**Author's response to reviews**

**Title:** Upregulation of heme oxygenase-1 in colorectal cancer patients with increased circulation carbon monoxide levels, potentially affects chemotherapeutic sensitivity

**Authors:**

Hongzhuan Yin (chad156@hotmail.com)
Jun Fang (fangjun@ph.sojo-u.ac.jp)
Long Liao (lyoulyuu@yahoo.co.jp)
Hiroshi Maeda (hirmaeda@ph.sojo-u.ac.jp)
Qi Su (chad156@hotmail.com)

**Version:** 4  
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Long Liao (lyoulyuu@yahoo.co.jp)
Hiroshi Maeda (hirmaeda@ph.sojo-u.ac.jp)
Qi Su (suqi100@hotmail.com)

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The Biomed Central Editorial Team

Object: MS: 2064028100119962 - Cardiac events in patients with idiopathic ventricular fibrillation excluding patients with the Brugada syndrome. Dr Hongzhuan Yin et al.

Thank you for consideration of our manuscript for publication in your journal. We gratefully appreciate the reviewers for their valuable comments and suggestions. According to their comments, we carefully checked the text and refined the English, as well as did some new experiments. All the corrections and revisions are highlighted in the revised MS. The following is the reply to each reviewer point by point.

* Please be noted that the page and line number may be changed after MS is changed to PDF file.

Reviewer 1 (Dr. Tomohiro Sawa)

The data presented are interesting, and the experiments were well performed. Only point that should be revised is that control experiments with ZnPP treatment in cell culture experiments. The authors examined the effects of ZnPP on drug resistance for cancer cell lines (Fig. 6). Does ZnPP treatment alone at this concentration (0.5 microM) affect cell viability? This notion should be included in the manuscript.

Thank you very much for your comments. We examined the cytotoxicity of ZnPP on C26 cells, and found it did not apparent affect the cell viability at the dose of 0.5 µM. This results are now included in the Supplement data (Fig. S2), and descriptions are added in the revised MS (P11L3-5).
Reviewer 2 (Dr. Takahiro Seki)

*However, there is an issue regarding manuscript that should be addressed. Authors showed that it is unlike to the impact of age, gender, metastasis and so on, except tissue character, to HO-1 expression analyzed by Fisher’s extract test, however, Table 2 showed that lymph node metastasis has a tendency of HO-1 expression level. It would be very nice to see multivariate statistics with different parameters.*

Thank you very much for your comments. We agree with the reviewer that multivariate statistics may be good to analyze the data, and we discussed with a statistician in our University, the suggestion from the statistician is that it is a little difficult to do such analysis partly because of the limited samples. So we are planning to continue this study by enrolling more CRC patients, and we hope do detailed analysis about HO-1 expression with CRC using multivariate statistics.

*Specific points, I appreciate that some typographical issues will be pick up by editors, but I would recommend reading through and checking them before resubmission, e.g. plops tissues on page 8 line 4.*

We carefully checked the text and corrected the typographical errors including the point you mentioned (P9L7). Thank you.

*Please add scale bars in Figure 1 and Figure 2.*

P5 line 1. Please describe formalin concentration.
P5 line 13. Please mention company name, not distributor, and product code.
P5 line 17. Please describe numbers of the pathologists who evaluated the staining samples.
P7 line 18. What does it meant of PBS (-)?

Done (revised Figure 1, 2, and P5L1, P5L13, P5L17-18, P8L6 of the revised text). Thank you.

*P6 line 10. Please add methods for quantification of the PCR band.*

Descriptions for quantification of PCR band is added in revised MS (P6L25-28).

P8 line 20. *No significant is right, however, there is a tendency in lymph node according to Table 2. Thus, it should be mentioned.*
Thank you very much for your comments. Descriptions about this issue are included in the revised MS (P9L23-25).

*P11 line 8. The sentence sounds not clear, therefore, it should be revised to clear what authors would like to tell.*

This sentence is revised (P12L30, P13L1). Thank you very much.

*Please provide internal genes, such as action and GAPDH, bands in Figure 6 a-c and revise y-axis to 100% of Figure 6d, allowing us to compare with other cell lines.*

Done. Thank you.
Reviewer 3 (Dr. Leo Otterbein)

Major

1.) Given the elevated CO - whether CO contributes to cancer survival/bioavailability should be tested. comparatives with the bile pigments would also be important to evaluate as additional products generated by HO-1. Are elevated bilirubin levels observed in the blood to correlate with COHb?

Thank you very much for your comments. As you suggested, we examined the effect of CO (by use of CORM2) on the viability of C26 cells under the treatment of THP, the data showed similar to that of HO-1 inducer hemin, suggesting CO is at least an important effector molecule of HO-1 mediated cytoprotection. This result is included in revised MS as Supplemental data (Fig. S3a), and descriptions and discussions regarding this issue are added in the revised text (P11L12-14, P14L13-16).

Also, we investigated the circulation bilirubin levels in CRC patients compared to non-tumor patients. However, no elevated bilirubin levels were observed (Supplemental data Fig. S1), which did not correlate with the results of COHb. This may probably due to that the circulation bilirubin level does not only reflect heme degradation, but is largely influenced by liver function, thus it may be difficult to evaluate in vivo HO-1 activity by circulation bilirubin levels. Descriptions and discussions regarding this issue are now included in the revised text (P7L19-24, P10L14-19, P13L29-30, P14L1-4).

2.) measures of proliferation in vitro should be compared to viability.

We added a cytotoxicity LDH assay to compare with the results of MTT assay, which showed consistent data in C26 cells (Supplemental data Fig. S4). Methods, results and discussions regarding this issue are added in the revised text (P7L7-9, P11L7, P11L16-17). Thank you.

3.) the use of Zn-PP is not ideal and it is unclear if Zn-PP has effects on viability independent of HO-1 activity. Complementary data with siRNA or perhaps the more HO-1 selective compounds generated and published by Dr. Nakatsu would be more selective.

We agree with the notions of the reviewer, accordingly we did the in vitro experiments with siRNA of HO-1 in C26 cells. We found similar effect on enhance the cytotoxicity of THP as showed in ZnPP treatment, and thus considered the effect of ZnPP in our study is mostly due to its
inhibitory effect on HO activity. Descriptions and discussions regarding this issue are added in the revised text (P7L10-18, P11L18-22). Thank you very much.

4.) While COHb reflects HO-1 activity, it is not direct proof that CO is arising via HO-1 in the tumor. It could reflect other pathology in the patient or if other stressors were present (e.g., chemotherapeutics) that could increase HO-1 in other non-cancerous tissues in the body and thereby increase COHb levels. Activity assays of the tumor material should be performed as well, given the amount of literature showing that increased expression does not necessarily correlate with activity.

We absolutely agree with the reviewer. HO-1 does not express only in tumor, it also reflect other pathological conditions such as stress and chemotherapy, So in this study patients with history of chemotherapy were excluded to minimize the external influence. As the results of CRC patients were compared with patients with similar characteristics (e.g., age, sex) but only without tumor, we considered the increased COHb was mostly due to the highly expressed HO-1 in tumor, however further investigations using different populations and patients are needed to clarify this point (P13L19-24).

Regarding the HO activity, because stocked CRC samples from the patients enrolled in the present study are currently not available, we could not carry out this study in clinical samples, and we hope we will so this assay in the future. But in stead, we measured the HO activity of C26 solid tumors and compared it with circulation levels as well as tumor growth, and we found that circulation levels were increased in parallel with HO-1 activity in tumor as well as tumor growth (revised Fig. 5, P13L25-26). The method of measuring HO activity is also included in the revised text (P8L13-27). Thank you very much.

**Minor**

1.) It seems the gels have been spliced in Fig 6. This should be repeated and presented in one gel or otherwise explained.

Thank you very much for your comments. We repeated this experiment and the data in Fig. 6 are revised accordingly.

2.) The authors take some liberty in labeling cell types in the tissue IHC in Fig 1 without positive
markers. Macrophage and endothelial cell specific markers should be used and preferably co-localization staining with HO-1.

Because the histological staining was done by clinical pathologists and no extra sample slides for other staining such as macrophage and endothelial cells were prepared, it is difficult to find the same sections for the markers staining. However, the cell types showed in Fig. 1 were analyzed and indentified by expert pathologists. But definitely you are right, and we will do such staining of positive markers in future study. Thank you very much for your consideration.