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The Association Between *PNPLA2* Methylation in Peripheral Blood and Early-Stage Lung Cancer in a Case–Control Study

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Purpose: Lung cancer (LC) brings great burden to the society worldwide. Exploring novel biomarkers in vitro for the early detection of LC would be of great importance.

Patients and Methods: We measured DNA methylation levels of 21 CpG sites within Patatin-like phospholipase domain containing 2 (*PNPLA2*) gene in the peripheral blood of 168 early-stage LC cases (94.0% LC at stage I) and 187 age- and gender-matched cancer-free controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression adjusted for covariates. Non-parametric tests were applied for the comparisons of stratified groups.

Results: Hypomethylation of PNPLA2_CpG_8,10 and hypermethylation of PNPLA2_CpG_9 were correlated to the early-stage LC with the ORs of 1.44 (95% CI: 1.06–1.96, $P = 0.018$) and 0.82 (95% CI: 0.69–0.98, $P = 0.029$), respectively. The associations were still significant for the very early-stage LC patients (stage I). Further gender- and age-stratified analyses indicated that the association between hypomethylation of PNPLA2_CpG_8,10 and LC existed only in females and in subjects younger than 55 years. In addition, the association between LC and hypermethylation of PNPLA2_CpG_6 and PNPLA2_CpG_9 was also observed in the younger population.

Conclusion: Taken together, our study has proved the hypothesis that the altered methylation in the peripheral blood may be correlated with the burden of cancer at an early stage. Here, we find a novel association between blood-based aberrant *PNPLA2* methylation and LC at a very early stage and particularly for women at a younger age.

Keywords: lung cancer, DNA methylation, peripheral blood, biomarker

Introduction

It has been estimated that lung cancer (LC) accounted for more than 2.09 million new cases and 1.76 million deaths worldwide in 2018, making it the leading cause of cancer-related incidence and mortality in men and women.[1] Data from the Global Burden of Disease study demonstrated that, from 1990 to 2017, the age-standardized death rates for LC showed a decreasing trend in men and an increasing trend in women worldwide, while raised in both men and women in China, indicating that LC in China represents a tremendous burden on the global epidemic.[2] For non-small cell lung cancer (NSCLC) patients, a decline in the 5-year survival rates with the clinical stages was well documented, ranging from 92% at the stage IA1 to 6% at the stage IV.[3]

Therefore, other than the development of treatment and therapy for LC, early detection may be an alternative option for the easing of LC burden in China.

A large randomized controlled trial showed an inspiring 20.0% decrease in mortality of persons at high risk for LC by low-dose computed tomographic (LDCT) screening, while the simultaneous high false-positive rate should also be concerned.[4] Besides, overdiagnosis, cost-effectiveness, radiation risks, and abnormal opacities caused by other diseases were also uncertainties to be considered before conducting a screening with LDCT.[5]

Adjunctive methods to improve the precision on the basis of LDCT are urgently needed. Epigenetics, characterized by regulating gene expression without changes in the DNA sequence, performs a critical function in initiation and progression of many diseases, thus attracting accumulative interests in recent decades.[6] DNA methylation is the most studied epigenetic modification. Altered DNA methylation in peripheral blood has been disclosed as a potential biomarker of LC, due to its superiority in biological stability and noninvasive sample collection.[7] For example, hypomethylation of *AHRR* and *F2RL3* gene in peripheral blood showed enhanced predictive value for LC risk in the prospective studies.[8] Our previous case-control study also indicated that peripheral blood-based *RAPSN* hypomethylation was associated with early-stage LC.[9]

Patatin-like phospholipase domain containing 2 (*PNPLA2*) gene, also known as *ATGL*, encodes an enzyme catalyzing the rate-limiting step in the hydrolysis of triglycerides.[10] *PNPLA2* is related to many diseases in human, including neutral lipid storage disease with myopathy, breast cancer, hepatocellular cancer, and pancreatic ductal adenocarcinoma.[11] *PNPLA2* may be involved in the process of cancers via p-AKT signaling pathway, AMP-kinase and mTOR signaling pathway and other lipolysis-related events.[11,12] Notably, a study reported that maternal prenatal folic acid supplementation may improve lipid metabolism in adult rats by elevated DNA methylation levels within *ATGL* and *LPL* promoter/first exon region.[13] However, there is no report about the association between blood-based *PNPLA2* methylation and cancers. In our present study, we aimed to investigate the association between altered *PNPLA2* methylation in the peripheral blood and early-stage LC in a case-control study in the Chinese population.

Patients and Methods
Study Design and Population
This was a case-control study design. A total of 168 LC cases, determined by thoracic surgery and pathology, were collected from Shanghai Chest Hospital in 2018–2019.

187 age- and gender-matched healthy controls were consecutively collected from a physical examination center at Jiangsu Province Hospital of Chinese Medicine during the year of 2018–2019. No further inclusion criteria were applied for the controls. The median (interquartile range, IQR) age of LC cases and healthy controls were 57.5 (53.3-63.8) and 55.0 (51.0–63.0) years old, respectively. All the LC patients in the present study were diagnosed at very early stage (158 at stage I and 10 at stage II), with 64.3% males and 35.7% females. Additional clinical information of the LC patients, such as the tumor size, the tumor length and the involved lymph nodes, was described in detail in Table 1.

This study was conducted in accordance with the Declaration of Helsinki. The present study was approved by the Ethics Committee of the Shanghai Chest Hospital and Jiangsu Province Hospital of Chinese Medicine in China. Written informed consents were obtained from all the participants.

Sample Collection and Processing
Ethylenediamine tetraacetic acid (EDTA) tubes were used to collect peripheral blood. For LC patients, blood samples were taken before surgery and before any LC-related treatments. All the blood samples were kept at 4°C for up to

Table 1 Clinical Characteristics of LC Cases

Sample Characteristics	Type	N	%
Age	≤ 55 years > 55 years	64 104	38.1 61.9
Gender	Female Male	60 108	35.7 64.3
Tumor subtype	Adenocarcinoma in situ Microinvasive adenocarcinoma Invasive adenocarcinoma	37 34 97	22.0 20.2 57.8
Tumor size	T1 T2 and T3	147 21	87.5 12.5
Tumor length	≤ 1 cm > 1 cm	68 100	40.5 59.5
Lymph node involvement	0 1 and 2	164 4	97.6 2.4
Tumor stage	Stage I Stage II	158 10	94.0 6.0

Abbreviations: LC, lung cancer; N, number.

24 hours after the blood extraction and stored at −80°C for future usage. DNA Extraction Kit (TANTICA, Nanjing, China) was used for the extraction of DNA from blood. All the cases and controls were processed in parallel.

Bisulfite Conversion

DNA of each sample was converted by a bisulfite reaction using EZ-96 DNA Methylation Gold Kit (Zymo Research, Orange, USA) according to the manufacturer's specifications. The bisulfite treatment converts non-methylated cytosine (C) at the CpG site to uracil (U), while methylated cytosine remains intact.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

Polymerase chain reaction (PCR) was used to amplify a 355 bp amplicon in *PNPLA2* gene covering 21 CpG sites. There are no single nucleotide polymorphisms (SNPs) located at the primer regions or overlapped with any of these CpG sites. The designed bisulfite-specific primers for *PNPLA2* gene are available upon request. The methylation levels of *PNPLA2* CpG sites were determined by MALDI-TOF mass spectrometry (Agena Bioscience, San Diego, California, US) in a quantitative manner as described before[14] and as presented in Supplementary Figure 1. In brief, the PCR products were incubated with Shrimp Alkaline Phosphatase (SAP) and treated by the T cleavage assay (Agena Bioscience, San Diego, California, U.S.), and further cleaned by Resin. The final products were transferred to a SpectroCHIP G384 and detected by the MassARRAY spectrometry (Agena Bioscience, San Diego, California, U.S.). The quantitative methylation level of each CpG site was collected by SpectroACQUIRE v3.3.1.3 software and visualized by EpiTyper v1.3 software. A total of 21 CpG sites were detected in the amplicon of *PNPLA2*, of which 20 CpG sites were measurable, and the PNPLA2_CpG_19 CpG site was excluded own to low quality of signal. These 20 measurable CpG loci yielded 13 signal peaks by the mass spectrometry, whereas most of the signal peaks contained only one CpG site. When two or more CpG sites were located at the same fragment, the methylation intensity was presented as the average intensity of these CpG sites, such as PNPLA2_CpG_11,12, PNPLA2_CpG_14,15,16,17 and PNPLA2_CpG_20,21. When two CpG sites shared the same mass, we showed their methylation intensities as combined briefly, for example, PNPLA2_CpG_2,3 stands for PNPLA2_CpG_2 and PNPLA2_CpG_3, and PNPLA2_CpG_8,10 stands for PNPLA2_CpG_8 and PNPLA2_CpG_10. The hypo- and hypermethylation was defined by the methylation levels of the cases compared to the controls. When the methylation levels of cases were lower than the controls, hypomethylation was referred. When the methylation levels of cases were higher than the controls, hypermethylation was referred.

Statistical Analyses

All statistical analyses were carried out using IBM SPSS Statistics 25.0 version (Chicago, IL, USA). Binary logistic regression was performed with the adjustment of age, gender and experimental batches. Odds ratios (ORs) and their 95% confidence intervals (CIs) were obtained to measure the association between the DNA methylation levels and LC. For comparison of clinical characteristics between two or multiple groups, we conducted non-parametric tests including Mann-Whitney *U*-test and Kruskal-Wallis test. All tests were two-sided, and $P < 0.05$ was considered to be statistically significant.

Results

The Association Between Aberrant *PNPLA2* Methylation in Peripheral Blood and Early-Stage LC

To explore the association between *PNPLA2* methylation and LC at an early stage, we conducted a binary logistic regression analysis in a case-control study. When controlling for age, gender and experimental batches, per 10% decrease in methylation level for PNPLA2_CpG_8,10 presented an OR of 1.44 (95% CI: 1.06–1.96, $P = 0.018$; Table 2). We also found an association between hypermethylation of PNPLA2_CpG_9 and LC with an OR of 0.82 (95% CI: 0.69–0.98, $P = 0.029$; Table 2). This aberrant methylation of PNPLA2_CpG_8,10 and PNPLA2_CpG_9 was also observed significantly in LC patients at very early stage (stage I) (for PNPLA2_CpG_8,10, OR = 1.42, 95% CI: 1.05–1.93, $P = 0.025$; for PNPLA2_CpG_9, OR = 0.80, 95% CI: 0.66–0.96, $P = 0.017$; Table 3). Apart from these, no significant associations were observed between any of the remaining *PNPLA2* CpG sites and LC or LC at stage I (*P* values > 0.05 for all, Tables 2 and 3).

Table 2 The Association Between *PNPLA2* Methylation and LC

CpG Sites	Controls (N = 187) Median (IQR)	LC Cases (N = 168) Median (IQR)	OR (95% CI)# per −10% Methylation	P-value#
PNPLA2_CpG_1	0.28 (0.17–0.40)	0.29 (0.19–0.43)	1.06 (0.87–1.28)	0.566
PNPLA2_CpG_2,3	0.17 (0.12–0.37)	0.18 (0.10–0.41)	0.96 (0.84–1.09)	0.495
PNPLA2_CpG_4	0.02 (0.02–0.04)	0.03 (0.02–0.04)	0.71 (0.27–1.88)	0.490
PNPLA2_CpG_5	0.04 (0.03–0.05)	0.04 (0.03–0.05)	0.87 (0.63–1.21)	0.414
PNPLA2_CpG_6	0.37 (0.32–0.43)	0.36 (0.27–0.48)	0.89 (0.77–1.03)	0.130
PNPLA2_CpG_7	0.23 (0.17–0.27)	0.22 (0.17–0.28)	1.08 (0.89–1.31)	0.453
PNPLA2_CpG_8,10	0.35 (0.31–0.38)	0.33 (0.28–0.38)	1.44 (1.06–1.96)	**0.018**
PNPLA2_CpG_9	0.28 (0.24–0.33)	0.28 (0.23–0.34)	0.82 (0.69–0.98)	**0.029**
PNPLA2_CpG_11,12	0.46 (0.38–0.53)	0.45 (0.35–0.51)	1.17 (0.97–1.42)	0.100
PNPLA2_CpG_13	0.15 (0.13–0.18)	0.15 (0.11–0.19)	0.85 (0.64–1.14)	0.288
PNPLA2_CpG_14,15,16,17	0.11 (0.08–0.13)	0.10 (0.07–0.14)	1.07 (0.62–1.85)	0.809
PNPLA2_CpG_18	0.12 (0.09–0.14)	0.11 (0.08–0.15)	0.94 (0.69–1.29)	0.718
PNPLA2_CpG_20,21	0.40 (0.34–0.45)	0.38 (0.30–0.46)	1.04 (0.85–1.28)	0.675

Notes: #Logistic regression, adjusted for age, gender and experimental batches. Significant *P*-values are in bold.
Abbreviations: LC, lung cancer; N, number; IQR, interquartile range; OR, odds ratio; CI, confidence interval.

Table 3 The Association Between *PNPLA2* Methylation and LC at Very Early Stage (Stage I)

CpG Sites	Controls (N = 187) Median (IQR)	LC Cases (N = 158) Median (IQR)	OR (95% CI)# per −10% Methylation	P-value#
PNPLA2_CpG_1	0.28 (0.17–0.40)	0.28 (0.18–0.40)	1.06 (0.88–1.29)	0.525
PNPLA2_CpG_2,3	0.17 (0.12–0.37)	0.18 (0.10–0.40)	0.96 (0.84–1.09)	0.507
PNPLA2_CpG_4	0.02 (0.02–0.04)	0.03 (0.02–0.04)	0.62 (0.23–1.67)	0.342
PNPLA2_CpG_5	0.04 (0.03–0.05)	0.04 (0.03–0.05)	0.87 (0.63–1.20)	0.394
PNPLA2_CpG_6	0.37 (0.32–0.43)	0.36 (0.27–0.48)	0.89 (0.76–1.03)	0.112
PNPLA2_CpG_7	0.23 (0.17–0.27)	0.22 (0.16–0.27)	1.08 (0.89–1.32)	0.432
PNPLA2_CpG_8,10	0.35 (0.31–0.38)	0.33 (0.28–0.38)	1.42 (1.05–1.93)	**0.025**
PNPLA2_CpG_9	0.28 (0.24–0.33)	0.29 (0.23–0.35)	0.80 (0.66–0.96)	**0.017**
PNPLA2_CpG_11,12	0.46 (0.38–0.53)	0.45 (0.35–0.51)	1.17 (0.97–1.42)	0.101
PNPLA2_CpG_13	0.15 (0.13–0.18)	0.15 (0.11–0.19)	0.84 (0.62–1.12)	0.238
PNPLA2_CpG_14,15,16,17	0.11 (0.08–0.13)	0.10 (0.07–0.14)	0.99 (0.57–1.73)	0.982
PNPLA2_CpG_18	0.12 (0.09–0.14)	0.11 (0.09–0.16)	0.91 (0.66–1.26)	0.578
PNPLA2_CpG_20,21	0.40 (0.34–0.45)	0.39 (0.30–0.47)	1.00 (0.81–1.23)	0.980

Notes: #Logistic regression, adjusted for age, gender and experimental batches. Significant *P*-values are in bold.
Abbreviations: LC, lung cancer; N, number; IQR, interquartile range; OR, odds ratio; CI, confidence interval.

The Association Between Blood-Based *PNPLA2* Methylation and Early-Stage LC Stratified by Gender and Age

To eliminate the confounding effects of gender and age, we further evaluated the association between *PNPLA2* methylation in peripheral blood and LC by stratified regression analyses. After stratified by gender, per 10% decrease in methylation for PNPLA2_CpG_8,10 conferred an 85% increase on the risk of LC in females (OR =1.85, 95% CI: 1.07–3.22, *P* = 0.029), but not in males (Table 4). The methylation of PNPLA2_CpG_9 showed a borderline

significant association with LC in males (OR =0.75, 95% CI: 0.56–1.00, *P* = 0.052), but not in females (Table 4). The rest 17 PNPLA2 CpG sites showed no association with LC in either gender (*P* values > 0.05 for all, Table 4).

In addition, subjects were stratified by the age of 55 years old. In participants less than 55 years old, hypermethylation of PNPLA2_CpG_6 and PNPLA2_CpG_9 was associated with LC (for PNPLA2_CpG_6, OR = 0.77, 95% CI: 0.63–0.95, *P* = 0.012; for PNPLA2_CpG_9, OR = 0.75, 95% CI: 0.60–0.94, *P* = 0.011; Table 5). The association between hypomethylation of PNPLA2_CpG_8,10 and LC was enhanced in this

Table 4 Gender-Stratified Association Between *PNPLA2* Methylation and LC

CpG Sites	Controls Median (IQR)	LC Cases Median (IQR)	OR (95% CI)[#] per −10% Methylation	P-value[#]
Female (63 controls vs 60 LC cases)				
PNPLA2_CpG_1	0.33 (0.19–0.52)	0.26 (0.15–0.48)	1.10 (0.86–1.41)	0.451
PNPLA2_CpG_2,3	0.14 (0.10–0.20)	0.17 (0.12–0.24)	0.80 (0.63–1.01)	0.063
PNPLA2_CpG_4	0.03 (0.02–0.03)	0.03 (0.02–0.05)	0.19 (0.02–1.95)	0.163
PNPLA2_CpG_5	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.95 (0.68–1.35)	0.786
PNPLA2_CpG_6	0.37 (0.33–0.43)	0.37 (0.29–0.48)	0.90 (0.71–1.13)	0.360
PNPLA2_CpG_7	0.24 (0.21–0.28)	0.24 (0.17–0.28)	1.21 (0.80–1.83)	0.375
PNPLA2_CpG_8,10	0.34 (0.32–0.37)	0.32 (0.28–0.36)	1.85 (1.07–3.22)	**0.029**
PNPLA2_CpG_9	0.27 (0.24–0.32)	0.26 (0.22–0.33)	0.87 (0.70–1.09)	0.235
PNPLA2_CpG_11,12	0.46 (0.39–0.53)	0.45 (0.32–0.50)	1.33 (0.99–1.78)	0.057
PNPLA2_CpG_13	0.15 (0.12–0.18)	0.14 (0.10–0.17)	0.90 (0.62–1.30)	0.566
PNPLA2_CpG_14,15,16,17	0.11 (0.09–0.13)	0.09 (0.07–0.13)	1.51 (0.57–4.01)	0.409
PNPLA2_CpG_18	0.12 (0.11–0.14)	0.11 (0.08–0.16)	0.94 (0.64–1.39)	0.761
PNPLA2_CpG_20,21	0.40 (0.35–0.47)	0.41 (0.30–0.47)	1.01 (0.72–1.43)	0.934
Male (124 controls vs 108 LC cases)				
PNPLA2_CpG_1	0.25 (0.17–0.37)	0.29 (0.22–0.40)	0.99 (0.73–1.34)	0.951
PNPLA2_CpG_2,3	0.29 (0.12–0.44)	0.25 (0.10–0.47)	1.07 (0.90–1.27)	0.446
PNPLA2_CpG_4	0.02 (0.01–0.04)	0.02 (0.01–0.04)	0.94 (0.32–2.76)	0.909
PNPLA2_CpG_5	0.04 (0.03–0.05)	0.04 (0.03–0.05)	0.68 (0.36–1.30)	0.243
PNPLA2_CpG_6	0.37 (0.31–0.42)	0.35 (0.27–0.46)	0.89 (0.74–1.08)	0.228
PNPLA2_CpG_7	0.21 (0.15–0.27)	0.22 (0.16–0.28)	1.04 (0.83–1.31)	0.743
PNPLA2_CpG_8,10	0.35 (0.30–0.38)	0.34 (0.28–0.39)	1.27 (0.87–1.84)	0.214
PNPLA2_CpG_9	0.29 (0.25–0.33)	0.29 (0.24–0.35)	0.75 (0.56–1.00)	0.052
PNPLA2_CpG_11,12	0.45 (0.35–0.53)	0.44 (0.36–0.54)	1.06 (0.82–1.38)	0.633
PNPLA2_CpG_13	0.15 (0.13–0.18)	0.15 (0.11–0.20)	0.80 (0.51–1.24)	0.316
PNPLA2_CpG_14,15,16,17	0.10 (0.08–0.12)	0.10 (0.07–0.14)	0.90 (0.46–1.77)	0.769
PNPLA2_CpG_18	0.12 (0.09–0.13)	0.11 (0.09–0.14)	0.95 (0.55–1.64)	0.858
PNPLA2_CpG_20,21	0.40 (0.34–0.45)	0.37 (0.30–0.46)	1.06 (0.82–1.37)	0.673

Notes: [#]Logistic regression, adjusted for age and experimental batches. Significant *P*-values are in bold.
Abbreviations: LC, lung cancer; IQR, interquartile range; OR, odds ratio; CI, confidence interval.

younger group with an OR of 2.43 (95% CI: 1.45–4.05, *P* = 0.001; Table 5). However, none of the 20 measurable CpG sites in *PNPLA2* was associated with LC in the subjects older than 55 years old (*P* values > 0.05 for all, Table 5).

The Correlation Between the Level of *PNPLA2* Methylation and the Clinical Characteristics of LC

To understand the correlation between methylation pattern of *PNPLA2* and the clinical characteristics of LC, the 168 early-stage LC cases were stratified by their clinical information and analyzed by nonparametric tests. Compared to the patients with smaller cancer (tumor length ≤ 1 cm), the LC patients with tumor larger than 1 cm have significantly higher methylation at PNPLA2_CpG_2,3 (median of

methylation: 0.15 vs 0.25, *P* = 0.006; Table 6), which indicated that hypermethylation of PNPLA2_CpG_2,3 might be associated with the progress of LC. Although PNPLA2_CpG_9 and PNPLA2_CpG_20,21 showed significantly different methylation between the stage I and stage II LC patients (Table 6), this result should be taken with caution own to the very limited samples of stage II tumor.

Discussion

In our present case-control study, we measured *PNPLA2* DNA methylation level in peripheral blood and found an association with LC in 168 early-stage LC cases and 187 age- and gender-matched healthy controls. *PNPLA2* gene is a rate-limiting enzyme catalyzing the first step of intracellular triglycerides hydrolysis in several tissues, and

Table 5 Age-Stratified Association Between *PNPLA2* Methylation and LC

CpG Sites	Controls Median (IQR)	LC Cases Median (IQR)	OR (95% CI)[#] per −10% Methylation	P-value[#]
≤ 55 years old (100 controls vs 64 LC cases)				
PNPLA2_CpG_1	0.32 (0.20–0.44)	0.24 (0.16–0.39)	1.19 (0.84–1.67)	0.327
PNPLA2_CpG_2,3	0.18 (0.11–0.39)	0.16 (0.08–0.40)	0.92 (0.78–1.10)	0.381
PNPLA2_CpG_4	0.02 (0.02–0.03)	0.02 (0.01–0.04)	0.82 (0.23–2.94)	0.758
PNPLA2_CpG_5	0.04 (0.02–0.05)	0.03 (0.02–0.06)	0.68 (0.38–1.25)	0.215
PNPLA2_CpG_6	0.39 (0.32–0.44)	0.39 (0.28–0.53)	0.77 (0.63–0.95)	**0.012**
PNPLA2_CpG_7	0.23 (0.18–0.27)	0.23 (0.16–0.28)	1.30 (0.92–1.84)	0.134
PNPLA2_CpG_8,10	0.35 (0.31–0.38)	0.33 (0.24–0.38)	2.43 (1.45–4.05)	**0.001**
PNPLA2_CpG_9	0.29 (0.24–0.33)	0.29 (0.23–0.39)	0.75 (0.60–0.94)	**0.011**
PNPLA2_CpG_11,12	0.45 (0.38–0.54)	0.45 (0.36–0.51)	1.28 (0.98–1.67)	0.073
PNPLA2_CpG_13	0.15 (0.13–0.18)	0.15 (0.11–0.21)	0.77 (0.50–1.18)	0.227
PNPLA2_CpG_14,15,16,17	0.11 (0.09–0.13)	0.11 (0.08–0.14)	1.38 (0.53–3.63)	0.512
PNPLA2_CpG_18	0.12 (0.10–0.14)	0.12 (0.09–0.16)	0.79 (0.52–1.21)	0.279
PNPLA2_CpG_20,21	0.39 (0.34–0.45)	0.39 (0.29–0.46)	1.00 (0.75–1.32)	0.983
> 55 years old (87 controls vs 104 LC cases)				
PNPLA2_CpG_1	0.23 (0.14–0.39)	0.30 (0.22–0.45)	0.86 (0.66–1.14)	0.293
PNPLA2_CpG_2,3	0.17 (0.12–0.36)	0.20 (0.12–0.42)	0.95 (0.77–1.17)	0.602
PNPLA2_CpG_4	0.02 (0.01–0.04)	0.03 (0.02–0.04)	0.53 (0.11–2.60)	0.430
PNPLA2_CpG_5	0.04 (0.03–0.05)	0.04 (0.03–0.05)	1.07 (0.60–1.91)	0.812
PNPLA2_CpG_6	0.36 (0.31–0.41)	0.35 (0.27–0.44)	0.97 (0.74–1.26)	0.801
PNPLA2_CpG_7	0.22 (0.17–0.28)	0.22 (0.17–0.28)	0.92 (0.69–1.22)	0.571
PNPLA2_CpG_8,10	0.34 (0.31–0.38)	0.33 (0.29–0.38)	0.84 (0.53–1.34)	0.464
PNPLA2_CpG_9	0.28 (0.25–0.32)	0.28 (0.23–0.34)	0.82 (0.58–1.17)	0.286
PNPLA2_CpG_11,12	0.47 (0.39–0.53)	0.44 (0.34–0.52)	1.08 (0.81–1.44)	0.606
PNPLA2_CpG_13	0.14 (0.12–0.17)	0.15 (0.10–0.18)	0.95 (0.62–1.45)	0.810
PNPLA2_CpG_14,15,16,17	0.10 (0.08–0.11)	0.10 (0.07–0.14)	0.79 (0.38–1.65)	0.540
PNPLA2_CpG_18	0.12 (0.09–0.13)	0.11 (0.08–0.15)	1.30 (0.70–2.43)	0.403
PNPLA2_CpG_20,21	0.40 (0.35–0.45)	0.37 (0.30–0.46)	1.15 (0.82–1.60)	0.425

Notes: [#]Logistic regression, adjusted for age and experimental batches. Significant *P*-values are in bold.
Abbreviations: LC, lung cancer; IQR, interquartile range; OR, odds ratio; CI, confidence interval.

subsequently affects the energy metabolism,[10] which is a typical feature of many cancer types.[15] Zagani et al found that increased *PNPLA2* activity could facilitate cell growth and attenuate apoptosis in NSCLC.[16] Intriguingly, *PNPLA2* gene is located on chromosome 11p15.5, which was known as a tumor suppressor gene region.[17] Loss of *PNPLA2* in the somatic tissue was found in 38% of lung cancer and in many other cancer types.[18] The mRNA and protein levels of PNPLA2 were lower in four lung adenocarcinoma cells than in normal cells, and the depletion of *PNPLA2* may cause triacylglycerol accumulation in lipid droplets and present an aggressive phenotype.[19,20]

DNA methylation is an early event of cancer. Our findings showed that the changed *PNPLA2* methylation level was associated with LC and even with LC at a very early stage (stage I). In our study, the DNA was extracted from the whole blood. The proportion of tumor DNA in

blood is minuscule compared with blood cell DNA (ratio about 1:1000),[21] and would be very unlikely to change the overall blood methylation values. Thus, the DNA obtained from the whole blood should mostly come from the leukocytes, as the platelet has no nuclear. Other studies have also revealed that the blood leukocyte DNA methylation is associated with LC.[22,23] Our previous studies have also disclosed that the breast cancer-associated altered methylation in the blood was mostly originated from the T cells,[14,24] which further confirmed that the leukocytes origination for the changed DNA methylation signatures in the whole blood. Unfortunately, there is no fresh blood available in this study to investigate which leukocytes subpopulation is the main contributor for the LC-associated *PNPLA2* methylation. So far, there is no direct study to explore the effects of *PNPLA2* methylation on its functions. Our target sequence is mainly located at the

Table 6 The Correlation Between *PNPLA2* Methylation and the Clinical Characteristics of LC Patients

Clinical Characteristics Group (N)	Median Methylation Level of Each CpG Site												
	CpG_1	CpG_2,3	CpG_4	CpG_5	CpG_6	CpG_7	CpG_8,10	CpG_9	CpG_11,12	CpG_13	CpG_14,15,16,17	CpG_18	CpG_20,21
Tumor subtype													
Adenocarcinoma in situ (37)	0.33	0.15	0.02	0.04	0.37	0.22	0.32	0.31	0.42	0.15	0.10	0.11	0.41
Microinvasive adenocarcinoma (34)	0.29	0.16	0.03	0.04	0.37	0.23	0.33	0.28	0.44	0.15	0.11	0.12	0.41
Invasive adenocarcinoma (97)	0.26	0.21	0.03	0.04	0.36	0.22	0.33	0.27	0.45	0.14	0.10	0.11	0.36
P-value*	0.377	0.193	0.912	0.892	0.781	0.976	0.895	0.284	0.990	0.833	0.500	0.752	0.275
Tumor size													
T1 (147)	0.29	0.18	0.03	0.03	0.36	0.22	0.33	0.28	0.45	0.15	0.10	0.11	0.39
T2 and T3 (21)	0.25	0.28	0.02	0.04	0.36	0.22	0.31	0.27	0.42	0.13	0.09	0.09	0.35
P-value$	0.791	0.780	0.545	0.331	0.948	0.639	0.812	0.146	0.167	0.486	0.481	0.058	0.206
Tumor length													
≤ 1 cm (68)	0.31	0.15	0.02	0.03	0.36	0.23	0.32	0.28	0.46	0.14	0.10	0.12	0.41
> 1cm (100)	0.25	0.25	0.03	0.04	0.37	0.22	0.33	0.27	0.43	0.15	0.10	0.11	0.36
P-value$	0.183	**0.006**	0.587	0.380	0.598	0.261	0.612	0.398	0.098	0.724	0.230	0.131	0.597
Lymph node involvement													
0 (164)	0.29	0.19	0.03	0.04	0.36	0.22	0.33	0.28	0.45	0.15	0.10	0.11	0.38
1 and 2 (4)	0.24	0.14	0.02	0.04	0.30	0.25	0.32	0.23	0.43	0.16	0.08	0.08	0.32
P-value$	0.818	0.530	0.058	0.946	0.443	0.578	0.776	0.067	0.818	0.935	0.396	0.236	0.119
Tumor stage													
Stage I (158)	0.28	0.18	0.03	0.04	0.36	0.22	0.33	0.29	0.45	0.15	0.10	0.11	0.39
Stage II (10)	0.43	0.31	0.02	0.04	0.37	0.25	0.31	0.25	0.42	0.12	0.08	0.11	0.32
P-value$	0.114	0.332	0.118	0.358	0.828	0.452	0.827	**0.018**	0.698	0.377	0.234	0.139	**0.008**

Notes: *P-value for Kruskal–Wallis test; $P-value for Mann–Whitney *U*-test; Significant P-values are in bold.
Abbreviations: LC, lung cancer; N, number; IQR, interquartile range; OR, odds ratio; CI, confidence interval.

promoter region, where the changed DNA methylation could regulate the expression of genes.[25] Here, we assumed that the changed methylation of *PNPLA2* may affect gene functions by modulating its expression and also call for functional studies in the future. The correlation between *PNPLA2* methylation and expression should also be investigated when RNA materials are available in further studies. In addition, it would also be interesting to investigate the methylation differences of *PNPLA2* among LC cases with different stages.

In gender-stratified analyses, the association of PNPLA2_CpG_8,10 hypomethylation and the risk of LC was observed only in women. On the one hand, it is well documented that inherent DNA methylation differences in peripheral blood between men and women exist in many CpG sites, which can be partly attributed to the difference in circulating sex hormones.[26] On the other hand, the evidence showed that female sex hormones including estrogen and its receptor (ER) are vital for lung development and related to the process of LC.[27] Both ERα and ERβ exerted a promoting effect on LC via multiple pathways.[28,29] Previous studies have identified that women tend to have higher incidence rates of LC, particularly at a younger age, which cannot be explained by smoking habits between gender disparity.[30] A comparative study implied that high sensitivity to the tobacco carcinogens in women was another reason for their increased LC morbidity.[31] Although tobacco consumption was not available in our study, gender-specific association should be marked.

Unlike our previous study in which the association between hypomethylation of *RAPSN* and LC was observed in subjects older than 55 years, the present age-stratified data analyses revealed a significant association between aberrant *PNPLA2* methylation and the risk of LC in subjects aged ≤ 55 years, highlighting that the effect of changed methylation on LC may be age-related.[9] Nevertheless, the sample size of our study is limited. The underlying mechanism of the age-related methylation pattern needs to be further explored in the future studies with large sample size and in a multi-center setting.

To our knowledge, this is the first study to assess the association between *PNPLA2* methylation in peripheral blood and LC. Our study recruited more than three hundred participants and 94% LC cases are at a very early stage. However, limited by hospital-based sample collection, information about lifestyle is not available. A prospective cohort and functional studies would be warranted to consolidate the relationship between changed methylation and LC.

Conclusion

In summary, our study disclosed the association between altered *PNPLA2* methylation in the peripheral blood and early-stage LC. Also, there are unmet needs for large-scale studies with comprehensive information to verify our findings and even functional experiments to elucidate the molecular mechanism.

Ethics Approval and Informed Consent

The present study was approved by the Ethics Committee of the Shanghai Chest Hospital and Jiangsu Province Hospital of Chinese Medicine in China. Written informed consents were obtained from all the participants.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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