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Tumor suppressor PTEN regulation by tobacco smoke in lung squamous-cell carcinoma based on bioinformatics analysis

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ABSTRACT

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), is a tumor suppressor inactivated in a variety of human cancers. PTEN alteration correlates with lung squamous-cell carcinoma (LUSC) histology. However, it is still unclear how tobacco smoke regulates PTEN in LUSC tissues. In this study, we used free online databases and online tools to analyze *PTEN* expression and the role of smoking on *PTEN* alteration in patients with LUSC. We validated bioinformatics data by performing RT-PCR analysis using LUSC patient samples. Our results showed a correlation between the downregulation of *PTEN* in LUSC tissues compared to normal tissues and smoking exposure. *In silico* results using online platforms suggest that hsa-mir-301a down-regulates *PTEN* expression level in smoking patients with LUSC. RT-PCR analysis demonstrated that the *PTEN* expression was significantly decreased, whereas expression of hsa-mir-301a was up-regulated in the smoker cohort of LUSC tissue compared to adjacent non-cancerous tissues. A significant negative correlation between *PTEN* and hsa-mir-301a levels was observed in tumour tissues in our cohort of LUSC patients. Our results suggest that the downregulation *PTEN* gene caused by tobacco smoke-mediated increase of hsa-mir-301a may play an important role in LUSC tumorigenesis.

1. Introduction

Lung cancer kills more people than many other types of cancer and has recently become one of the leading causes of death [1]. Non-small cell lung cancer (NSCLC, about 85%) accounts for the largest proportion of all lung cancer cases. NSCLC is subdivided into lung adenocarcinoma (LUAD), lung squamous-cell cancer (LUSC), large cell carcinoma, and mixed histotype, the largest proportion of which is occupied by LUAD (about 40%) and LUSC (about 30%) [2]. It is well known that among all risk factors for all histological types of lung cancer, smoking is the most important. At the same time, the role of smoking is strongest for LUSC and weakest for LUAD [3]. Moreover, never-smokers and smokers with lung cancer show significant differences in treatment responses [4]. Molecular targeted therapies based on genomic profiling are currently widely used in a subgroup of never smoking young LUAD patients [2]. Sensitive molecular biomarkers and molecular targets for therapy have not yet been identified for LUSC. As a result, there are no available targeted agents for treatment in LUSC patients, which is directly reflected in the poor clinical prognosis. Recent

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transcriptomic data indicate that the transcriptome profiles in LUSC and LUAD cells are significantly different [5]. Thus, it has been shown that LUSC and LUAD cells express differently cell proliferation regulation genes, which may indicate different regulatory settings for the pathways of malignant transformation. This fact may have a direct impact on the course and treatment of the disease.

The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is often inactivated in various human cancers, including melanoma, glioblastoma, colon, breast, ovarian, prostate and lung cancer [6]. PTEN activity can be altered by mutations, repression of transcription, and post-translational modifications of the PTEN protein. In cells, PTEN negatively regulates AKT signaling, which produces cell cycle arrest in G1-phase and apoptosis initiation. The AKT signaling pathway is one of the most frequently deregulated pathways in cancers, including lung cancer [7]. Most often, abnormalities in PTEN activity in cancer cells are due to genetic alterations. However, most previous studies demonstrated a low frequency of PTEN genetic changes in NSCLC tissues, while a decrease in PTEN levels was described in more than 40% of NSCLC cases [7]. The decrease of PTEN in NSCLC tissues is associated with a decrease in patient survival and significantly correlated with LUSC histology [8–10].

Although smoking is most strongly associated with LUSC, it is still unclear how tobacco smoke can affect PTEN levels in tumor tissues. Understanding the mechanism may be important in the diagnosis and treatment of LUSC patients. In this study, we used several free online databases and tools to analyze *PTEN* expression levels and the role of smoking on *PTEN* alteration in LUSC patients.

2. Materials and methods

2.1. TIMER analysis

DiffExp module of web server TIMER [11] (https://cistrome.shinyapps.io/timer/) was applied to examine the expression of the *PTEN* gene in a variety of cancers. DiffExp module allows users to study the differential expression between tumor and adjacent non-cancerous tissues for any gene of interest across all The Cancer Genome Atlas (TCGA) tumors.

2.2. TNMplot analysis

The Pan-cancer analysis module of the online platform TNMplot [12] (https://tnmplot.com/analysis/) was used to display the expression range for the *PTEN* gene between normal and cancer related data RNA Seq.

2.3. UALCAN analysis

The Expression module of UALCAN [13] (http://ualcan.path.uab.edu/analysis.html) was applied to analyze *PTEN* gene and miRNAs expression data in the TCGA dataset of LUSC tissues and normal lung tissues. The Methylation module of UALCAN was used to investigate the DNA methylation of the *PTEN* gene promoter. UALCAN provides the ability to assign patients to several subgroups using clinical patient data, including smoking status for patients with lung cancer.

Table 1
Clinicopathological characteristics of LUSC patients.

Patients' characteristics	Number of patients (%)
Age (years)	
≤65	28 (50)
>65	28 (50)
Smoking history	
Current smokers	56 (100)
Pack-years	
≤40 PYs	32 (57)
>40 PYs	24 (43)
Gender	
Male	55 (98)
Female	1 (2)
Tumors' characteristics	**Number of cases (%)**
Tumour size (TNM classification)	
T1	14 (25)
T2	32 (57)
T3	10 (18)
Regional lymph nodes (TNM classification)	
N0	34 (61)
N1	18 (32)
N2	4 (7)
AJCC	
I	22 (39)
II	24 (43)
III	10 (18)

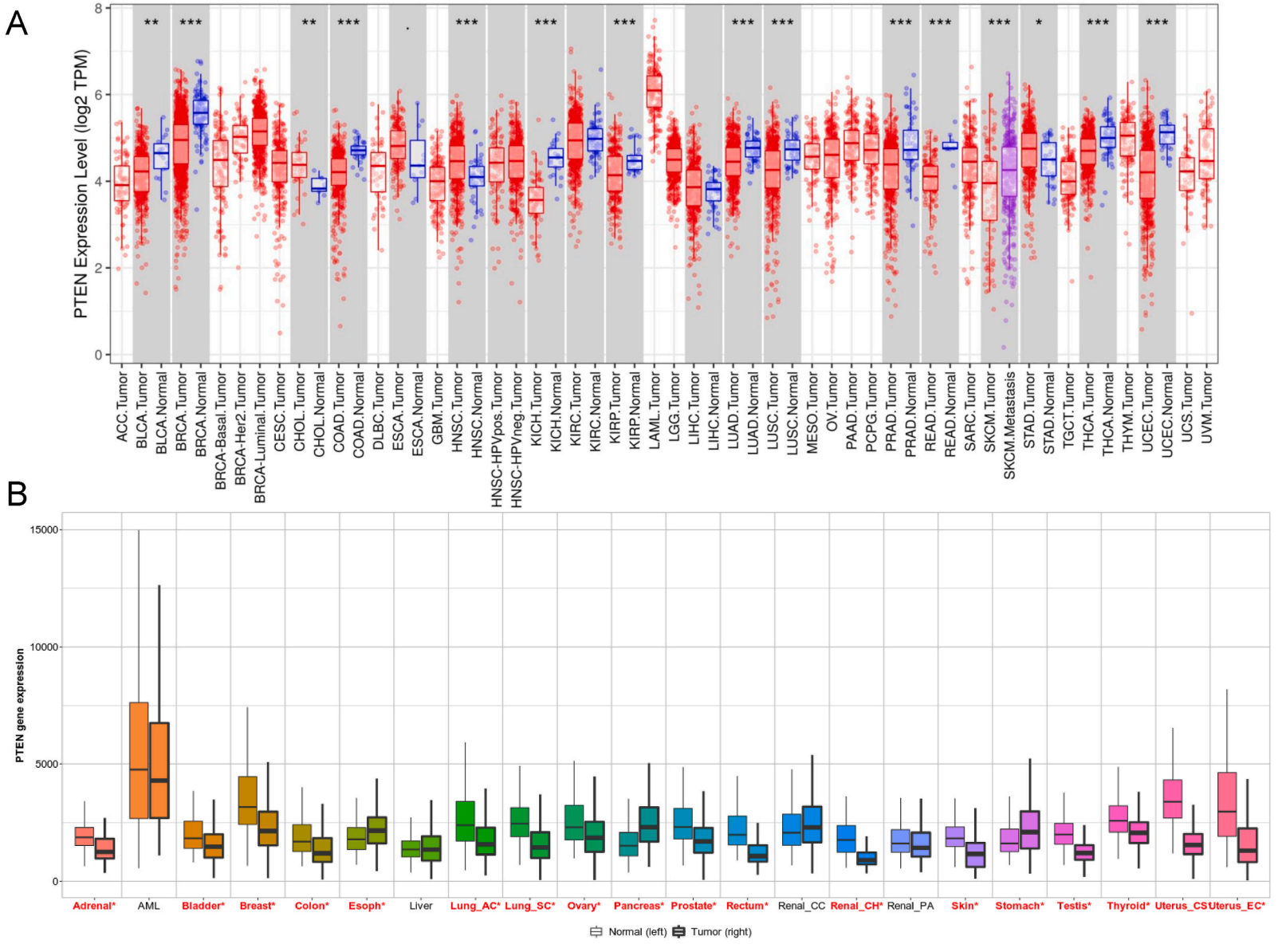

Fig. 1. *PTEN* gene expression in multiple cancers. A, PTEN mRNA levels from TIMER (DiffExp module). *p < 0.05, **p < 0.01 and ***p < 0.001. B, PTEN mRNA levels from TNMplot. Left box is normal, right box is tumor. *p < 0.01 (marked with red color).

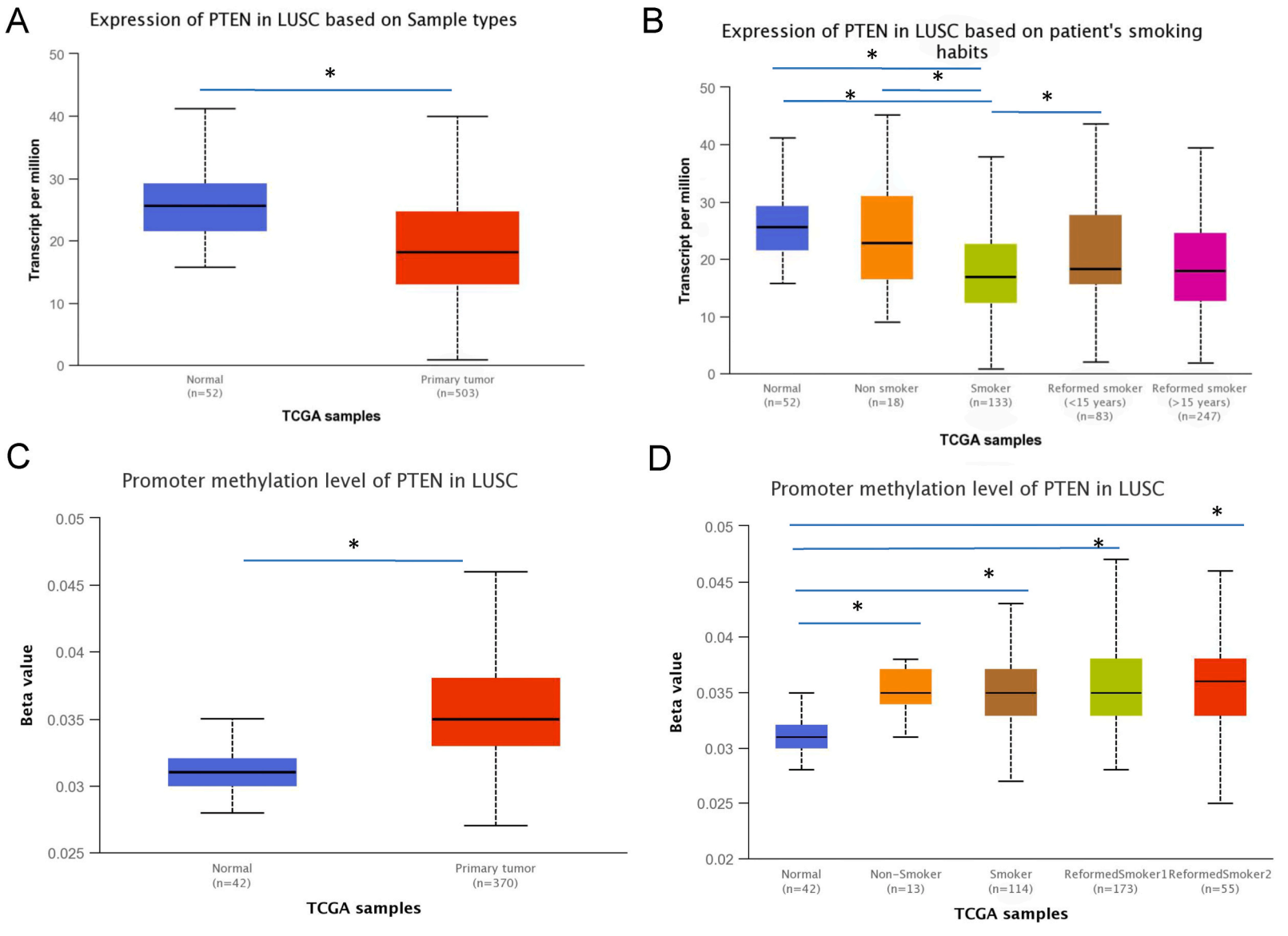

Fig. 2. Bars showing *PTEN* expression (A,B) and methylation of *PTEN* promoter region (C,D) in all primary tumors compared to normal tissues (A,C, UALCAN database) and subgroups based on patient's smoking habit (B,D). LUSC samples were categorized into four subgroups including smoker, non-smoker, reformed smoker 1 ($\leq$ 15 years), and reformed smoker 2 (>15 years). *p $<$ 0.05.

2.4. LinkedOmics database analysis

The LinkFinder module of the online platform LinkedOmics [14] (http://www.linkedomics.org/) was used to study differentially expressed miRNAs related to *PTEN* in the TCGA dataset of LUSC samples. The Spearman-correlation coefficient was used to statistically analyze the results.

2.5. TargetScan algorithm

The TargetScan algorithm (https://www.targetscan.org/vert_80/) was applied to find all miRNAs that can interact with *PTEN*.

2.6. Patients and tissue collection

Following the Helsinki Declaration of 1975, the study was approved by the Ethics Committee of the Federal Research Center for Fundamental and Translational Medicine (Novosibirsk, Russia, protocol code # 4/2021). Each of the 56 patients gave written informed consent. LUSC tissues and matched adjacent non-cancerous tissues were obtained during surgery at the Novosibirsk Regional Clinical Oncology Centre from 2021 to 2022. After resection, patient tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C. Verification of the diagnosis of each patient was carried out during a pathological examination. Table 1 contains the clinicopathological characteristics of LUSC patients.

2.7. Total RNA extraction and the real-time PCR

Previously published protocols were used for miRNA and total RNA isolation, miRNA and RNA reverse transcription, and real-time PCR to determine miRNA and mRNA levels [15]. Total RNA was isolated using the TRIzol™ reagent (Invitrogen, USA). RNA concentration and purity were determined spectrophotometrically using Agilent-8453 (Agilent Technologies, USA). RNA integrity was assessed by agarose gel electrophoresis. Reverse transcription was performed using the RT-*M*-MuLV-RH kit (BiolabMix, Russia). For stem-loop RT-PCR, total RNA was reverse-transcribed using a specific stem-loop primer. Ct values were measured by real-time PCR with HS-qPCR SYBR Blue (2×) kit (Biolabmix, Russia) for PTEN, 18SRNA and UDG HS-qPCR (2×) kit (Biolabmix, Russia) for hsa-mir-301a and U44 snRNA using a CFX96™ Detection System (Bio-Rad Laboratories, USA). The specific oligonucleotides were employed for reverse transcription reaction and PCR (Supplementary Table 1). Ct values for each reaction were normalised to 18SRNA (for *PTEN*) and U44 snRNA (for miRNA) values.

2.8. Statistical analysis

Experimentally obtained data were analyzed using non-parametric tests due to the non-normal distribution of the data. MedCalc v20.011 software was used to carry out statistical analysis using a non-parametric two-tailed Mann–Whitney *U* test. A Spearman-correlation analysis was used to examine the relationship between values. A p cut-off value of 0.05 was set.

3. Results

3.1. PTEN expression in diverse cancers

We first examined *PTEN* levels in a variety of cancers using TIMER and found that the *PTEN* gene has low expression in a variety of human cancers, including LUSC, compared to normal samples (Fig. 1A). In addition, we employed an online tool named TNMplot and found that *PTEN* gene is down-regulated in adrenal, bladder, breast, colon, lung, ovary, prostate, rectum, skin, testis, thyroid and uterus tumor samples compared to adjacent normal tissues, which are consistent with findings from the TIMER datasets (Fig. 1B).

3.2. PTEN expression in patients with LUSC

Next, we examined *PTEN* gene expression in LUSC compared to normal samples using the UALCAN online tool, which includes 503 LUSC cases. The results showed that *PTEN* levels were low in LUSC tissues compared with the normal group ($p = 1.72e{-}09$; Fig. 2A). We conducted a subgroup analysis based on patient's smoking habit of LUSC cases through UALCAN and found that *PTEN* gene expression in patients with smoker status was significantly lower compared with the patients with non-smoker status ($p = 1.32e{-}02$; Fig. 2B). Interestingly, no such dependence of *PTEN* gene expression on smoking status was found for LUAD patient samples (Supplementary Figure S1).

3.3. PTEN promoter methylation in patients with LUSC

Next, we examined the potential reason why *PTEN* is down-regulated in LUSC, especially in smokers. A large number of studies have convincingly demonstrated that DNA methylation can significantly regulate mRNA levels by modulating transcription. We applied the UALCAN to investigate the methylation of *PTEN* promoter and observed significantly increased methylation levels in LUSC tissues ($p = 1.62e\text{-}12$; Fig. 2C), which is consistent with the decreased levels of *PTEN* in tumors compared to normal tissue (Fig. 2A).

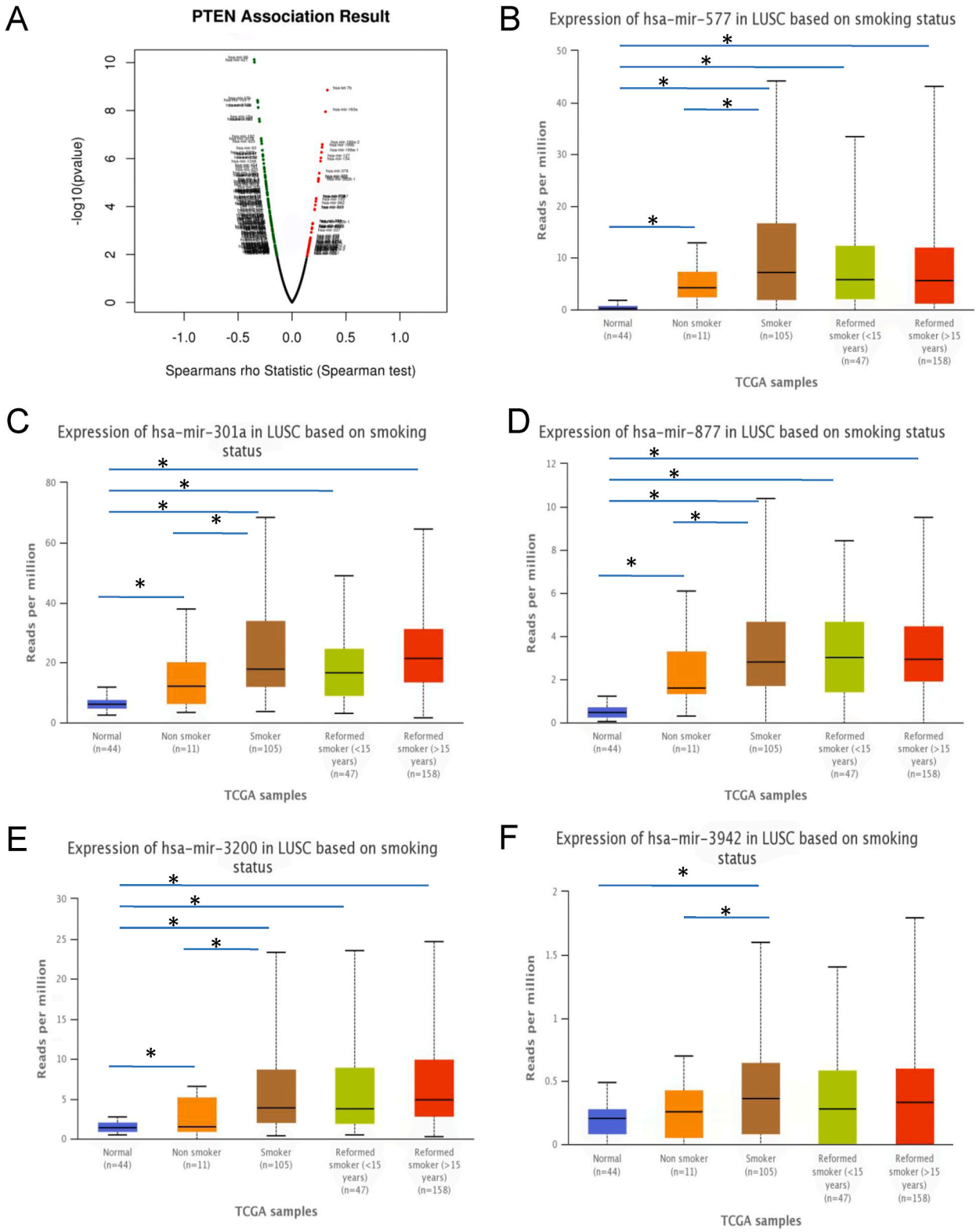

Fig. 3. miRNAs related to *PTEN*. A, Results of *PTEN* association in LinkedOmics (volcano graph). Bars showing the level of *PTEN*-correlated hsa-mir-577 (B), hsa-mir-301a (C), hsa-mir-877 (D), hsa-mir-3200 (E), hsa-mir-3942 (F) in subgroups based on patient's smoking habit. LUSC samples were categorized into four subgroups including smoker, non-smoker, reformed smoker 1 ($\leq$ 15 years), and reformed smoker 2 (>15 years). *p $<$ 0.05.

Interestingly, the methylation of *PTEN* promoter in patients with smoker status was not different from the level of the methylation in patients with non-smoker status (Fig. 2D), suggesting a complex mechanism in *PTEN* gene regulation are required upon exposure to tobacco smoke. MicroRNAs are unique regulators of gene and protein levels that interact with the 3′ untranslated region (3′UTR) of a target mRNA, thereby producing mRNA degradation or translational repression.

3.4. miRNA targets of PTEN in patients with LUSC

We hypothesized that miRNAs may significantly contribute to the decrease of *PTEN* levels in a subgroup of smokers with LUSC. MicroRNAs in tumor tissues of patients with LUSC were analyzed using the LinkFinder module in LinkedOmics. As shown in the volcano graph, there is a set of miRNAs that are significantly positively (dark red dots) and negatively (dark green dots) correlated with PTEN mRNA levels (Fig. 3A).

Next, we compared the level of microRNAs, which have the highest negative correlation with the level of the *PTEN* gene (Supplementary Table 2), between subgroups of current smokers and non-smokers using the online tool UALCAN. Five differentially expressed microRNAs (hsa-mir-577, hsa-mir-301a, hsa-mir-877, hsa-mir-3200, hsa-mir-3942) were identified, the levels of which were significantly increased in the subgroup of smokers compared with non-smokers (Fig. 3B–F).

TargetScan was used to isolate the list of microRNAs that can interact with the 3′UTR of *PTEN*. *PTEN* was predicted to be a target gene for 4 microRNAs (hsa-mir-577, hsa-mir-301a, hsa-mir-3200, hsa-mir-3942), the level of which significantly differed in smokers from non-smokers (Supplementary Table 3). Further investigations were carried out with hsa-mir-301a, which has a relatively higher context++ score percentile (90). The 3′UTR of the *PTEN* gene contains a putative complementary sequence for hsa-miR-301a-3p, namely at position 412–418. Correlation analysis using the LinkFinder module of the LinkedOmics database revealed a significant negative correlation ($r = -0.236$, $p = 1.55e\text{-}05$) between hsa-mir-301a and *PTEN* levels in LUSC tumor tissues (Fig. 4). Thus, the *in silico* results using online platforms suggest that hsa-mir-301a down-regulated *PTEN* expression by directly targeting its 3′UTR, especially in smoking patients with LUSC.

3.5. Expression level of hsa-miR-301a and PTEN in LUSC clinical samples

To validate bioinformatic data, we performed experiments using RT-PCR analysis and clinical tissue samples. RT-PCR results demonstrated that *PTEN* expression was significantly lower (Fig. 5A) and hsa-miR-301a level was significantly higher (Fig. 5B) in tumor tissues of patients with LUSC compared with non-cancerous tissues. In addition, Spearman-Correlation analysis revealed a significant negative correlation between *PTEN* and hsa-mir-301a (Fig. 5C), indicating the involvement of hsa-miR-301a and *PTEN* in the development of LUSC.

4. Discussion

Tobacco smoking is a key risk factor for lung cancer, and the percentage of smokers is higher in LUSC than that in LUAD [3,16]. However, how smoking affects the occurrence and promotion of LUSC is still not completely clear. Our results of bioinformatics

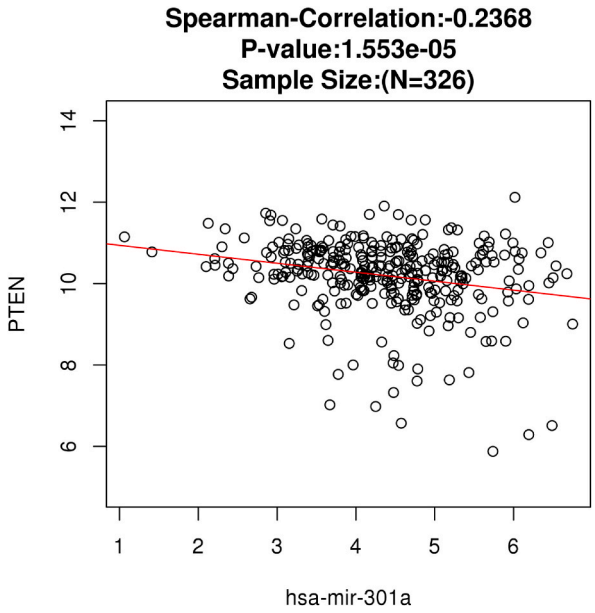

Fig. 4. Spearman-Correlation analysis of the correlation between *PTEN* and hsa-mir-301a. Data was obtained using LinkedOmics.

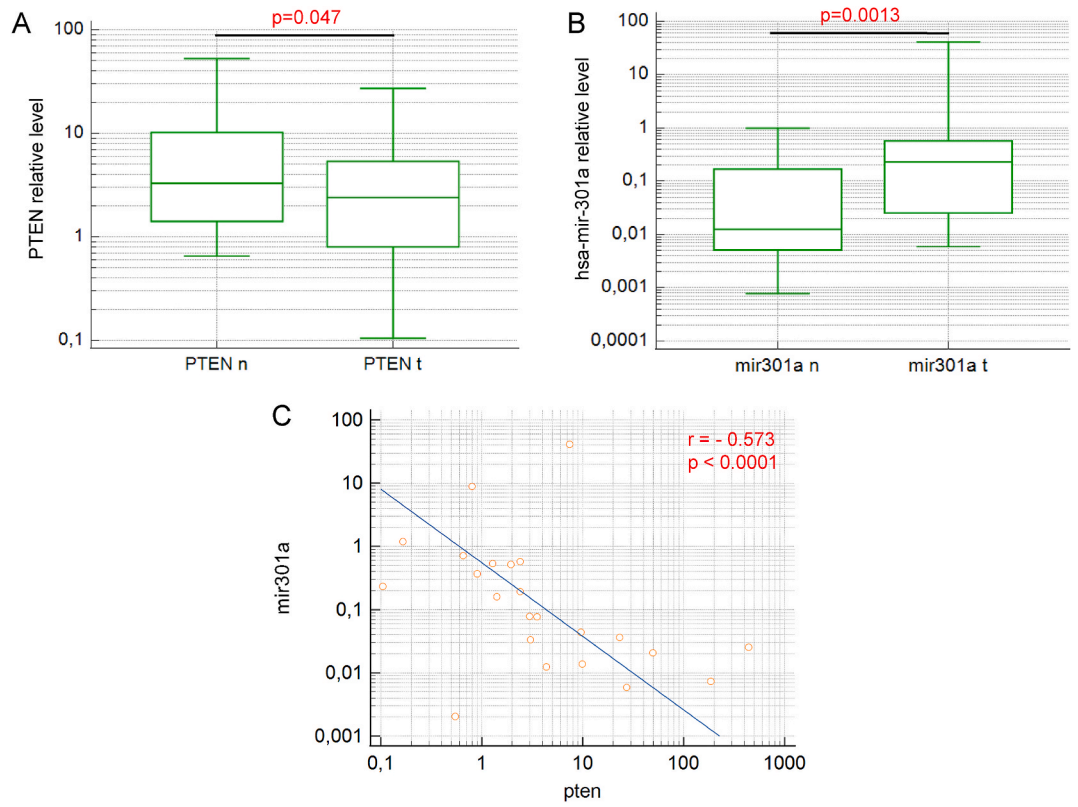

Fig. 5. The relative levels of *PTEN* (A), and hsa-mir-301a (B) in LUSC clinical tissue samples. Spearman-Correlation analysis of the correlation between PTEN and hsa-mir-301a (C). n - matched adjacent non-cancerous tissues, t – tumor tissues.

analysis showed a correlation between PTEN mRNA suppression and smoking exposure in LUSC patients. It is known that smoking causes not only genetic but also epigenetic anomalies, which can affect the change in the expression of a set of genes in the lungs [17, 18]. Previous research has shown that exposure to tobacco smoke induces changes in DNA methylation that are associated with lung cancer [17]. However, our current study indicated that although the level of *PTEN* promoter methylation in LUSC samples is significantly higher compared to normal tissue, there is no significant difference in the level of *PTEN* promoter methylation between smokers and non-smokers. This suggests the existence of an alternative regulatory network that produces downregulation of the *PTEN* gene upon exposure to smoking.

There is a huge amount of evidence that microRNAs play an important role in the development of cancer through the regulation of multiple genes [19]. Even a slight change in the level of one miRNA can have a significant impact on the global network of gene expression. Many previous studies have established correlations between the levels of some microRNAs and *PTEN* gene expression, as well as the important role of these relationships in the development and promotion of various tumors [7,20]. We hypothesized that the level of the *PTEN* gene in LUSC tissues of smokers can be regulated with the participation of microRNAs. To understand the role of microRNAs in PTEN regulation in LUSC tissues upon exposure to smoking, we analyzed TCGA databases using free online tools and found that *PTEN* and hsa-mir-301a expression are inversely associated in LUSC tissues of smokers. Our *in silico* data suggested that the upregulation of hsa-mir-301a induced by tobacco smoking might at least partially reflect the downregulation of *PTEN* expression.

It has been reported that hsa-mir-301a is a prognostic marker for the negative course of many human cancers. Accumulating evidence indicates that hsa-mir-301a can act as an oncogene to exert a wide range of effects on cell proliferation, apoptosis, and metastasis in several types of cancer [21–27]. It has been shown in a mouse model of lung cancer that deletion of mir-301a reduces lung tumor development and increases survival [28]. Moreover, it has been reported that hsa-miR-301a is overexpressed in lung cancer [29–32]. Thus, it was recently shown that hsa-mir-301a was detected at significantly higher levels in both LUAD and LUSC tissues compared to normal tissue. At the same time, one of the hsa-mir301a target genes, SMAD4, was reduced only in LUAD tissues, but not in LUSC [32]. This fact indicates that other potential hsa-mir-301a target genes are likely to be involved in the process of malignant transformation in the case of LUSC. Our results suggest that the downregulation of *PTEN* evoked by tobacco smoke-mediated increase of hsa-mir-301a may play an important role in LUSC tumorigenesis.

Recent studies demonstrated that the repressive effects of hsa-mir-301a on PTEN mRNA levels are characteristic of several cancers [25,33–35]. We also studied the expression of the *PTEN* gene and the *PTEN*-targeting hsa-mir-301a in clinical samples of LUSC patients and investigated the potential relationships between miRNA and *PTEN* levels. Our data demonstrated that the expression of the *PTEN* gene was significantly decreased, whereas the expression of hsa-mir-301a was increased in the smoker cohort of LUSC tissues

compared to adjacent non-cancerous tissues. Additionally, we observed a significant negative correlation between *PTEN* gene and the *PTEN*-targeting hsa-mir-301a levels in tumour tissues in our cohort of LUSC patients, suggesting that hsa-mir-301a could influence LUSC progression by the regulation of PTEN mRNA levels in smoker cohort. As shown in Table 1, 100% of patients recruited in this study were smokers. Unfortunately, due to the rare occurrence of LUSC in non-smokers, during the two years of replenishment of our biobank, we were unable to obtain a sufficient number of samples from non-smoking LUSC patients to form a separate subgroup for comparison. Since there is not any comparison between smokers and non-smokers, it cannot be concluded that the observed *PTEN* downregulation and hsa-mir-301a upreglation are specific to smoking patients in our cohort of LUSC. Nevertheless, we have shown for the first time that an inverse correlation between PTEN and hsa-mir-301a may be associated with the pathogenesis of LUSC. Another limitation of the present study is the lack of experimental data describing gender differences. In fact 98% of the patients recruited into the study were male. This distribution of patients into subcohorts by gender did not allow statistical comparison.

In conclusion, our results suggest that the PTEN/hsa-mir-301a axis may act as a therapeutic target for the prevention and treatment of LUSCs that develop with chronic exposure to tobacco smoke. Of course, more research is needed in the future to provide more evidence that the hsa-mir-301a/PTEN pathway is a target in the clinical application of LUSC treatment. In addition, further studies are needed to determine whether the hsa-mir-301a/PTEN regulatory network in smokers is associated with clinical outcomes in LUSC patients.

Author contribution statement

Vladimir O. Pustylnyak: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lyudmila F. Gulyaeva: Conceived and designed the experiments.

Efim Y. Alekseenok; Vadim V. Kozlov: Performed the experiments.

Alina M. Perevalova: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19044.

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