European Review for Medical and Pharmacological Sciences 2018; 22: 8731-8739

Elevated *PHD2* expression might serve as a valuable biomarker of poor prognosis in lung adenocarcinoma, but no lung squamous cell carcinoma

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Abstract. – OBJECTIVE: Using data from The Cancer Genome Atlas (TCGA), we aimed to explore the association between Egl nine homolog 1 (EGLN1/PHD2) expression and survival outcomes in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), respectively.

PATIENTS AND METHODS: A retrospective study was conducted based on the level-3 data in the Cancer Genome Atlas (TCGA)-LUAD and TCGA-LUSC and data from the Human Protein Atlas (HPA).

RESULTS: Both LUAD and LUSC had elevated PHD2 expression compared to their respective adjacent normal tissues. However, Kaplan-Meier survival curves showed that the high PHD2 expression LUAD patients had a significantly shorter overall survival (OS) and recurrence-free survival (RFS) (p=0.001 and p<0.001) compared to the low expression group. However, these differences were not observed in LUSC patients. Univariate and multivariate analysis showed that high PHD2 expression was an independent indicator of unfavorable OS (HR: 1.685, 95%CI: 1.251-2.269, p=0.001) and unfavorable RFS (HR: 2.008, 95%CI: 1.430-2.818, p<0.001) in LUAD patients. The methylation status of two CpG sites (cg07040244 and cg21875980) in PHD2 was at least moderately and negatively correlated with PHD2 expression. High methylation level of these two CpG sites was associated with better OS in LUAD patients.

CONCLUSIONS: Elevated PHD2 expression might only serve as a valuable biomarker of poor prognosis in LUAD, but no in LUSC. Cg07040244 and cg21875980 might be two CpG sites modulating PHD2 expression in LUAD.

Key Words
PHD2, Prognosis, Lung adenocarcinoma, Lung squamous cell carcinoma, Methylation.

Introduction

Egl nine homolog 1 (EGLN1) often known as PHD2, is a gene encodes hypoxia-inducible factor prolyl hydroxylase 2 (HIF-PH2), or prolyl hydroxylase domain-containing protein 2 (PHD2), which belongs to the superfamily of α-ketoglutarate/2-oxoglutarate-dependent hydroxylase[1,2]. PHD2 acts as an oxygen sensor and catalyzes prolyl hydroxylation of HIF1 under normoxia, which enables its binding with von Hippel-Lindau (VHL) and subsequent proteasomal degradation by ubiquitination[1].

Despite the importance of PHD2 in mediating polyubiquitylation of HIF1, its role in carcinogenesis and cancer development remains conflicting and incompletely elucidated. Some studies[3] found that in pancreatic cancer, PHD2 functions as a tumor suppressor gene that inhibits tumor growth and reduces tumor invasion by inhibiting angiogenesis. In colorectal cancer, low expression of PHD2 predicts poor survival independent of HIF-1α[4]. However, a recent study[5] found that PHD2 interacts with epidermal growth factor receptor (EGFR) and enhances its stability in some cancers. PHD2 heterozygous loss is associated with the reduced metastatic potential of tumor cells via decreasing cancer-associated fibroblasts (CAFs) activation, matrix production, and contraction by CAFs[6]. Another recent investigation[7] found that the combined loss of PHD2 in stromal and cancer cells sensitizes tumors to chemotherapy via promoting tumor vessel normalization and subsequent enhanced tumor response to chemotherapeutics. In triple-negative breast cancer cells, inhibition of PHD2 inhibits tumor growth affecting

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TGF-beta1 processing[8]. These findings suggest that the functional role of PHD2 might be highly contexted and cell-type dependent.

A previous study[9] found that *PHD2* upregulation was associated with unfavorable prognosis in non-small cell lung cancer (NSCLC). However, the histological subtypes of NSCLC, mainly lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), vary significantly in molecular and clinical features[10-13]. In this study, using data from The Cancer Genome Atlas (TCGA), we explored the association between PHD2 expression and survival outcomes in LUAD and LUSC patients, respectively.

Patients and Methods

Secondary Analysis Using Data From TCGA-LUAD and TCGA-LUSC

The level-3 data in TCGA-LUAD and TCGA-LUSC were downloaded using the UCSC Xena Browser (https://xenabrowser.net/). Only the primary tumor tissues from patients without historical neoadjuvant chemotherapy and with gene expression measured by RNA-seq (IlluminaHiSeq) were included in this study. Based on the criteria, 511 LUAD cases and 494 LUSC cases were included. Among LUAD patients, 499 cases had intact OS data, while 424 cases had intact RFS data. Among LUAD patients, 487 cases had intact OS data, while 380 cases had intact RFS data. Their genomic, clinicopathological and survival data, including RNA-seq data, DNA methylation data, age at initial diagnosis, gender, smoking history, pathological stage, primary therapy outcome success, the presence of residual tumors, canonical mutation in KRAS/EGFR/ALK, recurrence status, RFS in days, living status and OS in days, were collected. The DNA methylation data were obtained by using Illumina Infinium Human Methylation 450K BeadChip. Among the primary LUAD cases included, 450 cases had RNA-seq and DNA methylation data available at the same time. 439 cases had intact OS data recorded, while 384 cases had intact RFS data recorded. Primary therapy outcomes were defined as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD).

Data Mining in the Human Protein Atlas

PHD2 protein expression in respiratory epithelial cells of human bronchus and human LUAD and LUSC tissues was examined by using immunochemistry (IHC) data in the Human Protein Atlas (HPA) (http://www.proteinatlas.org/) [14,15]. In this database, IHC staining results were scored as not detected, low, medium and high, which is a combination of staining intensity and fraction of stained cells.

Ethical Approval

This study was approved by the Ethical Committee of the Shanghai Pulmonary Hospital Affiliated to Tongji University.

Statistical Analysis

Statistical analysis was performed using SPSS 25.0 software package (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Inc., La Jolla, CA, USA). The difference in *PHD2* expression between different groups was compared using Welch's unequal variances *t*-test. Kaplan-Meier survival curves were generated using GraphPad Prism 7.0 (GraphPad Inc., La Jolla, CA, USA), by separating the patients into two groups according to the Youden Index (optimal cutoff) of *PHD2* expression or its DNA methylation in the receiver operating characteristic (ROC) analysis for death and recurrence detection. The log-rank test was conducted to compare the difference between the survival curves. The difference in the clinicopathological parameters and survival outcomes between patients with high and low PHD2 expression (separated by the optimal cutoff in ROC analysis for death detection) in LUAD patients was assessed by the χ^2-test by two-sided Fisher's exact test. Univariate and multivariate Cox regression models were used to analyze the prognostic significance of PHD2 expression in terms of overall survival (OS) and recurrence-free survival (RFS). Regression analysis was performed to assess the Pearson correlation coefficiency between PHD2 expression and the methylation level of its CpG sites. $p < 0.05$ was considered statistically significant.

Results

PHD2 Expression Was Upregulated in Both LUAD and LUSC Compared With Adjacent Normal Tissues

To examine the expression profile of PHD2 in NSCLC, we used RNA-Seq data in both TCGA-LUAD and TCGA-LUSC. Only the primary tumor cases without the history of neoadjuvant chemotherapy were included. In TCGA-LUAD cohort, 511 LUAD cases and 58 adjacent normal tissues were subjected to RNA-seq study, while in TCGA-LUSC, 494 LUSC cases and 51 adjacent normal tissues were subjected to RNA-seq.

Results showed that both LUAD and LUSC had elevated PHD2 expression compared to their respective adjacent normal tissues (Figure 1A-D). Besides, PHD2 expression in LUSC cases was even higher than that in LUAD cases (Figure 1E). Then, we further examined PHD2 expression at the protein level in normal bronchus and NSCLC tissues, using IHC staining data from the HPA. Results showed that the respiratory epithelial cells in normal bronchus tissues usually had medium PHD2 staining (Figure 1F, left). In comparison, 6 LAUD cases and 5 LUSC cases examined all had medium to high staining (Figure 1F, right).

Elevated PHD2 Expression Was Associated With Unfavorable Survival Outcome in LUAD, But Not in LUSC Patients

Since we observed that PHD2 expression was significantly upregulated in both LUAD and LUSC compared to that in normal bronchus tissues, we next investigated its association with survival outcome in LUAD and LUSC patients respectively. Results showed that among LUAD patients, the deceased cases and the cases with recurrence had a significantly higher PHD2 expression, compared to their respective control groups (p<0.001 and p=0.005) (Figure 2A-B). In comparison, these associations were not confirmed in LUSC patients (p=0.95 and p=0.48) (Figure 2C-D). Kaplan-Meier survival curves were generated to compare OS and RFS between patients with high and low PHD2 expression. Results showed that in LUAD patients, the high PHD2 expression group had a significantly shorter OS and RFS (p=0.001 and p<0.001) (Figure 3A-B). However, these differences were not observed in LUSC patients (Figure 3C-D).

High PHD2 Expression Was an Independent Indicator of Unfavorable Survival in LUAD Patients, But Not in LUSC Patients

The clinicopathological parameters of patients with high or low *PHD2* expression (separated by the optimal cutoff in ROC for death detection) were summarized in Table I. Chi-square analy-

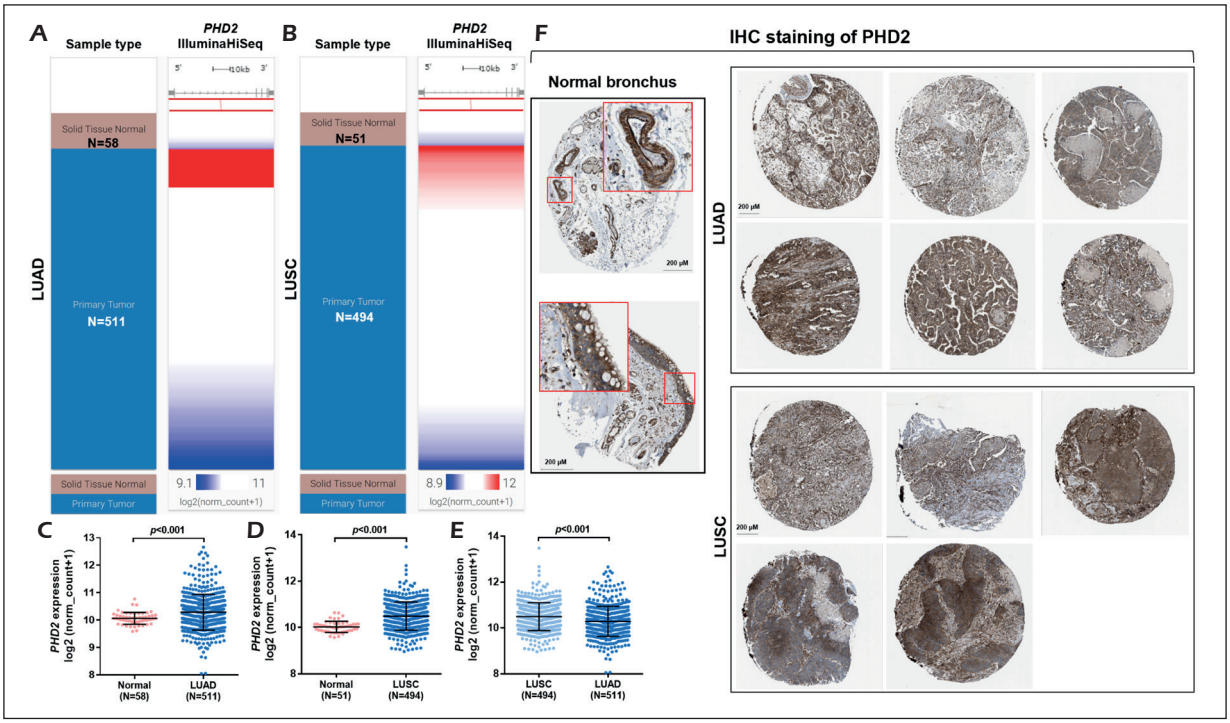

Figure 1. PHD2 expression was upregulated in both lung adenocarcinoma and lung squamous cell carcinoma compared with adjacent normal tissues. **A-B**, Heatmap showing the expression profile of PHD2 RNA in LUAD (**A**) and LUSC (**B**) tissues and in their respective adjacent normal tissues. **C-E**, Plots chart comparing the difference in PHD2 RNA expression in LUAD (**C**) and LUSC (**D**) tissues and in their respective adjacent normal tissues and between LUAD and LUSC tissues (**E**). **F**, IHC staining of PHD2 protein in normal respiratory epithelial cells *(left)* and in LUAD and LUSC tissues *(right)*. Image credit: Human Protein Atlas. Data summary images were obtained from: v18.proteinatlas.org, via: https://www.proteinatlas.org/ENSG00000135766-EGLN1/tissue/bronchus and https://www.proteinatlas.org/ENSG00000135766-EGLN1/pathology/tissue/lung+cancer#ihc

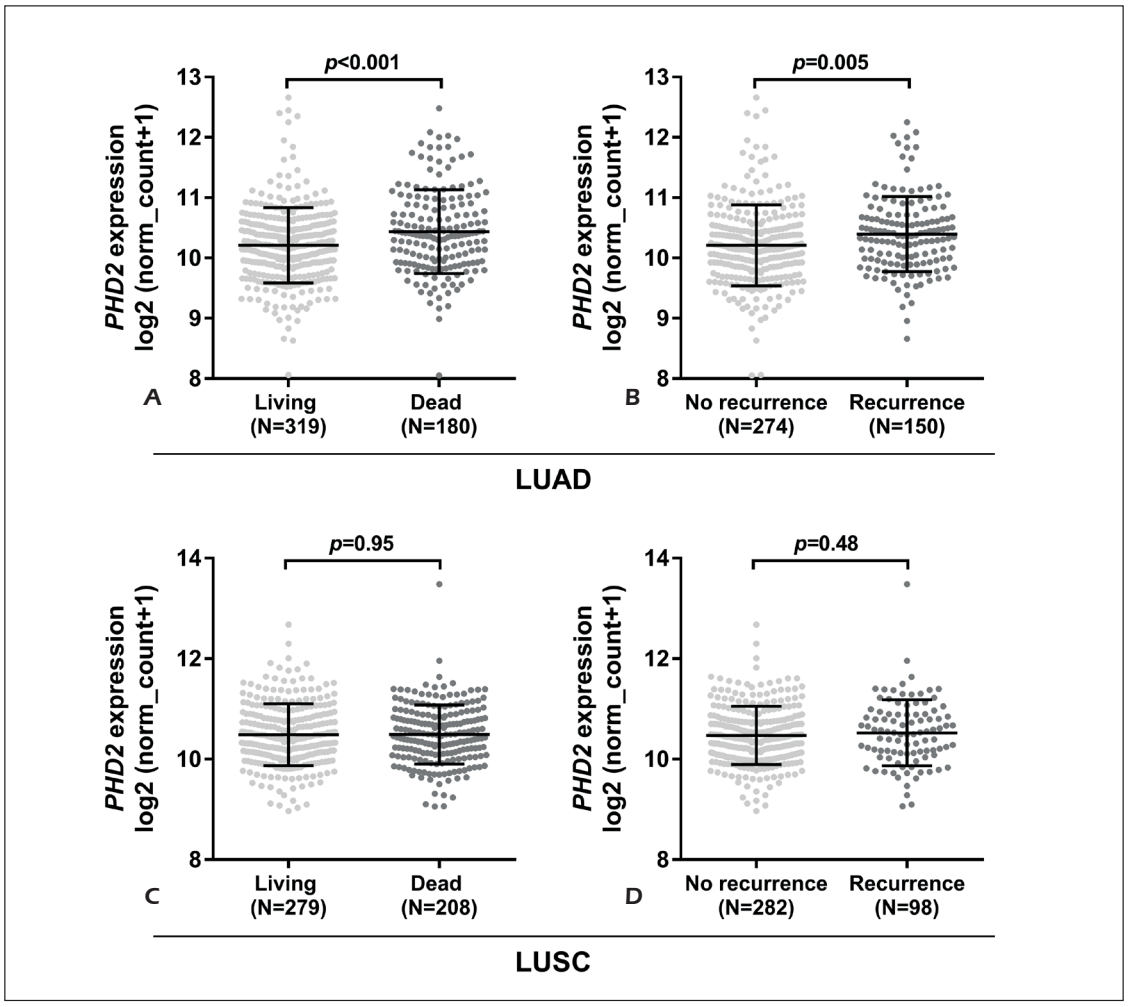

Figure 2. Elevated PHD2 expression was associated with unfavorable survival outcome in LUAD, but not in LUSC patients. **A**-**D**, Comparison of PHD2 expression in LUAD (**A** and **B**) and LUSC (**C**-**D**) patients grouped according to OS status (**A** and **C**) or RFS status (**B** and **D**).

sis showed that the high PHD2 expression group (N=238) had a significantly higher proportion of male patients (121/238 *vs.* 67/227, p=0.031), patients with recurrence after primary therapy (83/197 *vs.* 107/261, p=0.008) and death (103/238 *vs.* 77/227, p=0.002) (Table I). By performing univariate analysis, we found that advanced pathological stages, with residual tumors and high PHD2 expression, were associated with significantly shorter OS (Table II). Multivariate analysis confirmed that high PHD2 expression was an independent indicator of unfavorable OS (HR: 1.685, 95% CI: 1.251-2.269, p=0.001) (Table II). Besides, we also found that advanced pathological stages and high *PHD2* expression were associated with significantly shorter RFS (Table III). Multivariate analysis suggested that high PHD2 expression was an independent indicator of unfavorable RFS (HR: 2.008, 95%CI: 1.430-2.818, p<0.001) (Table III).

PHD2 Elevation Was Related to the Hypomethylation Status of its DNA in LUAD

Then, we checked the methylation status of PHD2 DNA and its correlation with PHD2 expression, among 450 LUAD cases with DNA methylation and RNA-Seq data available at the same time. Regression analysis suggests that the methylation status of two CpG sites (cg07040244 and cg21875980) were at least moderately and negatively correlated with PHD2 expression (Pearson's r=-0.41 and -0.44 respectively) (Figure 4A-B). Next, we examined the association between the methylation status of these two sites

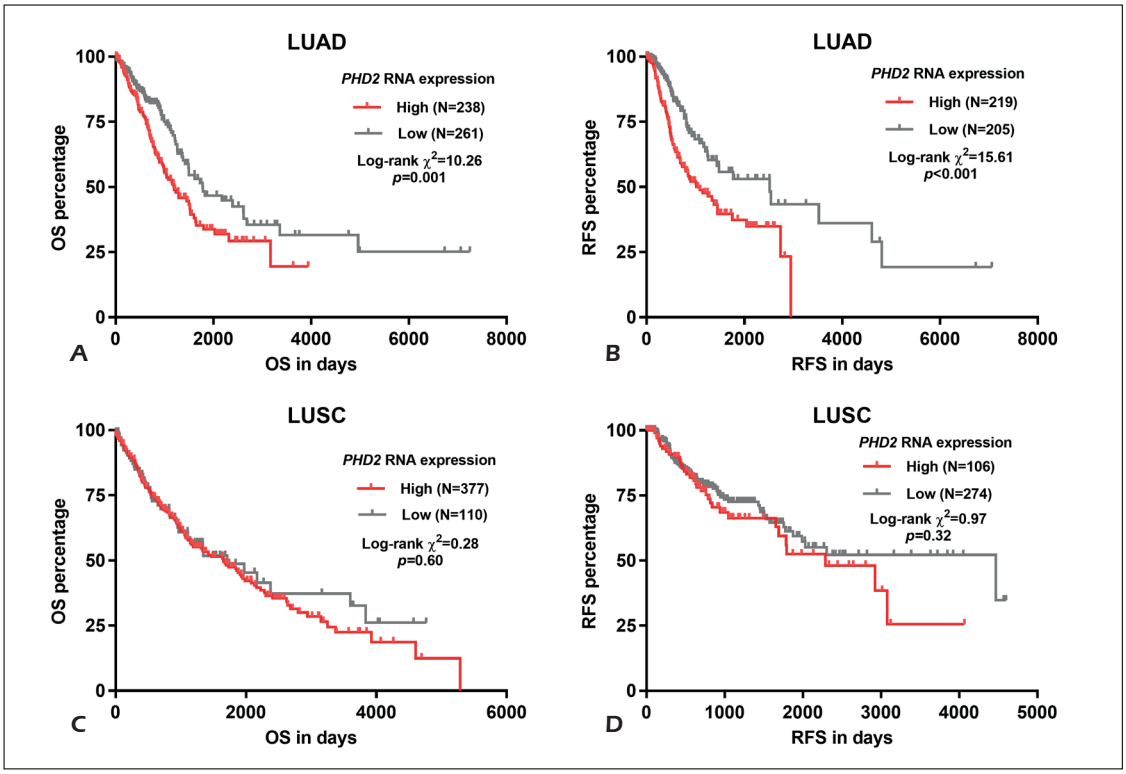

Figure 3. High PHD2 expression was an independent indicator of unfavorable survival in LUAD patients, but not in LUSC patients. **A**-**D**, Kaplan-Meier curves of OS (**A** and **C**) and RFS (**B** and **D**) in LUAD (**A**-**B**) and LUSC (**C**-**D**) patients. The patients were separated into two groups according to the best cutoff of PHD2 expression in ROC for death (**A** and **C**) or recurrence (**B** and **D**) detection respectively.

Table I. Comparison of the clinicopathological parameters and survival outcomes between patients with high and low EGLN1 expression.

Parameter		EGLN1 expression *p*-value		
			High (N=238)	Low (N=261)
Age (Mean ± SD)		65.53±9.30	65.15±10.48	0.67
Gender	Female	117	154	0.031
	Male	121	107	
Smoking History	1	40	32	0.16
	2/3/4/5	190	223	
	No data	8	6	
Clinical Stage	I/II	178	209	0.23
	III/IV	55	49	
	Discrepancy/no data	5	3	
Primary therapy outcome success	PD/SD	55	47	0.085
	PR/CR	133	171	
	Discrepancy/no data	50	43	
Residual tumors	R0	158	176	0.61
	R1/R2	6	10	
	RX/no data	74	75	
Mutation in KRAS/EGFR/ALK	No	64	61	0.58
	Yes	51	41	
	No data	123	159	

Table II. Univariate and multivariate analysis of OS in LUAD patients.

Parameters	Univariate analysis				Multivariate analysis			
	p	HR	95% CI (lower/upper)		*p*	HR	95% CI (lower/upper)	
Age (Continuous)	0.307	1.008	0.993	1.024				
Gender								
Male (N=228)		1.000						
Female (N=271)	0.843	0.971	0.724	1.302				
Smoking history								
1 (N=72)		1.000						
2/3/4/5 (N=413)	0.596	0.894	0.592	1.351				
Pathological stages								
III/IV (N=104)		1.000						
I/II (N=387)	**<0.001**	0.385	0.281	0.527	**<0.001**	0.409	0.297	0.565
Residual tumors								
Yes (N=16)		1.000						
No (N=334)	**<0.001**	0.248	0.139	0.443	**<0.001**	0.323	0.178	0.586
Mutation in KRAS/ EGFR/ALK								
Yes (N=92)		1.000						
No (N=125)	0.570	1.134	0.734	1.753				
ENLG1 expression								
Low (n=261)		1.000						
High (n=238)	**0.001**	1.636	1.215	2.203	**0.001**	1.685	1.251	2.269

and the survival of LUAD patients. Kaplan-Meier survival curves showed that the high methylation level of these two CpG sites was associated with better OS ($p<0.001$, Figure 4C). However, no significant association was observed in LUSC patients ($p=0.16$, Figure 4D).

Table III. Univariate and multivariate analysis of RFS in LUAD patients.

Parameters	Univariate analysis				Multivariate analysis			
	p	HR	95% CI (lower/upper)		*p*	HR	95% CI (lower/upper)	
Age (Continuous)	0.356	1.008	0.991	1.025				
Gender								
Male (N=191)		1.000						
Female (N=233)	0.516	1.114	0.805	1.541				
Smoking history								
2/3/4/5 (N=345)		1.000						
1 (N=65)	0.454	1.199	0.746	1.926				
Pathological stages								
III/IV (N=80)		1.000						
I/II (N=337)	**0.009**	0.599	0.407	0.882	**0.004**	0.566	0.384	0.834
Residual tumors								
Yes (N=12)		1.000						
No (N=273)	0.000	0.262	0.126	0.543				
Mutation in KRAS/ EGFR/ALK								
Yes (N=77)		1.000						
No (N=108)	0.787	1.063	0.684	1.653				
ENLG1 expression								
Low (n=205)		1.000						
High (n=219)	**<0.001**	1.952	1.392	2.738	**<0.001**	2.008	1.430	2.818

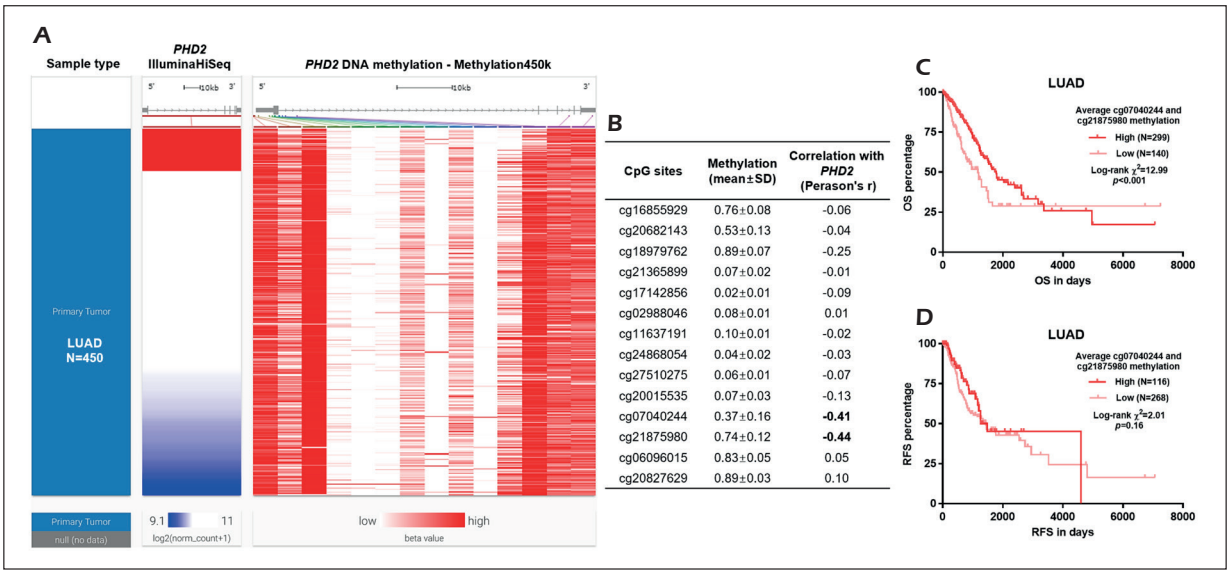

Figure 4. PHD2 elevation was related to the hypomethylation status of its DNA in LUAD. **A-B**, Heatmap and regression analysis showing the correlation between PHD2 DNA methylation and PHD2 expression in LUAD tissues. **C-D**, Kaplan-Meier curves of OS (**C**) and RFS (**D**) in LUAD patients. The patients were separated into two groups according to the best cutoff of the average methylation of cg07040244 and cg21875980 in ROC for death (**C**) or recurrence (**D**) detection, respectively.

Discussion

Although PHD2 was an oxygen sensor with key regulatory effects on the HIF-pathway, this enzyme has also been associated with the regulation of some other non-HIF proteins, such as epidermal growth factor receptor (EGFR)[5], nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[16], eukaryotic elongation factor 2 kinase (eEF2K)[17], and N-myc downstream-regulated gene 3 protein (NDRG3)[18]. Besides, based on available large databases, including Oncomine and TCGA, it was found that there are more tumor types with PHD2 overexpression compared to their normal histological counterpart than the tumor types with this gene downregulation[19]. Briefly, PHD2 is generally upregulated in brain and central nervous system cancer, head and neck cancer, kidney cancer, liver cancer, and lung cancer, but might be downregulated in colorectal cancer and pancreatic cancer[19]. Accordingly, previous studies reported that PHD2 might exert both pro- and anti-tumoral effect, depending on the cellular context. For example, in *in-vitro* and *in-vivo* models using Lewis lung carcinoma and melanoma cell lines, PHD2 inhibition retards tumor growth via the anti-proliferative activity of TGFβ, in a HIF-independent manner[20]. However, in CRC, PHD2 shows a protective role in patients with early-stage CRC and low PHD2 expression had significantly shorter survival compared to the patients who did not display this decrease[4]. Besides, inhibition of PHD2 in CRC xenografts resulted in facilitated tumor growth due to the activation of the NF-κB signaling and subsequent increased angiogenesis[21]. Therefore, the role of PHD2 in cancer progression is still controversial, and it is reasonable to speculate that PHD2 has diverse functions under different environment.

In this study, using RNA-seq data from TCGA and IHC staining data from the HPA, we found that both LUAD and LUSC tissues had significantly increased PHD2 expression compared with adjacent normal tissues. One previous study observed that PHD2 upregulation was associated with unfavorable prognosis in NSCLC[9]. Considering the significant difference in molecular and clinical features between LUAD and LUSC, we further performed subgroup analysis in LUAD and LUSC respectively. Results showed that PHD2 upregulation was only associated with unfavorable survival outcomes in LUAD patients, but not in LUSC patients. By generating Kaplan-Meier survival curves and univariate/multivariate analysis, we found that high *PHD2* expression was an independent indicator of unfavorable OS (HR: 1.685, 95% CI: 1.251-2.269, p=0.001) and RFS (HR: 2.008, 95% CI: 1.430-2.818, p<0.001) in LUAD patients, but not in LUSC patients. Therefore, PHD2 upregulation might serve as a valuable biomarker of poor prognosis in this subtype of NSCLC. In the future, it will be necessary to characterize the regulatory effects of PHD2 in LUAD.

The mechanisms underlying the dysregulation of PHD2 is quite complex and far from been fully illustrated. Its transcription might be suppressed by miR-182 in prostate cancer[22]. The PHD2 promoter contains a Hypoxia-Response Element (HRE) region and thus is positively regulated by the HIF pathway itself. Under hypoxic condition, HIF1α binds to the HRE region and induces *PHD2* transcription [23]. In this study, we explored the epigenetic dysregulation, typically methylation alterations associated with *PHD2* in 450 LUAD cases. Based on the results, we found two CpG sites (cg07040244 and cg21875980) were at least moderately and negatively correlated with *PHD2* expression. High methylation level of these two CpG sites was associated with better OS. These findings suggest that these two sites might play a critical role in modulating PHD2 expression in LUAD cases.

Conclusions

We found that the elevated PHD2 expression might only serve as a valuable biomarker of poor prognosis in LUAD, but no LUSC. Cg07040244 and cg21875980 might be two CpG sites modulating the PHD2 expression in LUAD.

Conflict of Interests

The authors have no conflict of interest.

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