (RR=1.325; p=0.361), while Takuya demonstrated that CAV1 was an independent prognostic factor (RR=3.858; p=0.0164).

These inconsistent results between Kentaro’s and Takuya’s as well as our own studies may be due to different analytic methods used (e.g., we employed real-time qMSP, while Kentaro and Takuya applied immunohistochemistry); or to different ethnic groups and sample sizes (we studied 260 specimens, while Kentaro and Takuya investigated 130 and 47 specimens, respectively).

In response to this important point, we have now added the following explanation in the Discussion section (p.13): “Two previous studies demonstrated that expression of CAV1 was elevated in ESCC compared to corresponding normal tissues, and its elevation was associated with malignant progression and poor survival [43, 44]. These inconsistent results may have been due to different analytic methods used, ethnic groups studied, and smaller sample sizes in the previous studies.”

Reviewers: 2
This study characterizes CAV1 gene in an important human malignancy. It is well written, and supported by carefully performed techniques and interpretation of results. Novel information is derived from this study with potential clinical application.
We greatly appreciate this reviewer’s insightful suggestions and are encouraged by his/her strong interest in our manuscript. Please find below our point-by-point responses to his/her comments:

However, I have the following concerns that should be addressed before accepting for publication:

1. How were these patients followed up to determine the outcome? The authors need to describe it more detail.

   In response to this helpful recommendation, we have now added the following explanation to our Tissue Samples section (p.6): “Outcome data were derived from a comprehensive database maintained by the institution’s cancer registry and from patients’ medical records at the University of Maryland and Baltimore Veterans Affairs Medical Centers.”

2. It is well-known that smoking and alcohol drinking are modifiers of DNA methylation. But it seems that the authors did not take this into account in data analysis. Smoking and alcohol drinking should be shown in Table 1. This issue should also be discussed.

   We thank the reviewer for this very astute suggestion. We added the information in the new Table 1 in the revised version of manuscript. As mentioned in p.11, in our study “No significant associations were observed between CAV1 promoter hypermethylation and patient age, survival (data not shown), smoking or alcohol consumption status, BE segment length, tumor stage or lymph node metastasis, histologic tumor differentiation, or histologic type of esophageal carcinoma (Table1).” Due to word limitations, therefore, we did not discuss this point further.

3. The expression of CAV1 on RNA and protein levels should be analyzed to demonstrate "hypermethylation of CAV1 promoter leading to gene silencing..." on patients' specimens. Post-transcriptional regulation may also exist.

   We, too, would have liked to perform these experiments, but unfortunately, due to the severely limited size of these small biopsy samples, there was no tissue remaining with which to perform these studies.

4. The cell line data is limited to one cell line and it is not surprising that a non-specific demethylating agent will increase CAV1 expression levels.

   Despite numerous attempts, we were unable to find additional cell lines with adequate silencing of CAV1 for these reactivation experiments, and unfortunately only very few of these types of cell lines are available.
5. How reproducible were these assays?

In response to this thoughtful question, we have now added the following sentence to the Results section (p. 9): “All assays in this study were performed in duplicate format, and data showed reproducible and concordant results.”

6. References for the formula of NMV and NRV are missing.

The reference supporting the formula for NMV and NRV has been added on p.8 and as reference 21.

Reviewers: 3
These are well-performed studies. They provide useful information about the understanding of the molecular mechanisms underlying esophageal carcinogenesis. The manuscript is suitable for the readership of BMC Cancer.

We are very pleased that this reviewer appreciated the value of our cohort study.

Thank you very much again for these highly thoughtful and timely reviews of our manuscript. If there are any further questions or comments pursuant to this letter, please contact us at 011-86-755-86671904 or 410-502-6057 or via email at zhejin1995@yahoo.com or smeltzer@jhmi.edu.

Sincerely yours,

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