*Cell Death & Disease* October 18th, 2018

Dear Professor Piacentini,

We appreciate for your letter about the information of our manuscript entitled “Epigenetic silencing of ZNF132 mediated by methylation sensitive Sp1-binding promotes cancer progression in esophageal squamous cell carcinoma (ESCC)”. The revised manuscript has benefited greatly from consideration and incorporation of the constructive and insightful comments from you and the reviewers. As for the reviewers’ comments, a point-by-point description of our responses is as follows, where the reviewers’ comments are in italics and our responses in Times New Roman type.

Enclosed is the revised version of the article with the title “Epigenetic silencing of ZNF132 mediated by methylation sensitive Sp1-binding promotes cancer progression in esophageal squamous cell carcinoma (ESCC)” and the response to the comments. The manuscript has been resubmitted to your journal. Please do not hesitate to contact me, if there is any further questions.

Sincerely,

Minghua Wang, Ph.D.

Professor,

Department of Biochemistry and Molecular Biology

Soochow University

**Reviewers’ Comments:**

**Referee #1**

This manuscript demonstrates that hypermethylation of ZNF132 promoter would be a novel biomarker in human esophageal squamous cell carcinoma (ESCC). The authors employed methylation target bisulfite sequencing (MTBS) to compare the methylation levels of cancer tissues to adjacent normal tissues in 91 ESCC patients, and found that the level is higher in cancer tissues. After demonstrating that the methylation level of ZNF132 promoter affect its expression, the authors explored the function of ZNF132 on tumor growth, invasion and metastasis in vitro. They also used xenograft model to further verify the role of ZHF132 in inhibiting tumor growth. Lastly, they suggest that hypermethylation interfere with Sp1 in binding to the ZNF132 promoter by ChIP analysis.

We thanks the great suggestion and comments.

1, Is there any difference in the methylation level of *ZNF132* in ESCC cell lines with different invasive and metastatic abilities?

We thanks this great question. We tried to compared the ZNF132 methylation with invasive and metastatic abilities with public database, such as NCI-60 methylation database ([Reinhold et al., 2017](#_ENREF_1)) and Richard’s human cell line methylome project ([Varley et al., 2013](#_ENREF_2)) , what a pity, we didn’t find multiple ESCC cell line methylation data. Meanwhile, we do not have enough ESCC cell line for methylation and invasive ability analysis. However, we still try to answer your question with our current dataset somehow. In our study, we applied target methylation sequencing to detect the methylation level of ZNF132 in CaEs-17 and Ec-109 ESCC cell, which have been used in our study. We found the DNA methylation status of ZNF132 promoter region are hyper-methylation in both CaEs-17 and Ec-109 (methylation level, average beta ~ 0.8 see the following figure). We found the methylation level of Ec-109 is a little bit higher than CaEs-17, even though the statistical different is not significant (P=0.07). Compared with Figure 3E and 3F, we can find Ec-109 is a little bit more aggressive than CaEs-17 which is consistent with that Ec-109 have a little bit higher methylation level. Therefore, it seems that the cell lines with hyper-methylation status with higher invasive ability, which is consistent with our further cell-biology and mice model experiments.

Figure 1. Methylation profile ESCC cell line of Ec-109 and CaEs-17. Methylation levels for 14 CpGs in the promoter region was detected by MTBS assay in Ec-109 and CaEs-17.

2, Fig4 suggests that overexpression of ZNF132 could inhibit tumor growth. How about using demethylation drugs in treating ESCC?

We thanks this great question. Yes, as we known, *Azacitidine* and [*Decitabine*](https://en.wikipedia.org/wiki/Decitabine) (5-aza-2′-deoxycytidine) have been applied in treatment of [myelodysplastic syndrome](https://en.wikipedia.org/wiki/Myelodysplastic_syndrome) since 2004. What’s more, demethylation drugs have been widely used in the treatment of sarcomas, blood and bone marrow cancers. Recent clinical application to [*Decitabine*](https://en.wikipedia.org/wiki/Decitabine) was applied in pancreatic cancer treatment together with Gemcitabine. [Decitabine](https://en.wikipedia.org/wiki/Decitabine) treatment in ESCC was still in the clinical trial (Phase I stage) by David S. Schrump from Warren Grant Magnuson Clinical Center and the last update time is 2015. We didn’t receive further news about the clinical trial yet. The efficiency for demethylation drug for solid cancer is not as good as circulating cancers, however, it is still a good choice when there is no better therapy approach, such as pancreatic cancers.

**References**

Reinhold, W. C., Varma, S., Sunshine, M., Rajapakse, V., Luna, A., Kohn, K. W., . . . Pommier, Y. (2017). The NCI-60 Methylome and Its Integration into CellMiner. Cancer Res, 77(3), 601-612. doi: 10.1158/0008-5472.CAN-16-0655

Varley, K. E., Gertz, J., Bowling, K. M., Parker, S. L., Reddy, T. E., Pauli-Behn, F., . . . Myers, R. M. (2013). Dynamic DNA methylation across diverse human cell lines and tissues. [Research Support, N.I.H., Extramural]. Genome Res, 23(3), 555-567. doi: 10.1101/gr.147942.112

**Referee #2**

In this manuscript, Jiang et al identified ZNF132 as a novel ESCC hypermethylation biomarker. They found that forced expression of ZNF132 in ESCC cells repressed cell growth, migration and invasion abilities in vitro, and xenograft tumor formation in vivo. They also found that hypermethylation of Sp1 biding site in ZNF132 promoter reduced Sp1 dependent transcription activation. Overall, this is an interesting piece of work which could be used as potential therapeutic targets in future. However, the manuscript should be improved by addressing the following concerns before publication in CDD is recommended.

We thanks the great suggestion and comments.

Minor concerns:

1. Forced expression of ZNF132 in each assays including transwell and xenograft mouse model should be validated by western blot instead of real-time PCR.

Thanks for the reviewer’s great suggestion. We have verified the expression of ZNF132 in a xenograft mouse model by western blot as suggested. We found the protein level of ZNF132 is significantly increased in ZNF132 treatment group compared with control great. The result is added in Figure 4F. In addition, regarding the transwell experiment, our aim was to observe the effects of ZNF132 overexpression on cell proliferation, cell migration and cell invasion of esophageal cancer cells by transferring ZNF132 gene into esophageal cancer cells. Before the formal experiment, we first determined the expression of ZNF132 cells and CaEs-17 cells in Ec-109 by q-PCR and Western blotting as shown in Figure 3A and Figure 3C. We hope this explanation could solve your questions.

2. It will be nice to examine the protein expression pattern of ZNF132 in clinical ESCC samples besides the real-time PCR examination.

We thanks for the reviewer’s great suggestion. It is really a great idea. In the stage of study design, our initial plan is to identify novel DNA methylation based diagnosis biomarkers for esophageal squamous cell carcinoma by two stages investigation approach including: 1) genome-wide methylation screening and 2) clinical sample validation. With this method, we have reported our finding in Front Genetics (2018)1 and Clinical Epigenetics (2017)2 as we mentioned in the manuscript. ZNF132 is another interesting finding to be frequently hyper-methylated in cancer patients. Interesting thing is when find the hyper-methylation can significantly decrease the gene expression of ZNF132 and that is the reason why we started this project. However, since our initial project is focus on DNA methylation biomarker, we only collected DNA and RNA from the clinical samples without the protein. We will conduct this assay in our further investigation, especially, when we collect enough samples with more clinical and demographic information. We hope to get the understanding and to report the further result in our next study.

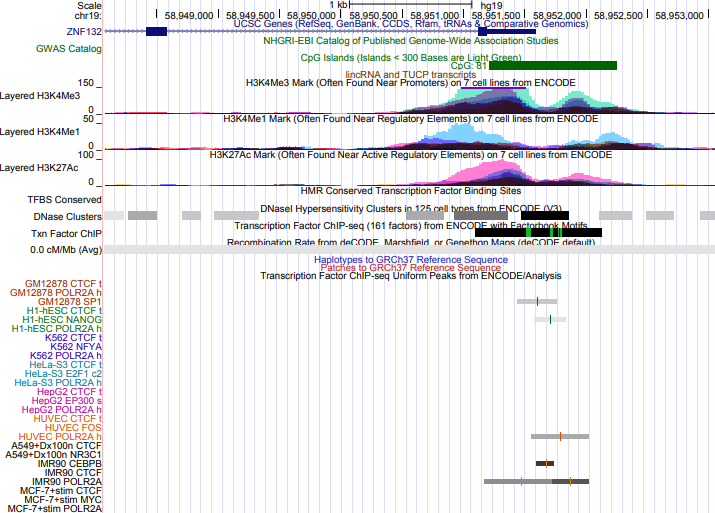
1. Wang, C., W. Pu, D. Zhao, Y. Zhou, T. Lu, S. Chen, Z. He, X. Feng, Y. Wang, C. Li, S. Li, L. Jin, S. Guo, J. Wang, and M. Wang. "Identification of Hyper-Methylated Tumor Suppressor Genes-Based Diagnostic Panel for Esophageal Squamous Cell Carcinoma (ESCC) in a Chinese Han Population." Front Genet 9, (2018): 356.
2. Pu, W., C. Wang, S. Chen, D. Zhao, Y. Zhou, Y. Ma, Y. Wang, C. Li, Z. Huang, L. Jin, S. Guo, J. Wang, and M. Wang. "Targeted Bisulfite Sequencing Identified a Panel of DNA Methylation-Based Biomarkers for Esophageal Squamous Cell Carcinoma (ESCC)." Clin Epigenetics 9, (2017): 129.

3. The scientific logic or references for analyzing Sp1 binding ability should be described in detail in P.13 after “As CpG was in silico predicted to be harbored in transcriptional activator Sp1-binding site at ZNF132 promoter”

We thanks for the reviewer’s great suggestion. SP1 belongs to the [Sp/KLF family](https://en.wikipedia.org/wiki/Sp/KLF_family) of transcription factors and it contains a [zinc finger protein](https://en.wikipedia.org/wiki/Zinc_finger) motif, by which it binds directly to DNA and enhances gene transcription. Its zinc fingers are of the Cys2/His2 type and bind the [consensus sequence](https://en.wikipedia.org/wiki/Consensus_sequence) 5'-(G/T)GGG**CG**G(G/A)(G/A)(C/T)-3'. CpG is located in the center of the SP1 binding motif and therefore we tried to investigate the effect of methylation status to the binding influence to SP1 in the ZNF132 promoter. We also add the following Figure as the Supplementary Figure 1 to help the reader figure out the binding regions of SP1.

We update the follow contents into the main manuscript in the page 12.

“In order to demonstrate the ZNF132 gene regulation mechanism, we tried to investigate the transcriptional factors binding status in the ZNF132 promoter region. We found the 6-base-pair (GGGCGG) SP1-binding motif located in the CpG-island of ZNF132 promoter region and this binding region was supported by ENCODE Transcription Factors ChIP-seq data (See the following Figure 2). We then try to determine whether methylation of Sp1-binding site play a role in ZNF132 expression regulation”



Supplementary Figure 1. ENCODE ChIP-seq data to show SP1 bindings in CpG-island of ZNF132 promoter region. Integrated Regulation from ENCODE Tracks based on hg19 was captured from UCSC Genome Browses. Transcription Factor ChIP-seq (161 factors) from ENCODE with Factorbook Motifs  Data version: ENCODE Mar 2012 Freeze was used in this study. .

4. How the promoter region of ZNF132 in ESCC is hyper-methylated should be further discussed.

We thanks for the reviewer’s great suggestion. Definitely, this question is quite interesting and important. Cancer is a complex disease with wide range abnormal from mutation, genome structure instability, methylation profile change, miRNA profile change and protein network change. Actually, DNA methylation changes is caused by the interaction between environment exposures (such as smoking, drinking) and genetic variations and gene expression abnormal (such as transcriptional factor expression change). DNA methylation abnormal change is an easy way to check the epigenetic abnormal in cancer and other diseases, however, the mechanism for the methylation abnormal might have different reasons for each individual or tissue types.

We add the following contents in the discussion section to illustrate it:

As the DNA methylation abnormal theoretically caused by the harmful environment and genetic variants exposure, such as smoking, drinking as well as risk allele carrying and it might be different environment triggers since they contact different environment. Since DNA methylation can be reversed by the appropriate treatment, DNA methylation is considered to be promising diagnosis and prognosis biomarker.