Professor Mikhail V. Blagosklonny, MD, PhD

Co-Editor-in-Chief

*Oncotarget*

Dear Professor Mikhail,

We appreciate for your letter about the information of our manuscript entitled “Aberrant methylation of *FHIT* can be a diagnostic biomarker for NSCLC in Asian population”. 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 black colour and our responses are highlighted in red.

Enclosed is the revised version of the article with the new title “Quantitative assessment of the diagnostic role of *FHIT* promoter methylation in non-small cell lung cancer”.

Thank you so much for handling with our manuscript for us!

Sincerely,

Jiucun Wang, Ph.D.

Professor,

School of Life Sciences

Fudan University

**Reviewer #1 (Comments to the Author (Required)):**

**Question 1**: Were the two compartments (i.e. tissue and plasma) combined or analyzed separately?

**Answer 1**: Yes, you are quite right. Combined and separated analysis were both conducted in current study to indicate the utilization in non-invasive diagnosis.

**Question 2**: All Figures are not numbered and have no legend.

**Answer 2**: Thanks very much for pointing out our neglect. In the revised version, we have updated all of the figures with numbers and legends according to your comments.

**Question 3**: I am not convinced that an aberrant FHIT gene methylation is a clinically useful marker for the detection/diagnosis of lung cancer in an Asian population.

**Answer 3**: We are appreciated for your concerns. Actually, we tried to make full use of published literature and microarray data to make deep insight of the potential role of FHIT methylation in diagnosis. Our study might make the conclusion with the largest sample size, including 2119 NSCLC samples (1244 Asian samples and 819 Caucasian samples as well as 56 Egyptian samples, respectively), and thus there might be several confounding factors which could affect the results in different ethnic samples. Although we have found the difference between Caucasian and Asian samples, because it needs further clinical validation to make a final conclusion that “Aberrant methylation of FHIT can be a diagnostic biomarker for NSCLC in Asian population”, we changed the title of the manuscript to “[Quantitative assessment of the diagnostic role of FHIT promoter methylation in non-small cell lung cancer](https://scholar.google.com/citations?view_op=view_citation&hl=en&user=4tIViCAAAAAJ&citation_for_view=4tIViCAAAAAJ:-jrNzM816MMC)”.

**Reviewer #2 (Comments to the Author (Required)):**

This is a well written article about a trendy topic for lung cancer. Its strengths are the number of patients recorded in the study as well as the differential findings between distinct populations. However, it falls short of expectations in aspects that need major overhaul.

**Question 1**: This article does not expand on its major point: FHIT as a diagnostic biomarker. Indeed, the manuscript only reports statistical associations regarding alterations of FHIT promoter methylation and diagnosis of lung cancer in Asian patients. The observations of aberrant methylation of FHIT promoter in lung cancer has already been expanded upon in several papers since 2004. I would expect that this manuscript expands on the value of FHIT as biomarker per se. Notably, it is not explained whether this association is also present in other cancer subtypes, or whether other conditions lead to alterations of FHIT promoter methylation. These are crucial questions to assess the relevance of these alterations in clinical practice, and as such, their value as a biomarker. In addition, it is not explained how the alterations of FHIT methylation can be used in clinical practice. What is their added value compared to standard pathological and immunohistochemistry analysis? Which techniques might be used now or in the foreseeable future to take advantage of these alterations, at a patient's scale?

Unless those questions are answered, I feel that the article will not be able to prove its point, and would not go much further than reports from historical studies. In summary, its content and its conveyed message should be importantly modified.

**Answer 1**: We are appreciated for your concerns and recognition. Our current study is the latest un-biased quantitatively evaluation to the association between FHIT and NSCLC with large number of lung cancer samples (1304 solid tissues and 815 plasma samples). it is quite important to provide the quantitative evidence/evaluation to decide whether this biomarker is valuable enough for future research in prospective cohort which would be cost huge number of funds since it would be conducted in plasma for the non-invasive diagnosis. And you are quite right, the diagnosis role of FHIT has been investigated since 2004, however, the contradiction was frequently occurred in different studies. That’s the reason why we should conduct this study as soon as possible.

To answer your second question, we check the methylation status in other 8 cancer types (8 cancer type, totally 1034 samples) in TCGA datasets and we found no significant difference of FHIT promoter methylation status in the 8 cancer types. We have added these result as the supplementary Table 4. However, because nearly all samples included in these microarray datasets were Caucasian, while the differential methylation of FHIT promoter methylation was found only in Asian samples, thus we need to do further research on the methylation status in Asian samples with high-throughput technology due to its robustness. Because it needs further clinical validation to make a final conclusion that “Aberrant methylation of FHIT can be a diagnostic biomarker for NSCLC in Asian population”, we changed the title of the manuscript to “Quantitative assessment of the diagnostic role of FHIT promoter methylation in non-small cell lung cancer. As for your third question, in our subgroup analysis, we found no significant difference between the tissue subgroup and non-tissue subgroup (mainly with serum/plasma), providing the robustness to test the differential methylation status of FHIT promoter in serum rather than in tissues, which can be utilized in the newly developed liquid biopsy methods. When compared with the traditional methods like pathological and immunohistochemistry analysis, the liquid biopsy can detect the occurrence of cancer with only peripheral blood but not the tissues, making the cancer detection much more convenient and reduce the patients’ cost. By detect the methylation status of FHIT promoter or a panel including other biomarkers, we could detect the cancer patients earlier and cheaper thus making the cancer treatment more effective.

As for the remaining questions, all of them are quite important concerns. We believe the reviewer provide the most constructive suggestions to the application of FHIT. We have mentioned these problem in the discussion section as the perspective. In next phase or our next study, we will conduct a study to explain how the alterations of FHIT methylation can be used in clinical practice and what is their added value compared to standard pathological and immunohistochemistry analysis. We also need to answer which techniques might be used now or in the foreseeable future to take advantage of these alterations, at a patient's scale. However, for current manuscript, we hope it can be terminated in this stage.

**Question 2**: The discussion should not be a reformulation of the results section.

**Answer 2**: Thanks very much for your suggestion. In our revised version, we have carefully re-written the discussion part and removing the repeated sentences and paragraphs with the results section.

**Question 3**: I would be interested to know more about the role of FHIT and its complex as tumor suppressors

**Answer 3**: Thanks very much for your question and we have added the description of FHIT as tumor suppressor into introduction part of the revised manuscript FHIT has been long considered as a tumor suppressor. The FHIT gene, located at FRA3B (3p14.2), is a large ten-exon gene[[1](#_ENREF_1)]. FHIT gene is about 1.5 Mb in length, however, the length of its message RNA is only about 1.1 kb. The core of FRA3B overlaps FHIT’s exon 4-6 and it is also the most frequently altered region of FRA3B[[2](#_ENREF_2)]. FHIT has been recently seen as a genome caretaker which is of great importance for genome stability. In general, FHIT loss or protein reduction have been demonstrated in several cancer types, including esophagus, liver, stomach, cervix etc. In addition, multiple studies have found the reduction of FHIT expression in precancerous lesions, indicating its potential suppressing role in carcinogenesis [[3-8](#_ENREF_3)].

The FHIT -/- mice were more prone to develop carcinogen-induced tumors as well as the spontaneous tumors than wild type mice[[9](#_ENREF_9), [10](#_ENREF_10)]. And FHIT viral gene therapy was found to be able to prevent and reverse carcinogen-induced tumors in a gastric cancer mouse model[[11](#_ENREF_11)]. Moreover, recent studies have found that FHIT can also function as the tumor suppressor by inhibiting EMT[[12](#_ENREF_12), [13](#_ENREF_13)].

In conclusion, Fhit loss is occurred at the precancerous stage and can inhibit the EMT process. Further, Fhit can decrease the fragility of CFSs and help to stabilize genome so as to function as a tumor suppressor gene.

**Question 4**: Avoid "and so on" in the introduction, regarding the interests of the study of DNA methylation status. Every features of interest should be stated to prove the point. I hope this will be helpful for your work.

**Answer 4**: Thanks very much for your suggestion. We have deleted the “and so on” in Line54-55 and added the phrases “and low requirements for sample quality” in Line 54-55.

**Reviewer #3 (Comments to the Author (Required)):**

Comments: In current study, Geng [et.al](http://et.al/). demonstrated that FHIT methylation was associated with the incidence of NSCLC by meta-analysis of previous studies but not from TCGA and GEO data. The authors concluded that the diagnostic role of FHIT gene in NSCLC is limited in the Caucasian but may be useful in the Asians. However, evidence of their conclusion remained extremely weak. Authors claimed that TCGA and GEO database are based on only Caucasian, however, these facts are not clearly demonstrated by any objective evidence. Additionally, meta-analysis for FHIT methylation showing similar outcome with this article has already been reported in Feb, 2016 (Wu, et. al, Drug Des Devel Ther. 2016).

**Answer:**We agree with your concerns. In our current study, we collected huge number of NSCLC samples (N=2119) from publish studies and genome-wide array data so that we hope to make an un-biased and stable quantitative evaluation to the diagnosis role of FHIT in NSCLC. Our result showed interesting and surprising results since all the published paper claimed FHIT is significantly hyper-methylated in NSCLC, however, we found the pooled OR is actually not quite high and un-biased microarray data don’t support the significant difference between NSCLC and normal samples. On the other side, we found the significant large OR for Asian samples compared with Caucasian samples with huge number of sample size (11244 Asian samples and 819 Caucasian samples as well as 56 Egyptian samples, respectively). We highly respected your suggestions and therefore we change the title of the manuscript to “[quantitative assessment of the diagnostic role of FHIT promoter methylation in non-small cell lung cancer](https://scholar.google.com/citations?view_op=view_citation&hl=en&user=4tIViCAAAAAJ&citation_for_view=4tIViCAAAAAJ:-jrNzM816MMC)”.As for the sample sources of TCGA and GEO datasets, we have added the Supplementary Table 5-6 to show the actual population sources of the datasets used in our paper. In TCGA datasets, there is only 1 Asian samples but 58 Caucasian samples. And the samples of the three GEO datasets are all from the Europe. Thus, with this exact annotations, we can safely say that the datasets used in our work are based on only Caucasian. We noticed that there are little common things between our study with previous Wu’s study, however, our study is significant different with it. We have much more sample size (2,119 vs 1,717), totally different analysis strategy (two stage vs one stage), analysis depth and width and even the conclusion is different. In Wu’s research, they found that FHIT hypermethylation were significantly differed both in the Caucasian samples and in the Asian samples, while we found no significant difference between cases and controls on the methylation status of FHIT promoter in Caucasian samples. In addition, we also found that the hypermethylation of FHIT was also correlated with the age. In the revised manuscript, we have quoted Wu’s paper in Line 229 and we believe we have better analysis method and quite different significance compared with previous study.

**Question 1**: Authors should investigate about the prognostic impact of FHIT methylation.

**Answer 1**: Thanks for your suggestion. We have seriously done the research from PubMed and Web of Science database to investigate the prognostic value of FHIT methylation in NSCLC and found only 2 papers to be related (PMID: 15042681; 16598757). Maruyama R et al found that FHIT hypermethylation could be a promising prognosis marker for NSCLC, while Nakata S et al found no significant association between FHIT promoter methylation and prognosis of NSCLC. To summary, we felt that the evidence was not enough and the conclusions from the two papers are totally different, making it further hard to generate a robust conclusion on this issue. Due to the lack of supportive evidence, we didn’t mention the prognostic impact of FHIT methylation in the manuscript.

**Question 2**: In the "Result; study characteristic" section, I recommend to add a flow chart showing selection process of eligible studies, which would help readers to understand if studies that do not fit the criteria has been appropriately excluded from the analysis.

**Answer 2**: Thanks for your suggestion. We have added the flow chart showing the criteria for excluding the studies from our analysis as Figure 1 in our revised version.

**Question 3**: Abbreviations should be noted at first, and use only abbreviations afterward.

**Answer 3**: Thanks very much for your correction. In the revised version, we have noted the abbreviations at first and use the abbreviations only afterward.

**Question 4**: I recommend the authors to inform the data for smoking history, and conduct subgroup analysis according to the history, because FHIT has been reported to be related to smoking history not to cancer in one of the article cited in this paper.

**Answer 4**: Thanks very much for your suggestion. We have conducted the smoking subgroup analysis and added the Supplementary Figure 7 and also added the subgroup analysis results to Supplementary Table 2. And we find that there is no significant difference between the smoker% >=68% and smoker% <68% (p –value = 0.46). And 68% is the median of the smoker percent of the total samples and thus was taken as the dividing criteria. And we have added the sentences as following “FHIT has been reported to be related with smoking history but not with cancer, thus we conducted the subgroups of the percentage of smoking samples. And we found no significant difference between the smoker%<68% and smoker% >=68% subgroups (Supp Figure S7)” in Line 119-122.

**Question 5**: Databases noted to be used in this analysis in the "Methods" section do not consistent with that in the "Result" section.

**Answer 5**: Thanks very much for pointing out our neglect. We have corrected it in the Line 249 in the revised manuscript.

**Question 6**: The authors often use the abbreviation "2" for "to". I recommend not to use this abbreviation which is not common to the readers.

Answer 6: Thanks very much for your suggestion. We have thoroughly checked the manuscript as well as the tables and figures and changed the abbreviation “2” with “to”.

**Question 7**: In the first paragraph of "Discussion" section, the authors should excuse the high rate of heterogeneity which is decreasing the reliability of the outcome. Also, the authors should note the limitations of this analysis (e.g. publication bias, English bias) in the "Discussion" section.

**Answer 7**: Thanks very much for your kindly suggestion. We have excused the high rate of heterogeneity in Line 219-223 of the Discussion part. And we also noted the limitations of our analysis in Line 234-242 in the revised version.

**Question 8**: Lots of typos, and errors of sited mismatched figure is crucial, declining the reliability of the article.

**Answer 8**: Thanks very much for your correction. We have updated all of our figures and tables as well as the main text in the revised manuscript. And we believe that there is little mismatches and errors in the revised version.

**Question 9**: Word of 'white population' is inappropriate term in scientific article.

**Answer 9**: Thanks very much for your correction. We have corrected this error in the revised manuscript and replaced it with “Caucasian population”. The changes have been highlighted in red.

**Question 10**: Figure 2D is not attached in this article.

**Answer10**: Thanks very much for pointing out our neglect. In the revised manuscript, we have attached and updated the Figure 2D.

1. Huebner, K., et al., *The role of the FHIT/FRA3B locus in cancer.* Annu Rev Genet, 1998. **32**: p. 7-31.

2. Durkin, S.G., et al., *Replication stress induces tumor-like microdeletions in FHIT/FRA3B.* Proc Natl Acad Sci U S A, 2008. **105**(1): p. 246-51.

3. Guler, G., et al., *Concordant loss of fragile gene expression early in breast cancer development.* Pathol Int, 2005. **55**(8): p. 471-8.

4. Michael, D., et al., *Frequent deletions of FHIT and FRA3B in Barrett's metaplasia and esophageal adenocarcinomas.* Oncogene, 1997. **15**(14): p. 1653-9.

5. Sozzi, G., et al., *Loss of FHIT function in lung cancer and preinvasive bronchial lesions.* Cancer Res, 1998. **58**(22): p. 5032-7.

6. Ahmadian, M., et al., *Analysis of the FHIT gene and FRA3B region in sporadic breast cancer, preneoplastic lesions, and familial breast cancer probands.* Cancer Res, 1997. **57**(17): p. 3664-8.

7. Mori, M., et al., *Altered expression of Fhit in carcinoma and precarcinomatous lesions of the esophagus.* Cancer Res, 2000. **60**(5): p. 1177-82.

8. Yuge, T., et al., *Loss of FHIT expression in squamous cell carcinoma and premalignant lesions of the larynx.* Ann Otol Rhinol Laryngol, 2005. **114**(2): p. 127-31.

9. Fong, L.Y., et al., *Muir-Torre-like syndrome in Fhit-deficient mice.* Proc Natl Acad Sci U S A, 2000. **97**(9): p. 4742-7.

10. Zanesi, N., et al., *The tumor spectrum in FHIT-deficient mice.* Proc Natl Acad Sci U S A, 2001. **98**(18): p. 10250-5.

11. Huebner, K. and C.M. Croce, *FRA3B and other common fragile sites: the weakest links.* Nat Rev Cancer, 2001. **1**(3): p. 214-21.

12. Joannes, A., et al., *Fhit regulates invasion of lung tumor cells.* Oncogene, 2010. **29**(8): p. 1203-13.

13. Joannes, A., et al., *Fhit regulates EMT targets through an EGFR/Src/ERK/Slug signaling axis in human bronchial cells.* Mol Cancer Res, 2014. **12**(5): p. 775-83.

14. Hosseini, S.A., et al., *Common chromosome fragile sites in human and murine epithelial cells and FHIT/FRA3B loss-induced global genome instability.* Genes Chromosomes Cancer, 2013. **52**(11): p. 1017-29.

15. Waters, C.E., et al., *The FHIT gene product: tumor suppressor and genome "caretaker".* Cell Mol Life Sci, 2014. **71**(23): p. 4577-87.