Peer Review File

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Reviewer A

Comment1: It is an interesting study. Please specify the baseline characteristics of the study population including smoking exposure, comorbidities, staging. It is not clear what subpopulations are compared.

Reply1: Thank you for your interest in our research and your valuable suggestions. The baseline characteristics of the study population were described in Table1 (please refer to Page 17, Line 322-328/Attachment Table1), which concluded in section "The negative correlation between *p16* expression and *p16* Cgi D-shore methylation was verified by the TCGA database and in LUAD tissues"

Change in the text: To further investigate the potential inverse correlation between *p16* expression and Cgi D-shore methylation, we examined the levels of *p16* expression, *p16* Cgi methylation and *p16* Cgi D-shore methylation in 15 paired LUAD and paracancerous tissues (the clinical pathological features of these samples are presented in Table 1). However, these clinical characteristics were not significantly associated with the level of *p16* Cgi D-shore methylation ($p>0.05$) (Table 1).

Comment2: Please clarify Clinical implications of these findings. I suggest to include the following references for discussion about the role of p16 in lung cancer

-Thorac Cancer. 2020 Nov;11(11):3060-3070.

-Anticancer Res. 2020 Feb;40(2):983-990

Reply2: Thank you for your comments. The clinical implications of these findings were presented in the "discussion" section, where two promising study designed proposed by Aldo Pezzuto et al. were also discussed (please refer to Page 18, Line 340-359).

Change in the text: Aldo Pezzuto et al. investigated the prognostic value of *p16* in 256 patients with NSCLC who underwent curative surgery. The research findings indicated that *p16* expression was associated with tumor grading and staging ($p<0.05$) and had an impact on overall survival (OS). The average OS was 36 months, but after stratifying patients based on *p16* expression levels, the OS increased to 54 months. Staging stratification showed significant prognostic value for early-stage *p16* expression ($p<0.014$). *P16* significantly influenced prognosis, particularly in early-stage cases, along with other variables such as tumor grading and staging. Although this study did not find a significant relationship between *p16* methylation levels and other variables due to the relatively small sample size ($p>0.05$), a significant regulatory effect of *p16* methylation on its expression level was observed. Methylation-mediated changes in *p16* expression levels may play a crucial role in affecting the prognosis of NSCLC.In their review, Aldo Pezzuto et al. summarized that aberrant expression of the *p16* gene is mainly observed in NSCLC, with *p16* gene methylation being the most common. High methylation of the *p16* gene, along with *p53* and *KRAS* mutations, has been reported to promote lung cancer development in smokers. Furthermore, the promoter

hypermethylation of the *p16* gene leads to gene silencing, which is of great significance in confirming the downregulation of *p16* protein expression in NSCLC.

Reviewer B

Comment1: The style of your writing sound good at the beginning through the end of the article, but it needs more clarify the title of your article.
For example, the transcription activity of OTX2 on p16 expression is significantly blocked by methylation of CpG shore in non-promoter of Lung cancer cell lines.

Reply1: Thank you very much for your interest in our research and your valuable comments. Based on your suggestions, we have revised the title to better clarify the theme of this study (please refer to Page 1, Line 1-2).

Change in the text: The transcription activity of *OTX2* on *p16* expression is significantly blocked by methylation of CpG shore in non-promoter of Lung cancer cell lines

Comment2: Introduction part is too long so I cut some sentence out as label in yellow band (as seen in the attached pdf file).

Reply2: Thank you for bringing attention to this deficiency. We have removed the highlighted content to streamline the Introduction section (please refer to Page 7, Line 105-113/ Page 8, Line 125-127).

Change in the text: ~~Our previous studies have found that exposure to polycyclic aromatic hydrocarbons (PAHs) in coke oven workers can lead to an increase in p16 Cgi shore methylation levels. Cytokinesis blocking micronucleus (CBMN) assay confirmed that the increase of p16 Cgi shore methylation level in peripheral blood lymphocytes (PBL)was related to DNA damage. An increase in the level of methylation in this region and a decrease in the expression of p16 were also found in Bap-induced malignant transformed HBER cells(15).~~

~~Using this method, the function of DNA methylation in a specific region was defined, and the causal relationship between DNA methylation and gene expression was determined.~~

Comment3: Need more discuss about why LUAD tissues is less expressed p16 than the cell lines. May be LUAD tissues have variants mRNA (fusions, mutants) than the cell lines.

Reply3: Upon considering your suggestion, we agree that genetic variants, such as mRNA fusions and mutants, in LUAD tissues might contribute to the differential expression of *p16* compared to cell lines (please refer to Page 22, Line 433-444).

Change in the text: LUAD is known for its significant genetic heterogeneity, and different patients may harbor distinct genetic alterations. This variability in genetic profiles among LUAD tissues might result in diverse *p16* expression patterns. The tumor microenvironment in LUAD tissues can influence gene expression levels. Interactions between tumor cells and surrounding stromal and immune cells can affect the expression of various genes, including *p16*. Regulatory mechanisms, such as mRNA

stability, alternative splicing, and microRNA-mediated regulation, can impact gene expression levels. These mechanisms may differ between LUAD tissues and cell lines, leading to varied *p16* expression. Epigenetic changes, including DNA methylation and histone modifications, can affect gene expression. Differences in epigenetic patterns between LUAD tissues and cell lines may contribute to the observed variation in *p16* expression. While cell experiments may not fully replicate the human environment, they can offer valuable insights to guide subsequent animal and clinical studies.

Comment4: Anyway, the another Transcription factors Zfp148gt/gt a also involve the expression of p16 expression.
Reply4: Indeed, we thoroughly discussed this issue in the Discussion section (please refer to Page 20, Line 394-399).
Change in the text: Several studies have consistently shown that *ZNF148* plays a crucial role as a transcription factor in regulating *p16* expression. However, the impact of methylation on *ZNF148*'s function remains uncertain. The binding sites of *ZNF148* on the *p16* gene are predominantly concentrated in the promoter region and its upstream regions. Methylation alterations downstream of the *p16* promoter may not significantly influence *ZNF148*'s ability to regulate *p16* expression.

Comment5: Another limitation of this article is no control with other pulmonary disease tissue to compare level of p16 expression.
Reply5: We appreciated the reviewer's positive evaluation of our work and agree with the comments regarding the limitations of our study. The study does have the limitation that *p16* expression levels in other lung disease tissues were not validated for comparison. To obtain a more comprehensive and extensive understanding of the regulation of downstream methylation of the *p16* promoter, it is essential to study various types of lung diseases. Such investigations will provide valuable insights into the potential significance of early diagnosis and targeted treatment strategies (please refer to Page 23, Line 446-448).
Change in the text: The impact of *p16* promoter methylation on its expression should be investigated in larger sample sizes and across various disease types to offer more comprehensive guidance for early clinical diagnosis and targeted therapy.