



The expression patterns and prognostic significance of pleckstrin homology-like domain family A (PHLDA) in lung cancer and malignant mesothelioma

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Background: Pleckstrin homology domain family A (PHLDA) genes play important roles in cancer cellular processes, including inhibiting Akt activation, repressing growth factor signaling, inhibiting the negative feedback of EGFR/ErbB2 signaling cells, and inducing apoptosis. However, the prognostic significance of PHLDA in non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MM) remains unclear. The present study investigates the associations between PHLDA expression patterns and their prognostic value in lung adenocarcinoma (LUAD) and MM.

Methods: We analyzed PHLDA family members at the genomic level *in silico* to explore their mRNA expression pattern and predictive significance in LUAD and MM. We then created a PHLDA–drug interaction network and a protein–protein interaction (PPI) network using different databases. Finally, we immunohistochemically assessed the protein expression of each PHLDA family member on tissue microarrays (TMAs) in both LUAD and MM cohorts with long-term follow-up.

Results: While *PHLDA1* mRNA expression in both LUAD and MM was lower than that of normal tissue, *PHLDA2* mRNA was significantly overexpressed in LUAD, and *PHLDA3* mRNA was overexpressed in MM. In NSCLC, both low *PHLDA1* mRNA expression and high *PHLDA3* mRNA expression correlated with worse overall survival (OS) ($P < 0.01$), whereas high *PHLDA2* mRNA expression was associated with better OS ($P < 0.01$). In MM, patients presenting high *PHLDA1* and *PHLDA2* mRNA expression had poor OS ($P = 0.01$ and $P < 0.01$, respectively). In addition, the PHLDA–drug interaction network indicated that several common drugs could potentially modulate PHLDA expression, and the PPI network suggested that *PHLDA1* interacts with Notch family members, whereas *PHLDA3* interacts with TP53. Our results also showed that the expression of *PHLDA2* and *PHLDA3* was significantly higher in LUAD and MM than that of *PHLDA1* ($P < 0.05$) and was associated with the risk of death. While patients with *PHLDA2* > 85.09 cells/mm² had a low risk of death ($P = 0.01$) and a median survival time of 48 months, those with *PHLDA3* < 70.38 cells/mm² had a high risk of death ($P = 0.03$) and a median survival time of 34 months.

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Conclusions: We shed light on the role of the PHLDA family as promising predictive biomarkers and potential therapeutic targets in LUAD and MM.

Keywords: Morphometry; data mining; malignant mesothelioma; lung adenocarcinoma; pleckstrin homology-like domain family A (PHLDA)

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Introduction

Non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MM) are pathologies that share the common fact that both are potentially highly aggressive tumors, and their prognosis is highly dependent on the histological subtype, lymph node status, and the pathological TNM stage of the disease (1-4). In addition, these characteristics end up influencing the decision-making decision regarding the therapeutic strategy. For both, surgery is the standard treatment for patients with early disease, however, the overall 5-year survival rate remains below 50% (1-3).

Today, the major difference between these pathologies is the broader range of therapeutic possibilities available for NSCLC, while MM does not yet have a broad arsenal. Therefore, new biomarkers are much necessary that could assist in more individualized treatment for both pathologies. Since conventional therapeutic protocols have been limited in their effects on treatment outcomes, innovative strategies for treating NSCLC and MM are now being explored, especially those that are molecularly targeted.

The pleckstrin homology-like domain, family A (PHLDA) is a family of genes consisting of 3 members: PHLDA1, PHLDA2, and PHLDA3 (5). The genes in the PHLDA family have a similar organization, involving one coding exon and one noncoding exon separated by a small intron (6). All three genes encode for PH domain-containing proteins. These proteins share high similarity within their PH domains and are detectable using immunohistochemistry screening with variable sensitivity and specificity (5). Cumulative evidence has shown that PHLDA is a family of tumor oncogenes that play important roles in cancer cellular processes, including competing with Akt signaling (7-9), repressing growth factor signaling (10), inhibiting negative feedback of EGFR/ErbB2 signaling cells (8), inducing apoptosis (6), and recent reports suggest the PHLDA3 gene acts as a tumor suppressor in human neuroendocrine tumors (11,12).

Thus, the current study seeks to support the clinical value of PHLDA genes as potential agnostic biomarkers and to point at new targetable therapeutic pathways for patients with NSCLC and MM. We comprehensively analyzed the expression patterns of PHLDA family genes and their prognostic value in LUAD and MM using *in silico* data-mining investigation methods combined with clinical immunohistochemistry on tissue microarrays.

We present the following article in accordance with the REMARK reporting checklist (available at <http://dx.doi.org/10.21037/jtd-20-2909>).

Methods

PHLDA mRNA expression

We used UALCAN (RRID:SCR_015827), a user-friendly web resource for analyzing data from The Cancer Genome Atlas (TCGA; RRID:SCR_003193) (13), to investigate the relative mRNA expression of *PHLDA* family genes in lung adenocarcinoma (LUAD) and normal samples. The mRNA expression level of *PHLDA* genes was normalized to transcript per million reads, and only a P value of no more than 0.01, according to a Student's *t*-test, was to be significant. We selected the Genomic Spatial Event (GSE) database (14) and GSE51024 (15) downloaded from NCBI GEO to analyze the mRNA expression of *PHLDA* family genes in MM and normal tissues according to adjusted $P < 0.01$ and having $|\log FC| > 2$ as the threshold. The OncoPrint database (RRID:SCR_007834), the world's largest cancer microarray database and web-based data mining platform for extracting cancer gene information (16,17), was then used to determine the differences in the mRNA expression of each *PHLDA* family member between different cancer specimens and normal controls. The P value for these differences in expression was established through a Student's *t*-test. We set the threshold at 2.0-fold change, $P = 0.05$, and top 10% gene rank.

The prognostic significance value of the mRNA expression of *PHLDA* genes in NSCLC was evaluated using the Kaplan-Meier plotter database (RRID:SCR_018753) (18). Only validated probes were used to access the overall survival (OS), first progression (FPS), and post-progression survival (PPS) curves. Patient samples were divided into two cohorts, according to the median expression of each gene (high vs. low expression). The effects of different patterns of *PHLDA* expression on OS were estimated through univariate and multivariate Cox proportional hazards models, with or without adjustments for confounding factors. Variables including gender, TNM stage, and age were further adjusted during the evaluation. The Kaplan-Meier plotter database calculated the log-rank P value and hazard ratio (HR) with 95% confidence intervals (CI). $P < 0.05$ was considered significant. The UCSC Xena (RRID:SCR_018938) (19) browser (<http://xena.ucsc.edu/>) was used to analyze OS in Mesothelioma (TCGA, Firehose Legacy) data.

Interaction networks

We searched within the Comparative Toxicogenomics Database (CTD; RRID: SCR_006530) (20) for *PHLDA* genes in cases employing drugs or chemicals that could decrease/increase their mRNA or protein expression and used this data to create a *PHLDA*-drug interaction network. This network was then generated using Cytoscape (RRID:SCR_003032) (21). Finally, we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; RRID:SCR_005223) database (22) to explore the protein-protein interaction (PPI) between *PHLDA* family members.

***PHLDA* protein expression**

Tissue samples

Specimens were obtained from archived formalin-fixed paraffin-embedded (FFPE) histological sections of 102 primary tumors from patients with stage I–IV who had been treated at Clinicas Hospital of University of São Paulo Medical School (HC-FMUSP), the Heart Institute (InCor) and São Paulo Cancer Institute (ICESP) between 1995 and 2018. Their histologic diagnoses were reviewed and confirmed by two pathologists who specialize in lung cancer. The study included 41 LUAD and 61 MM (epithelioid and sarcomatoid histotypes), placed in tissue microarrays (TMAs), none of which had received neoadjuvant therapy. Each TMA was constructed from the primary resected

tumors using three 1.5 mm tissue cores obtained from the center, middle, and periphery of the most representative tumor areas that were selected and marked on hematoxylin-eosin (HE) stained slides. Normal liver and kidney tissues were used for control and slide orientation purposes. The previous histological assessment of each analysis area was performed to ensure that the tumor tissue (at least 85% malignant cells) was included in the selected intratumoral region. Twelve slides containing 1,197 tumor cores were evaluated.

The Internal Ethics Committees of all participant institutions approved this study protocol.

Immunohistochemistry analysis

Firstly, the immunostains were tested on 10 whole tissue sections and 18 TMA to ensure that the stain was evenly and not patchy. Four-micron-thick sequential whole tissue sections and TMA sections were stained with immunoperoxidase. The staining was performed with antibodies against *PHLDA1* (sc6142, clone M-20, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, EUA; RRID:AB_2163292), *PHLDA2* (polyclonal antibody, CAT#: TA344404, dilution 1:50; Origene, OriGene Technologies, Rockville, USA), *PHLDA3* (polyclonal antibody, CAT#: TA315261, dilution 1:100; Origene, OriGene Technologies, Rockville, USA) and tumor suppressor P53 (clone 318-6-11, dilution 1:200; DAKO, Via Real Carpinteria, CA, USA). Cell expression of all markers was detected using a Novocastra Bond Polymer Refine Detection kit (Leica Microsystems) with a diaminobenzidine reaction to detect antibodies labeling and hematoxylin counterstaining. For *PHLDA*s expression, cytoplasmic with perinuclear accentuation and dots patterns were considered positive. Nuclear expression was considered positive for P53.

Immunohistochemical staining for the *PHLDA*s family showed homogeneous cytoplasmic staining with perinuclear accentuation and cytoplasmic staining in dots in all 10 MM and 18 LUAD whole mount slides and tumor cores. In addition, immunohistochemical staining for *PHLDA* family antibodies showed homogeneous cytoplasmic staining with perinuclear accentuation and cytoplasmic staining in dots in 40 (40%) MM and 60 (60%) LUAD tumor cores (Figure S1).

Image analysis quantification

To measure the IHC expression of each different marker and quantify protein expression, the TMA slides were digitally scanned at $\times 40$ magnification using a Panoramic

Table 1 Frequency of clinicopathologic characteristics of the patients included in the study

Characteristic	Lung adenocarcinoma (N=41)	Malignant mesothelioma (N=61)
Age (years), median	65	59
Sex, n (%) ^a		
Female	17 (41.5)	18 (29.5)
Male	22 (53.7)	43 (70.5)
Tumor stage [†] , n (%)		
I	17 (41.5)	–
II	14 (34.1)	–
III	5 (12.2)	–
IV	5 (12.2)	61 (100.0)
Nodal status [†] , n (%) ^b		
N0	21 (51.2)	–
N1	15 (36.6)	–
Status, n (%)		
Death	26 (63.4)	36 (59.0)

[†], according to the International Association for the Study of Lung Cancer (1,23,24). ^a, in 2 patients with LUAD we did not find information about your gender; ^b, in 5 patients with LUAD we did not find information about the lymph nodes status.

250 whole slide scanner (3DHistech, Budapest, Hungary). The stained TMA sections were disarrayed within QuPath version 0.2.0-m429, 30 (Centre for Cancer Research & Cell Biology, University of Edinburgh, Edinburgh, Scotland; RRID:SCR_018257), an open-source image analysis platform. All cores were evaluated during the scoring process to manually exclude invalid cores (less than 10% of tumor per core or artefacts).

The TMAs were quantified on QuPath using a simple automated, semi-assisted method. First, each scanned TMA slide underwent a series of automated evaluations, starting with a staining vector analysis, followed by total tissue area detection, tumor separation from non-tumor areas, and cellular detection. Next, we established the threshold of positivity for each one of our four markers through trial and error, and positive cells were submitted to validation by an expert pathologist before being applied to the full array. QuPath then exported the result of this analysis as the number of positive cells per mm² of tissue.

Based on each marker's threshold of positivity, we

generated a script used to automatically detect positive and negative cells in each slide and export the results in image format be validated by a pathologist who was blind to patient outcome. A membrane and cytoplasmic algorithm quantified PHLDA immunohistochemical expression, whereas a nuclear algorithm quantified P53 expression. The average number of positive nuclei was expressed as positive cell density per mm² and used as the median value to which specific samples were compared. We refer to low expression as positive cell density equal or below the median expression level, and high expression as positive cell density above this median.

Clinical data and outcome

Clinical data of MM and LUAD patients was derived from an existing prospectively maintained database. Patient and tumor characteristics were retrospectively collected including age, sex, smoking history, tumor size, tumor stage (1,23,24), and follow-up information for OS rates (Table 1). OS served as the primary outcome and was defined as the interval from surgery to death or last contact. Secondary outcomes included associations with clinicopathological factors.

Statistical analysis

According to protocols of the tools employed in the *in silico* analyses, the mRNA levels of PHLDA in NSCLC, MM, and normal tissues were analyzed in each individual dataset using the Student's *t*-test and the patients' OS distribution was estimated using the Kaplan-Meier method. A log-rank test was performed to determine the difference in survival between the groups. For the clinical data, either the Chi-square test or the Fisher exact test was used to examine differences in categorical clinical variables, and the Wilcoxon rank-sum test and Kruskal-Wallis test were used to detect differences in continuous immunohistochemistry variables between groups of patients. A regression analysis of the risk of death (OR) was performed using univariate and multivariate Cox proportional hazards models. The criteria to include the variables in multivariate analysis was: (I) using the P value obtained in Cox's univariate analysis, or (II) using classically accepted criteria with impact on the risk of death and OS, namely staging and histological types, as co-dependent variables of the significant variables obtained in the univariate analysis. If no statistical differences were found for any clinicopathologic and immunohistochemistry variables, multivariate analysis will be performed to

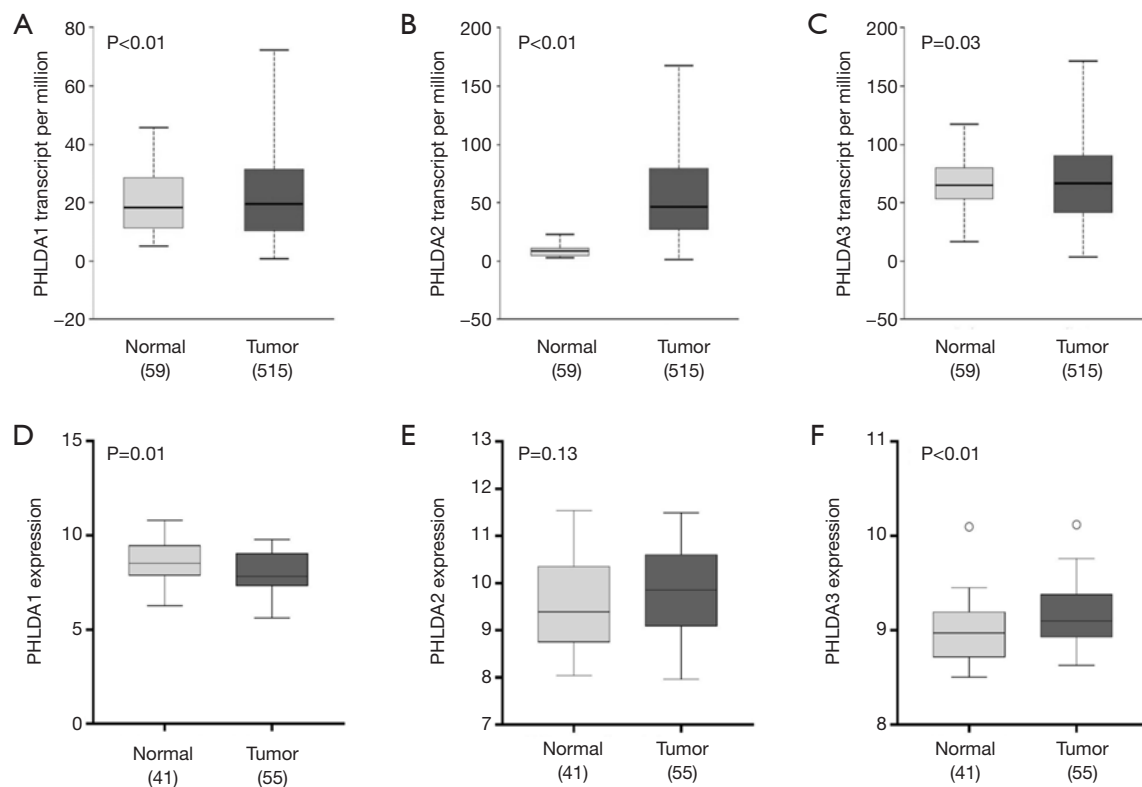


Figure 1 The relative expression of pleckstrin homology domain family A (PHLDA) genes in normal and lung adenocarcinoma (LUAD) tissues according to The Cancer Genome Atlas (TCGA) datasets and the relative expression of PHLDA genes in normal and malignant mesothelioma (MM) tissues according to GSE51024 raw data. (A) *PHLDA1*, (B) *PHLDA2*, and (C) *PHLDA3* in LUAD subtype tissue, (D) *PHLDA1*, (E) *PHLDA2*, and (F) *PHLDA3* in MM tissue. (A) All *PHLDA* genes were highly expressed in LUAD when compared to normal tissue. (D) *PHLDA1* gene expression in MM was lower than in normal tissue; (F) *PHLDA3* gene expression was significantly higher than in normal tissue; (E) no statistical difference was found for *PHLDA2* expression between MM and normal samples.

determine if the immunohistochemistry variables would depend on the control of the model (co-dependency) by the classically accepted variables with an impact on the risk of death and OS, such as staging and histological types.

The statistical software programs IBM SPSS (version 22; Armonk, NY, USA; RRID:SCR_002865) and S-Plus (version 8.04; TIBCO, Palo Alto, CA, USA) were used to perform the computations for all analyses. P value P<math>< 0.05</math> was considered significant.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the national ethics committee of n° 3.729.622 and individual consent for this retrospective analysis was waived.

Results

In silico data mining

PHLDA mRNA expression

The UALCAN analysis showed that *PHLDA1*, *PHLDA2*, and *PHLDA3* mRNA expression levels were statistically significant in LUAD samples compared to normal tissues ($P<0.01$, $P<0.01$, and $P=0.03$, respectively; *Figure 1A,B,C*). There was a significant correlation between LUAD squamoid alveolar lineage type and *PHLDA1* ($P=0.02$), *PHLDA2* ($P<0.01$), and *PHLDA3* ($P<0.01$) genes. No statistical differences were found for the other parameters (*Table 2*).

The GSE51024 raw data on MM showed that *PHLDA1* mRNA expression was lower than that of normal tissue ($P=0.01$), *PHLDA2* expression was not statistically significant ($P=0.13$), and *PHLDA3* expression was

Table 2 Clinicopathological characteristics of the patients with lung cancer from TCGA database (Chi-square test; P value<0.05)

Parameters	PHLDA1					PHLDA2					PHLDA3				
	High		Low		P value	High		Low		P value	High		Low		P value
	N	%	N	%		N	%	N	%		N	%	N	%	
Age															
≤50 years	15	5.8	22	8.5	0.23	21	8.1	16	6.2	0.34	21	8.1	16	6.2	0.44
>50 years	234	90.7	227	87.6		224	86.8	237	91.5		231	89.5	230	88.8	
NA	9	3.5	10	3.9		13	5.0	6	2.3		6	2.3	13	5.0	
Sex															
Female	140	54.3	137	52.9	0.76	149	57.8	128	49.4	0.06	145	56.2	132	51.0	0.23
Male	118	45.7	122	47.1		109	42.2	131	50.6		113	43.8	127	49.0	
Race Category															
American Indian or Alaska native	1	0.4	0	0.0	0.88	1	0.4	0	0.0	0.44	1	0.4	0	0.0	0.19
Asian	4	1.6	4	1.5		3	1.2	5	1.9		2	0.8	6	2.3	
Black or African American	24	9.3	29	11.2		30	11.6	23	8.9		31	12.0	22	8.5	
White	194	75.2	195	75.3		187	72.5	202	78.0		196	76.0	193	74.5	
NA	35	13.6	31	12.0		37	14.3	29	11.2		28	10.9	38	14.7	
Patient smoking															
Non smoker	28	10.9	48	18.5	0.07	42	16.3	34	13.1	0.40	40	15.5	36	13.9	0.36
Reformed smoker (≤15 years)	91	35.3	78	30.1		88	34.1	81	31.3		82	31.8	87	33.6	
Reformed smoker (>15 years)	72	27.9	63	24.3		62	24.0	73	28.2		72	27.9	63	24.3	
Smoker	56	21.7	63	24.3		54	20.9	65	25.1		51	19.8	68	26.3	
NA	11	4.3	7	2.7		12	4.7	6	2.3		13	5.0	5	1.9	
Stage															
I/II	205	79.5	194	74.9	0.11	195	75.6	204	78.8	0.47	206	79.8	193	74.5	0.14
III/IV	47	18.2	63	24.3		58	22.5	52	20.1		48	18.6	62	23.9	
NA	6	2.3	2	0.8		5	1.9	3	1.2		4	1.6	4	1.5	
ALK translocation status															
No	106	41.1	105	40.5	0.45	107	41.5	104	40.2	0.18	113	43.8	98	37.8	0.58
Yes	20	7.8	15	5.8		22	8.5	13	5.0		17	6.6	18	6.9	
NA	132	51.2	139	53.7		129	50.0	142	54.8		128	49.6	143	55.2	
KRAS mutation															
No	20	7.8	20	7.7	0.13	20	7.8	20	7.7	0.87	20	7.8	20	7.7	0.87
Yes	16	6.2	7	2.7		11	4.3	12	4.6		12	4.7	11	4.2	
NA	222	86.0	232	89.6		227	88.0	227	87.6		226	87.6	228	88.0	

Table 2 (continued)

Table 2 (continued)

Parameters	PHLDA1					PHLDA2					PHLDA3				
	High		Low		P value	High		Low		P value	High		Low		P value
	N	%	N	%		N	%	N	%		N	%	N	%	
Expression subtype [†]					0.02					<0.01					<0.01
Bronchioid	48	18.6	41	15.8		36	14.0	53	20.5		56	21.7	33	12.7	
Magnoid	25	9.7	38	14.7		20	7.8	43	16.6		19	7.4	44	17.0	
Squamoid	49	19.0	29	11.2		60	23.3	18	6.9		43	16.7	35	13.5	
NA	136	52.7	151	58.3		142	55.0	145	56.0		140	54.3	147	56.8	

[†], according to Song *et al.* [2018] (25). PHLDA, pleckstrin homology domain family A; NA, not available.

significantly increased compared to normal samples ($P < 0.01$) (Figure 1D,E,F). Lower *PHLDA3* mRNA expression was associated with lymph node status ($P = 0.02$), tumor size ($P = 0.01$), and *TP53* mutational status ($P < 0.01$). No correlation was found between *PHLDA1* and *PHLDA2* expression and clinicopathological characteristics (Table 3).

To further validate and cross-examine the observations made in the TCGA and GEO databases, the OncoPrint data was used to evaluate the differential mRNA levels of the *PHLDA* genes in both LUAD and MM. The mRNA expression levels of *PHLDA1*, *PHLDA2*, and *PHLDA3* were collected from 457, 436, and 360 different types of tumor studies, respectively (Figure S2). Of these, 90 studies showed a statistical difference for the *PHLDA1* gene, 56 for the *PHLDA2* gene, and 6 for the *PHLDA3* gene. From these data groups, we selected three datasets: Hou Lung (203476_at), Selamat Lung (ILMN_1687978), and Su Lung (217996_at) to investigate the expression of these genes in LUAD histotype and normal tissues (Figure S3).

The Hou Lung and Selamat datasets showed no significant difference in the expression of *PHLDA1* mRNA (fold change = 1.18 and -1.03) (Figure S3A,B). However, the Su dataset indicated a trend for *PHLDA1* mRNA under-expression in LUAD (fold change = -1.71) (Figure S3C). In all three databases, *PHLDA2* mRNA was overexpressed (fold change = 2.21, 2.39, and 3.63, respectively) in LUAD compared to normal tissue (Figure S3D,E,F). Conversely, none of the datasets showed changes in the mRNA levels of *PHLDA3* (fold change = -1.14, 1.29, and 1.03, respectively) (Figure S3G,H,I). Finally, the Gordon Mesothelioma dataset was used to assess *PHLDA* mRNA expression in MM (Figure S4), and neither *PHLDA1* nor *PHLDA3* mRNA showed a significantly different expression in tumor tissue compared

to normal tissue (fold change = -1.09 and 1.25, respectively) (Figure S4A,C). *PHLDA2*, however, trended toward under-expression in MM (fold change = -1.92) (Figure S4B).

Prognostic significance of *PHLDA* mRNA expression

A Kaplan–Meier plotter analysis was used to find the correlation between *PHLDA* mRNA levels and OS, FPS, and PPS in NSCLC patients (Figure 2). Low *PHLDA1* mRNA expression was correlated with worse OS (HR = 0.77; 95% CI: 0.63–0.94; $P < 0.01$; Figure 2A), FPS (HR = 0.66; 95% CI: 0.47–0.93; $P = 0.02$; Figure 2D), and PPS (HR = 0.43; 95% CI: 0.26–0.72; $P < 0.01$, Figure 2G); whereas high *PHLDA2* mRNA expression was significantly associated with better OS (HR = 0.84; 95% CI: 0.74–0.96; $P < 0.01$; Figure 2B), though not with FPS (HR = 1.14; 95% CI: 0.92–1.40; $P = 0.24$; Figure 2E) or PPS (HR = 0.82; CI: 0.62–1.08; $P = 0.16$; Figure 2H). Finally, patients with high *PHLDA3* mRNA expression had poor OS (HR = 1.25; 95% CI: 1.1–1.42; $P < 0.01$; Figure 2C), FPS (HR = 1.78; 95% CI: 1.41–2.24; $P < 0.01$; Figure 2F), and PPS (HR = 1.37; 95% CI: 1.02–1.83; $P = 0.03$; Figure 2I).

Next, we correlated OS with clinicopathological data (Figure S5). Low *PHLDA1* mRNA expression was significantly related to worse survival in male patients (HR = 0.56; 95% CI: 0.37–0.86; $P < 0.01$), female patients (HR = 0.52; 95% CI: 0.34–0.77; $P < 0.01$) and smokers (HR = 0.37; 95% CI: 0.2–0.67; $P < 0.01$) (Figure S5A,B,D); high *PHLDA2* mRNA expression was significantly related to poor survival in male patients (HR = 1.52; 95% CI: 1.08–2.14; $P = 0.01$) and in non-smokers (HR = 4.42; 95% CI: 1.83–10.67, $P < 0.01$) (Figure S5E,G); and high *PHLDA3* mRNA expression was correlated with short survival in male patients (HR = 1.46; 95% CI: 1.01–2.11; $P = 0.04$), female patients (HR

Table 3 Clinicopathological characteristics of the patients with malignant mesothelioma from TCGA database (Chi-square test; P value <0.05)

Parameters	PHLDA1					PHLDA2					PHLDA3				
	High		Low		P value	High		Low		P value	High		Low		P value
	N	%	N	%		N	%	N	%		N	%	N	%	
Age					0.23					0.05					0.05
≤50 years	2	4.7	5	11.4		6	14.0	1	2.3		6	14.0	1	2.3	
>50 years	41	95.3	39	88.6		37	86.0	43	97.7		37	86.0	43	97.7	
Race category					1.00					1.00					1.00
Asian	1	2.3	0	0.0		0	0.0	1	2.3		1	2.3	0	0.0	
Black or African American	0	0.0	1	2.3		0	0.0	1	2.3		0	0.0	1	2.3	
White	42	97.7	43	97.7		43	100.0	42	95.5		42	97.7	43	97.7	
Gender					0.29					0.61					0.09
Female	6	14.0	10	22.7		7	16.3	9	20.5		11	25.6	5	11.4	
Male	37	86.0	34	77.3		36	83.7	35	79.5		32	74.4	39	88.6	
Tumor size					0.72					0.60					0.01
1	7	16.3	7	15.9		6	14.0	8	18.2		3	7.0	11	25.0	
2	12	27.9	14	31.8		16	37.2	10	22.7		18	41.9	8	18.2	
3	18	41.9	14	31.8		15	34.9	17	38.6		18	41.9	14	31.8	
4	5	11.6	8	18.2		6	14.0	7	15.9		4	9.3	9	20.5	
TX	1	2.3	1	2.3		0	0.0	2	4.5		0	0.0	2	4.5	
Lymph node status					0.73					0.06					0.02
Negative	22	51.2	22	50.0		25	58.1	19	43.2		27	62.8	17	38.6	
Positive	18	41.9	21	47.7		14	32.6	25	56.8		14	32.6	25	56.8	
NX	3	7.0	1	2.3		4	9.3	0	0.0		2	4.7	2	4.5	
Metastasis status					0.55					0.47					0.63
Negative	27	62.8	30	68.2		27	62.8	30	68.2		33	76.7	24	54.5	
Positive	1	2.3	2	4.5		2	4.7	1	2.3		2	4.7	1	2.3	
MX	15	34.9	12	27.3		14	32.6	13	29.5		8	18.6	19	43.2	
Stage					0.61					0.53					0.05
I	6	14.0	4	9.1		6	14.0	4	9.1		3	7.0	7	15.9	
II	6	14.0	10	22.7		10	23.3	6	13.6		12	27.9	4	9.1	
III	24	55.8	21	47.7		20	46.5	25	56.8		23	53.5	22	50.0	
IV	7	16.3	9	20.5		7	16.3	9	20.5		5	11.6	11	25.0	
TP53 status					0.39					0.73					<0.01
Wild type	38	88.4	36	81.8		36	83.7	38	86.4		41	95.3	33	75.0	
Mutated	5	11.6	8	18.2		7	16.3	6	13.6		2	4.7	11	25.0	

PHLDA, pleckstrin homology domain family A.

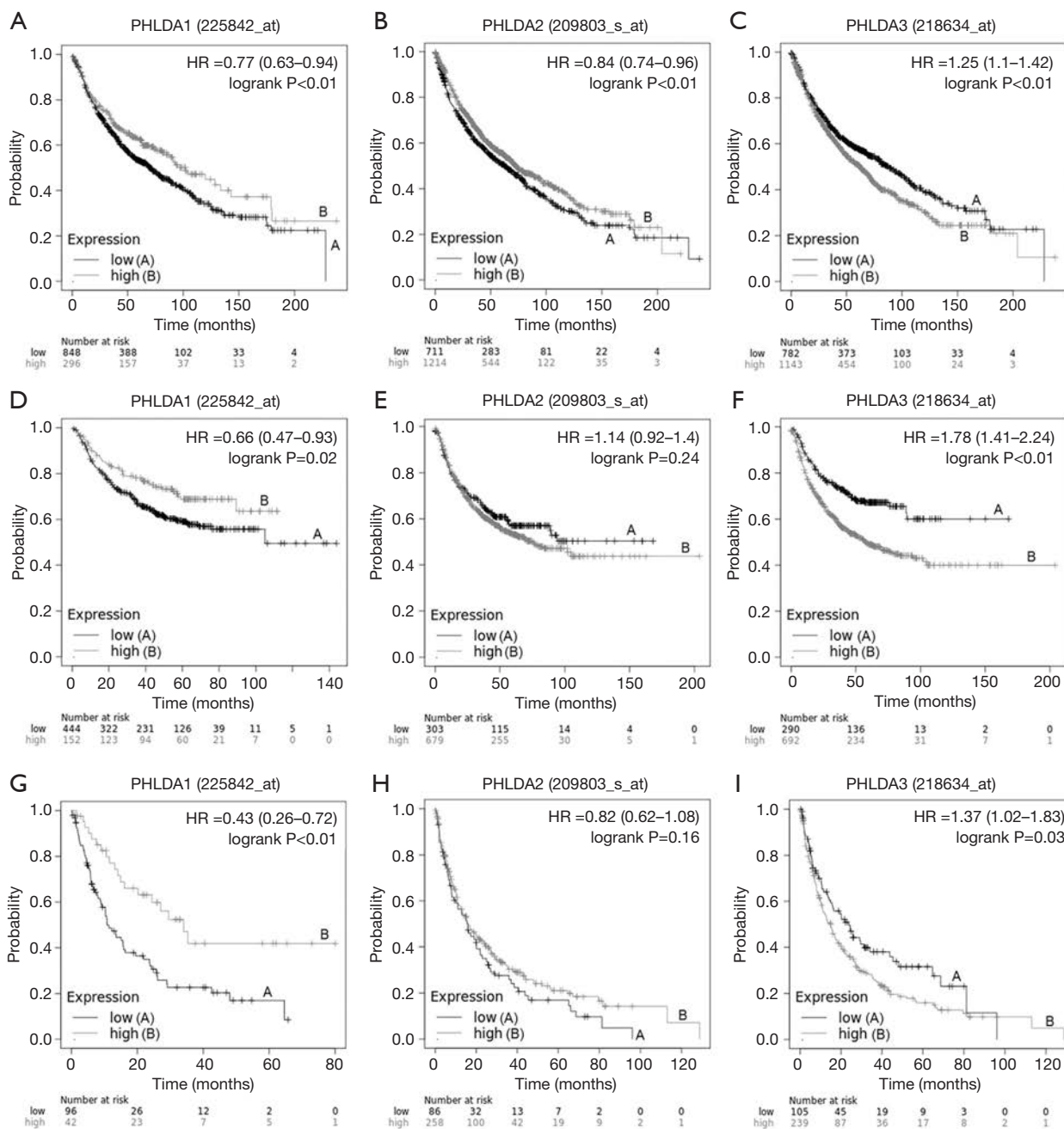


Figure 2 This Kaplan Meier survival curve shows the correlation between pleckstrin homology domain family A (PHLDA) genes expression and overall survival (OS), first progression survival (FPS) and post progression survival (PPS) in lung cancer patients. (A) Low expression of the *PHLDA1* gene was correlated with worse OS (HR = 0.77, 95% CI: 0.63–0.94; P < 0.01); (B) high expression of the *PHLDA2* gene was significantly associated with better OS (HR = 0.84, 95% CI: 0.74–0.96; P < 0.01); (C) high expression of the *PHLDA3* gene also correlated with worse OS (HR = 1.25, 95% CI: 1.1–1.42; P < 0.01); (D) low expression of the *PHLDA1* gene showed a significant correlation with worse FPS (HR = 0.66, 95% CI: 0.47–0.93; P = 0.02); (E) *PHLDA2* gene expression was not statistically significant on FPS (HR = 1.14, 95% CI: 0.92–1.40; P = 0.24); (F) high expression of the *PHLDA3* gene was significantly correlated with worse FPS (HR = 1.78, 95% CI: 1.41–2.24; P < 0.01); (G) low expression of the *PHLDA1* gene showed a significant correlation with worse PPS (HR = 0.43, 95% CI: 0.26–0.72; P < 0.01); (H) *PHLDA2* gene expression was not statistically significant on PPS (HR = 0.82, 95% CI: 0.62–1.08; P = 0.16); (I) high expression of the *PHLDA3* gene was significantly correlated with worse PPS (HR = 1.37, 95% CI: 1.02–1.83; P = 0.03).

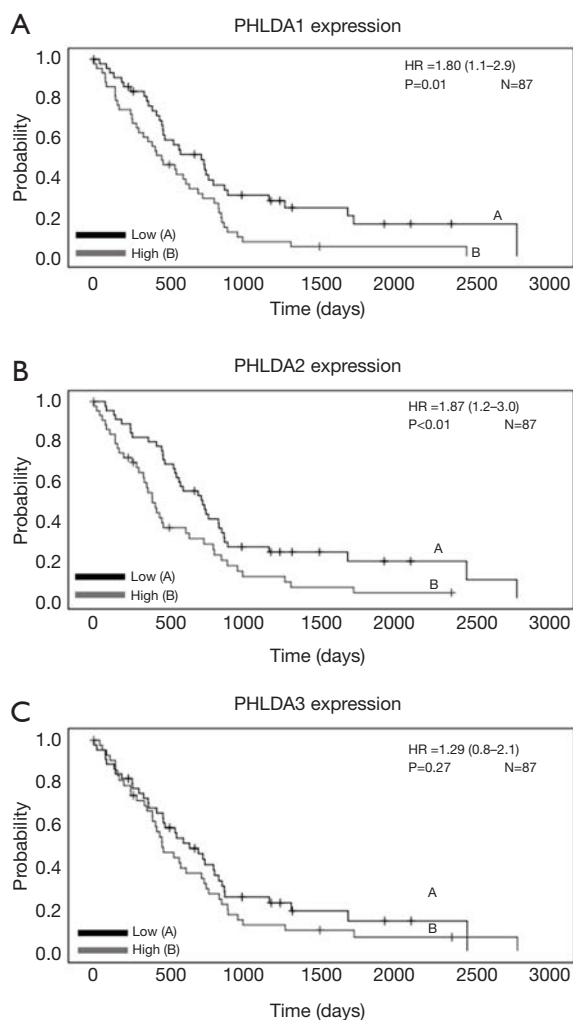


Figure 3 This Kaplan Meier survival curve shows the correlation between pleckstrin homology domain family A (PHLDA) genes expression and overall survival (OS) in malignant mesothelioma patients according to the The Cancer Genome Atlas (TCGA) Firehose Legacy datasets. (A) High expression of the *PHLDA1* (HR=1.80, 95% CI: 1.1–2.9; P=0.01) and (B) *PHLDA2* (HR=1.87, 95% CI: 1.2–3.0; P<0.01) genes was significant correlated with worse OS; (C) *PHLDA3* gene expression was not correlated with survival (HR =1.29, 95% CI: 0.8–2.1; P=0.27).

=1.86; 95% CI: 1.24–2.8; P<0.01), non-smokers (HR =2.82; 95% CI: 1.26–6.3, P<0.01), and smokers (HR =1.83; 95% CI: 1.13–2.95; P=0.01) (Figure S5I,J,K,L).

The Kaplan-Meier curves in MM (n=87 samples) were generated using Mesothelioma (TCGA, Firehose Legacy) data from the UCSC Xena tool (Table S1). While patients with MM presenting high *PHLDA1* and *PHLDA2* mRNA

expression had poor OS (HR =1.80; 95% CI: 1.1–2.9; P=0.01, and HR =1.87; 95% CI: 1.2–3.0; P<0.01, respectively; Figure 3A,B), their *PHLDA3* mRNA expression showed no correlation with survival (HR =1.29; 95% CI: 0.8–2.1; P=0.27; Figure 3C). A multivariate Cox analysis that controlled for age, sex, lymph node metastases, stage, and *TP53* confirmed that *PHLDA1* and *PHLDA2* mRNA expression were independent predictors of worse OS (HR =1.64; 95% CI: 1.0–2.6; P=0.04 and HR =1.71, 95% CI: 1.1–2.7; P=0.03; Table S1).

Interaction network

We used the Comparative Toxicogenomics Database to investigate the possibility of interaction and modulation between PHLDA genes and potential chemical drugs. Our PHLDA–drug interaction network indicated that a number of commonly used drugs could modulate the mRNA or protein expression of PHLDA. Specifically, chemotherapy agents, including cisplatin, potentially decrease *PHLDA1* levels; whereas carcinogenic substances, such as tetrachlorodibenzodioxin, were able to increase the expression of all PHLDA family members (Figure 4).

Figure 5 shows the significant PHLDA-PPI network that was designed using the STRING database. Its molecular organization can be visualized as a network of differentially connected nodes. Each node stands for a protein and its edges represent dynamic interactions. The *PHLDA1*-PPI network had 31 nodes and 100 edges (P=2.06e-08), and its most significant biological processes and pathways were associated with positive regulation of the transcription of both the Notch receptor target (GO:0007221; Purple color) and Notch signaling pathway (GO:0007219; Red color) (Figure 5A). The *PHLDA2*-PPI network had 11 nodes and 40 edges (P=1.42e-12) and was related to the activity of cyclin-dependent protein serine/threonine kinase (GO:0004693; Red color) and the p53 signaling pathway (hsa04115; Purple color) (Figure 5B). Finally, the *PHLDA3*-PPI network had 21 nodes and 82 edges (P=8.31e-12) and was mainly associated with signal transduction by p53 class mediator (Figure 5C).

Taken together, the above *in silico* results suggest that the PHLDA genes may be reliable prognostic indicators for malignant thoracic tumors, including MM and NSCLC.

PHLDA protein expression

Protein expression and histotypes

PHLDA protein expression was assessed using immunohistochemistry on tissue microarrays (TMAs) in a well-characterized cohort of 41 LUAD and 61

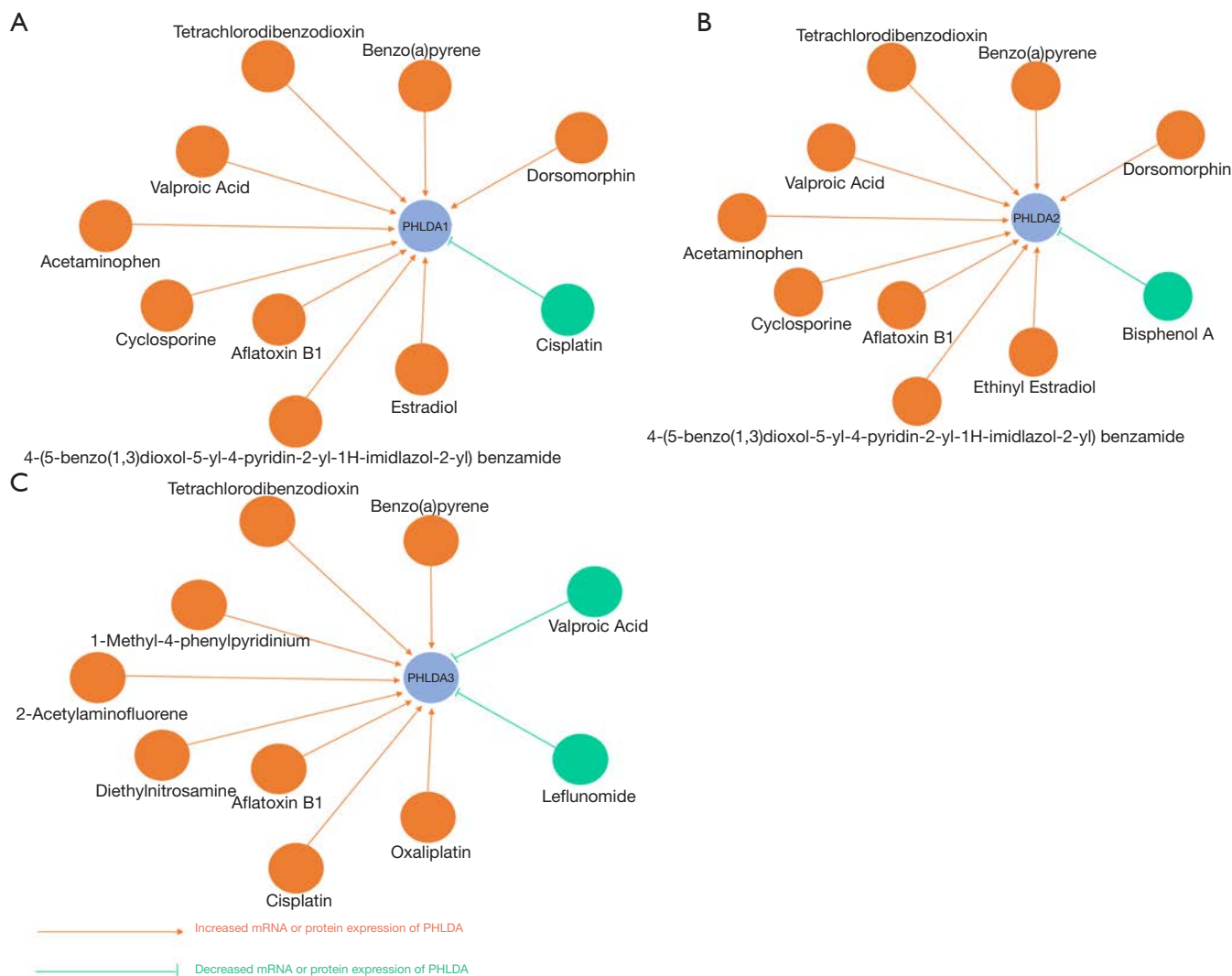


Figure 4 The PHLDA-drug interaction network obtained from the Comparative Toxicogenomics Database. (A) PHLDA1-drug interaction network; (B) PHLDA2-drug interaction network; (C) PHLDA3-drug interaction network. This network shows that several common drugs could potentially modulate the mRNA or protein expression of PHLDA. PHLDA, pleckstrin homology domain family A.

MM. *Figure 6* brings representative images of LUAD and MM stained by HE (*Figure 6A,B*), and subjected to immunohistochemistry for PHLDA1 (*Figure 6C,D*), PHLDA2 (*Figure 6E,F*), PHLDA3 (*Figure 6G,H*) and P53 (*Figure 6I,J*). The PHLDA1 staining created a cytoplasmic expression in dots pattern in mild number of MM and LUAD malignant cells (*Figure 6C,D*). In contrast, the PHLDA2 staining showed strong and diffuse expression in cytoplasm with perinuclear accentuation in numerous MM cells (*Figure 6E*), while a mild number of LUAD cells showed a predominance of cytoplasmic dots pattern (*Figure*

6F). Regarding PHLDA3 staining, a mild and diffuse cytoplasmic expression in numerous MM cells (*Figure 6G*), comparing to the cytoplasmic with perinuclear accentuation expression in mild number of LUAD (*Figure 6H*). TP53 staining was predominantly found in LUAD (*Figure 6J*), and almost absent in MM (*Figure 6I*).

Table 4 shows the protein expression frequency of PHLDAs and P53 in 41 LUAD and 61 MM samples. The expression of PHLDA2 and PHLDA3 was significantly higher in LUAD (median 88.16 cells/mm² and 84.90 cells/mm², respectively) than in MM (median 79.51 cells/mm² and

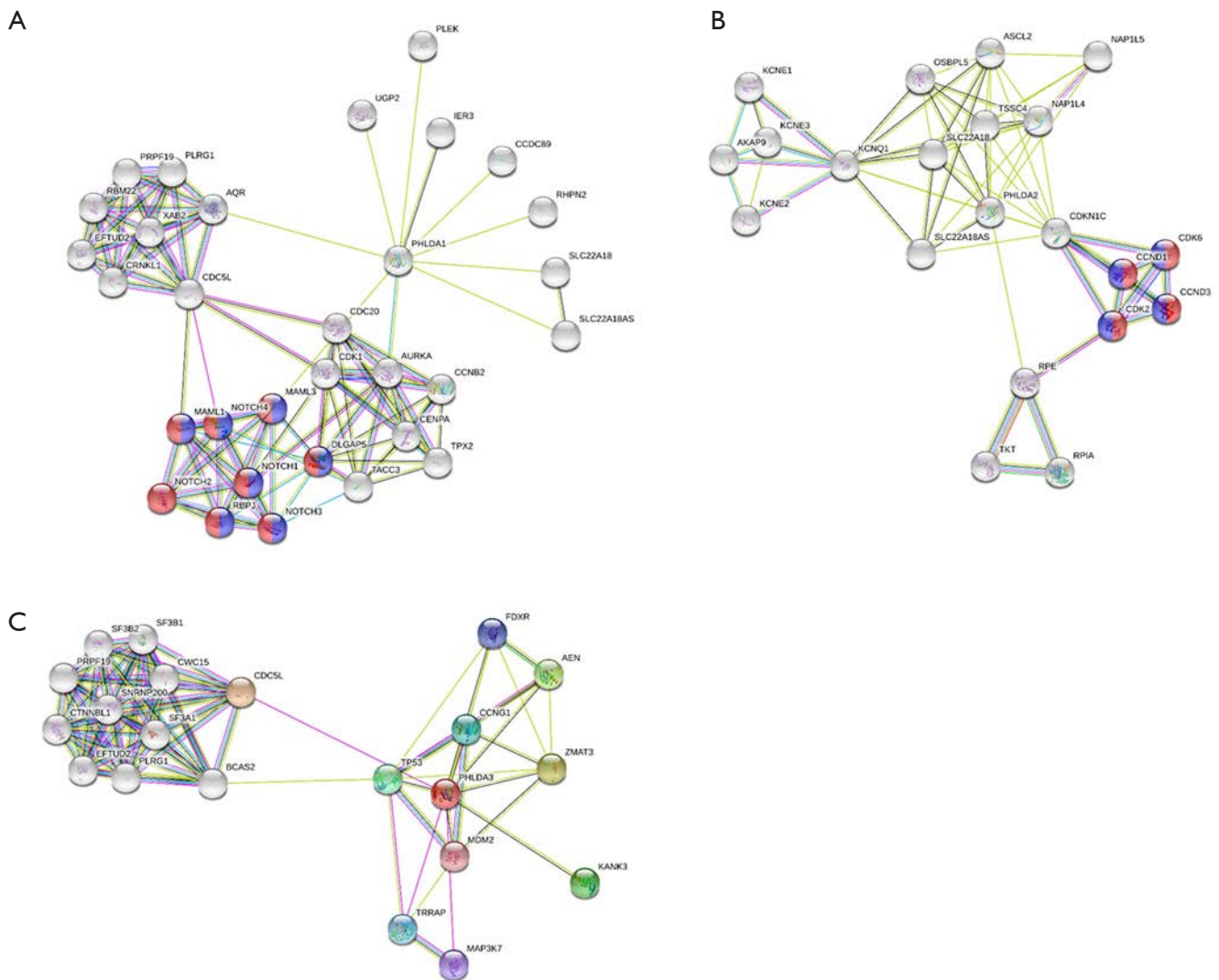


Figure 5 Cluster analysis of the PPI network. (A) PHLDA1-PPI network. (B) PHLDA2-PPI network, and (C) PHLDA3-PPI network. PHLDA, pleckstrin homology domain family A; PPI, protein-protein interaction.

67.80 cells/mm² respectively). PHLDA1 expression was not statistically significant ($P=0.37$). 23 cases of LUAD (56.1%) and 31 cases of MM (50.8%) presented values above the median expression of PHLDA1 ($P=0.49$). For PHLDA2 protein expression, 26 cases (63.4%) of LUAD and 28 cases (45.9%) of MM showed higher cell density than the median ($P=0.02$), and the PHLDA3 protein was expressed above the median in 33 cases (80.5%) of LUAD, and 27 cases (44.3%) of MM ($P<0.01$).

Protein expression and clinicopathological parameters

The demographic data and clinicopathological characteristics

of the LUAD and MM cohorts were stratified according to PHLDA and P53 expression (*Table 5*). Specimens from 29 (28.4%) patients with LUAD presented higher expression of PHLDA3 compared to 22 (21.6%) patients with MM ($P<0.01$). Among patients in clinical stage IV, PHLDA3 expression was lower in 41 patients, and high in 25 patients ($P<0.01$). A significant correlation was demonstrated among 31 (31.6%) patients with high PHLDA1 expression ($P=0.03$), high PHLDA2 expression ($P=0.01$), high PHLDA3 expression ($P<0.01$) and high P53 protein expression (median expression value of 2.9 cells/mm²). No statistical differences were found for the other parameters.

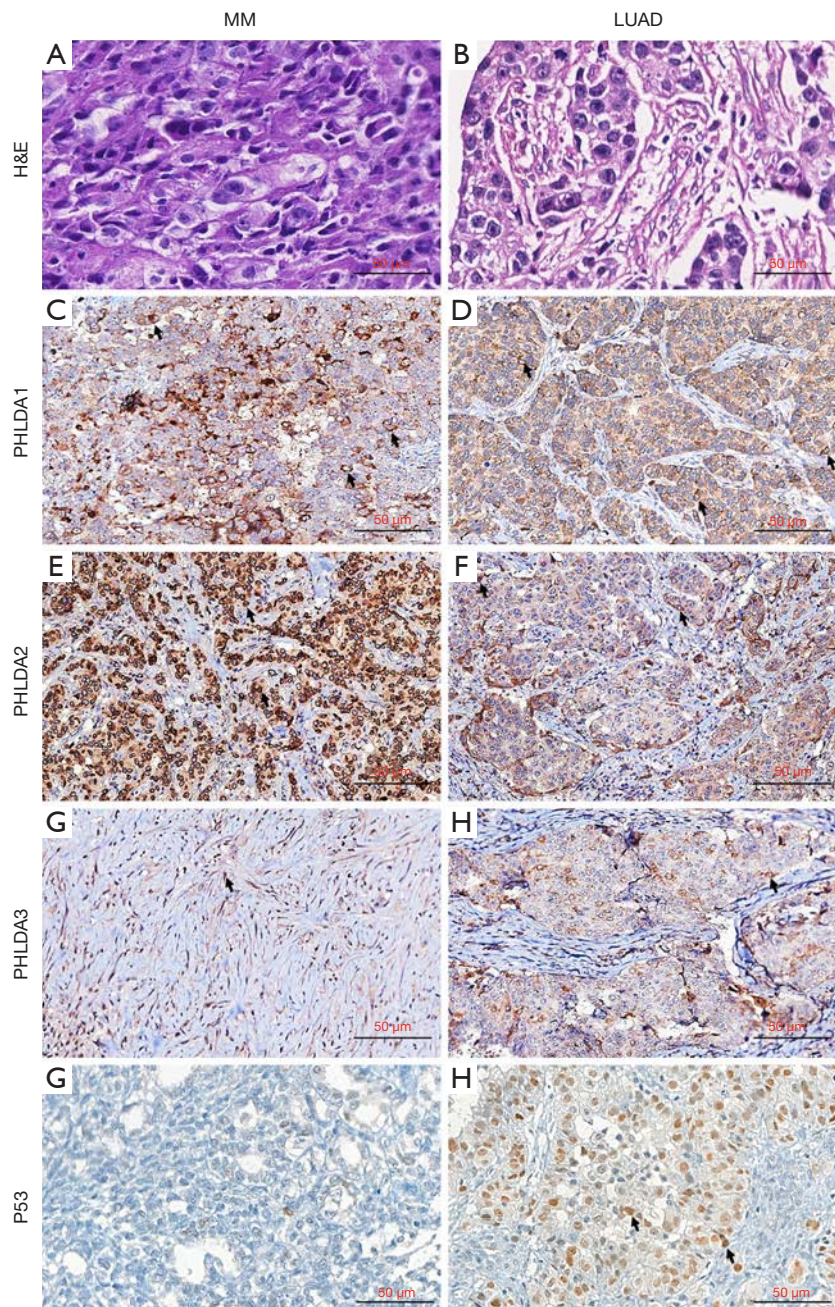


Figure 6 PHLDA protein expression using immunohistochemistry on tissue microarrays (TMAs) in MM and LUAD. H&E staining of an epithelioid MM section/showing blocks of epithelioid cells infiltrating sparse desmoplastic stroma (A); H&E staining of a representative solid subtype LUAD composed of cohesive and poor differentiated malignant cells, aggregated in blocks and immersed in exuberant desmoplastic stroma (B). Immunohistochemistry: PHLDA1 staining of mild number of epithelioid MM cells (arrows, C) and LUAD cells (arrows, D); PHLDA2 with strong and diffuse cytoplasmic staining with perinuclear accentuation in numerous MM malignant cells (arrows, E), contrasting with diffuse cytoplasmic staining in a mild number of LUAD malignant cells (arrows, F); PHLDA3 with diffuse cytoplasmic expression in MM malignant cells (arrows, G), and cytoplasmic staining with perinuclear accentuation in a mild number of LUAD malignant cells (arrows, H); strong and diffuse P53 nuclear staining in LUAD (arrows, J) and weak, almost absent, in MM (I). PHLDA, pleckstrin homology domain family A; H&E, hematoxylin & eosin; LUAD, lung adenocarcinoma; MM, malignant mesothelioma.

Table 4 Median densities of PHLDAs and TP53 according to histological type (N=102) (Mann-Whitney test; P value <0.05)

Protein (cells/mm ³)	Malignant mesothelioma		Lung adenocarcinoma		P value
	Mean	Median	Mean	Median	
PHLDA1	52.76	51.47	55.49	56.74	0.37
PHLDA2	74.73	79.51	86.17	88.16	0.01
PHLDA3	64.99	67.80	80.71	84.90	<0.01
TP53	2.64	1.11	239.67	48.40	<0.01

PHLDA, pleckstrin homology domain family A.

Table 5 PHLDA and p53 protein expression and clinicopathologic characteristics of the patients with lung cancer and malignant mesothelioma (Chi-square test; P value <0.05)

Characteristic	PHLDA1 (cell/mm ²)			PHLDA2 (cell/mm ²)			PHLDA3 (cell/mm ²)		
	Low	High	P value	Low	High	P value	Low	High	P value
Age (years)			0.42			0.11			0.11
>61.5	27 (27.6%)	22 (22.4%)		29 (29.6%)	20 (20.4%)		29 (29.6%)	20 (20.4%)	
≤61.5	22 (22.4%)	27 (27.6%)		20 (20.4%)	29 (29.6%)		20 (20.4%)	29 (29.6%)	
Sex			0.09			1.0			0.09
Female	13 (13.0%)	22 (22.0%)		17 (17.0%)	18 (18.0%)		13 (13.0%)	22 (22.0%)	
Male	37 (37.0%)	28 (28.0%)		33 (33.0%)	32 (32.0%)		37 (37.0%)	28 (28.0%)	
Tumor type			0.42			0.22			<0.01
LUAD	18 (17.6%)	23 (22.5%)		17 (16.7%)	24 (23.5%)		12 (11.8%)	29 (28.4%)	
MM	33 (32.4%)	28 (27.5%)		34 (33.3%)	27 (26.5%)		39 (38.2%)	22 (21.6%)	
Tumor stage [†]			0.61			0.30			<0.01
I	7 (7.0%)	8 (8.0%)		7 (7.0%)	8 (8.0%)		3 (3.0%)	12 (12.0%)	
II	7 (7.0%)	7 (7.0%)		5 (5.0%)	9 (9.0%)		5 (5.0%)	9 (9.0%)	
III	1 (1.0%)	4 (4.0%)		2 (2.0%)	3 (3.0%)		1 (1.0%)	4 (4.0%)	
IV	35 (35.0%)	31 (31.0%)		36 (36.0%)	30 (30.0%)		41 (41.0%)	25 (25.0%)	
Status ^a			0.36			0.20			0.58
Alive	13 (13.3%)	20 (20.4%)		12 (12.2%)	21 (21.4%)		17 (17.3%)	16 (16.3%)	
Dead	34 (34.7%)	28 (28.6%)		35 (35.7%)	27 (27.6%)		30 (30.6%)	32 (32.7%)	
Overall survival			0.28			0.83			0.83
>19.9 months	25 (29.4%)	18 (21.2%)		22 (25.9%)	21 (24.7%)		22 (25.9%)	21 (24.7%)	
≤19.9 months	19 (22.4%)	23 (27.1%)		20 (23.5%)	22 (25.9%)		23 (27.1%)	19 (22.4%)	
p53			0.03			0.01			<0.01
≤2.9 cell/mm ²	30 (30.6%)	19 (19.4%)		31 (31.6%)	18 (18.4%)		32 (3.7%)	17 (17.3%)	
>2.9 cell/mm ²	18 (18.4%)	31 (31.6%)		18 (18.4%)	31 (31.6%)		18 (18.4%)	31 (31.6%)	

[†], according to the International Association for the Study of Lung Cancer (1,23,24). ^a, 3 cases could not define the patient's status. PHLDA, pleckstrin homology domain family A; LUAD, lung adenocarcinoma; MM, malignant mesothelioma.

Table 6 The univariate and multivariate analysis employed a Cox proportional hazards model

Clinicopathological characteristics	Univariate analysis ^a			Multivariate analysis ^b	
	HR (95% CI)	HR	P value	HR (95% CI)	P value
Age (years)					
>65					
≤65 (reference)	1.01 (0.99–1.03)	0.01			
Gender					
Male					
Female (reference)	0.83 (0.45–1.51)	0.19			
Histologic types					
Sarcomatoid MM (reference)					
Epithelioid MM	0.67 (0.35–1.29)	0.23	0.30	0.22 (0.03–0.66)	0.12
LUAD	0.75 (0.44–1.29)	0.28	0.30	0.17 (0.04–0.76)	0.02
Overall stage					
I			0.50		0.01
II	0.76 (0.34–1.71)	0.28	0.14	0.13 (0.02–0.65)	0.13
III	1.68 (0.85–3.32)	0.52	0.41	0.33 (0.08–1.36)	0.05
IV (reference)	0.43 (0.06–3.15)	0.84	0.26	0.08 (0.01–1.01)	0.06
PHLDAS and p53 (≤ median vs. > median)					
PHLDA1+ cells/mm ²					
>51.230	1.27 (0.74–2.17)	0.24	0.38	0.99 (1.00–1.01)	0.58
PHLDA2+cells/mm ²					
>85.09	1.25 (0.73–2.14)	0.23	0.40	0.98 (0.96–0.99)	0.01
PHLDA3+cells/mm ²					
<70.38	0.90 (0.57–1.69)	0.01	0.96	1.02 (1.00–1.05)	0.03
P53+cells/mm ²					
>2.87	1.13 (0.65–1.96)	0.12	0.66	1.00 (1.00–1.00)	0.38

^a, univariate analysis was carried out without any adjustment in order to generate hazard ratios with confidence intervals for individual risk for each of the parameters on survival; ^b, multivariate analysis was carried out to analyze the effects of several risk parameters on survival. PHLDA, pleckstrin homology domain family A; HR, hazard ratio (β coefficient); CI, confidence interval; MM, malignant mesothelioma; LUAD, lung adenocarcinoma.

Correlation between protein expression and clinical outcome

The Cox model analysis of our cohort appears in *Table 6*. While no statistical differences were found for any clinicopathologic parameters on the risk of death in univariate analysis, a multivariate Cox model analysis demonstrated that LUAD and epithelioid MM histotypes, and overall stage I were significantly related to low risk of death ($P=0.02$, $P=0.01$, $P=0.01$, respectively). For the

quantitative tumor staining of PHLDA2 and PHLDA3 staining, multivariate analysis showed a significant association with the risk of death ($P=0.01$ and $P=0.03$). Patients that presented PHLDA2 >85.09 cells/mm² had a low risk of death (HR =0.98; 95% CI: 0.96–0.99; $P=0.01$) and median survival time of 48 months. In contrast, those with PHLDA3 <70.38 cells/mm² presented a high risk of death (HR =1.02; 95% CI: 1.00–1.05; $P=0.03$) and median survival time of 34 months. The multivariate Cox analysis

confirmed that LUAD, epithelioid MM, overall stage I, reduced PHLDA2 protein expression, and increased PHLDA3 protein expression are independent risk factors.

Discussion

Based on our previous studies, we found that the PHLDA family acts as an immediate target downstream of the EGFR/ErbB2 signaling pathway in breast cancer (6,26,27). Moreover, due to the fact that PHLDA genes are activated in various cancer types, the PHLDA family members have been increasingly perceived as promising targets for therapies against cancers (28). However, their detailed expression patterns, prognostic value, potential function, and drug interaction network in NSCLC, and especially in MM, remain largely unclear.

Several studies have reported decreased *PHLDA1* mRNA expression in melanoma, breast carcinoma, oral carcinoma, gastric adenocarcinoma, and cholangiocarcinoma (6,26). Interestingly, for *PHLDA2* expression, studies on lung cancer have shown controversial reports: while Wang *et al.* (8) found increased levels of *PHLDA2* in lung cancer, results from Hsu *et al.* (29) reached the opposite conclusion. In addition, in human neuroendocrine tumors, PHLDA3 was shown to act as a tumor suppressor gene in cases with increased *PHLDA3* mRNA expression (11,12). In agreement with these data, we noticed this variation of results during data mining, in which the data obtained in the TCGA for LUAD revealed significant mRNA expression of all members of the PHLDA family compared to normal tissue, and in Oncomine we observed underexpression of *PHLDA1* mRNA and *PHLDA2* mRNA overexpression, when compared to normal tissue. The same happened in the results for MM, where the GEO data showed lower expression of *PHLDA1* and the expression of *PHLDA3* significantly higher than normal samples, contrasting with the data from Oncomine in which both *PHLDA1* and *PHLDA3* mRNA were not significantly expressed in a tumor tissue compared to normal samples.

Regarding the prognostic significance of PHLDA genes in NSCLC and MM, we observed that low *PHLDA1* expression was responsible for shorter OS, FPS, and PPS in NSCLC; high *PHLDA2* mRNA expression was significantly associated with better OS; and high *PHLDA3* mRNA expression led to poorer OS. Using Mesothelioma (TCGA, Firehose Legacy) data, we demonstrated that patients with MM presenting high *PHLDA1* and *PHLDA2* mRNA expression had poor OS. In contrast, Muroi *et al.* (30)

demonstrated that low expression of *PHLDA3* is associated with poor outcome in patients with esophageal squamous cell carcinomas.

We also used the Comparative Toxicogenomics Database to show that a number of commonly used drugs were able to modulate PHLDA. For example, while cisplatin may decrease PHLDA1 levels, a few carcinogenic substances, such as tetrachlorodibenzodioxin and benzo(a)pyrene, seem to increase its expression (Figure 4). Moreover, the protein-protein interaction network that resulted from the STRING analysis revealed intricate interrelationships among PHLDA family members (Figure 5). For instance, PHLDA1 acts on the Notch signaling pathway, PHLDA2 impacts cyclin-dependent protein serine/threonine kinase activity, and PHLDA3 relates to signal transduction by p53 class mediator. Thus, there is great relevance in the study of the PHLDA family members for future applications as we suggested in this study.

Additionally, to our knowledge, this is the first reported case of a PHLDA protein investigation using a quantitative approach in NSCLC and MM. In our experimental study, we found that median protein expression for PHLDA1 was similar between LUAD and MM, whereas PHLDA2 and PHLDA3 protein levels were significantly higher in LUAD than in MM, though increased PHLDA3 protein levels were dependent on an advanced stage of the disease. P53 density was also higher in LUAD when compared with MM. Increasing evidence demonstrates that PHLDA protein levels, based on immunohistochemistry, were associated with diagnosis, prognosis, and targeted therapy in different cancer cells (31-35), including lung cancer (8). In addition, IHC staining markers were quantified using a cytoplasmic and nuclear algorithm to analyze PHLDA. LUAD and MM showed predominant cytoplasmic staining of PHLDA1 and PHLDA2, while PHLDA3 staining demonstrated a strong and diffuse nuclear and cytoplasmic expression. Viúdez *et al.* (32) presented a strong correlation between disease free survival and PHLDA3 nuclear expression, where a higher expression of PHLDA3 was associated with worse clinical outcomes.

The multivariate Cox model analysis of our cohort showed that higher levels of PHLDA2 and PHLDA3 in patients with stage I and stage III LUAD were also significantly associated with risk of death. While patients with PHLDA2 >85.09 cells/mm² had lower risk of death and a median survival time of 48 months, those with PHLDA3 <70.38 cells/mm² had higher risk of death and a median survival time of 34 months. These inconsistent

associations of PHLDA expression levels with prognosis in lung cancer and MM between our study and previous studies might be due to differences in racial composition, population and sample size, and methods employed to measure the expression of PHLDA.

Nevertheless, this study has some limitations. First, our research consists of an analysis based on previous data; therefore, additional experimental studies are still needed to confirm its results. Second, the clinical sample size of immunohistochemistry on tissue microarrays was not large enough and may have resulted in false negatives, a well-described problem in real-world oncology (36). Finally, as a retrospective cohort, none of our patients received a targeted therapy that evaluated the role of PHLDA in the therapeutic outcome. In summary, we suggest that more well-designed studies be carried out to support our findings.

However, the present study validated PHLDA1 underexpression in LUAD patients and its involvement on worse survival outcomes. On the other hand, both PHLDA2 and PHLDA3 were highly expressed in LUAD and MM according to our bioinformatics analysis and clinical tissue microarray. We therefore conclude that PHLDA family members might be adopted as promising predictive biomarkers and potential therapeutic targets in LUAD and MM.

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Footnote

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uniform disclosure form (available at <http://dx.doi.org/10.21037/jtd-20-2909>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the national ethics committee of nº 3.729.622 and individual consent for this retrospective analysis was waived.

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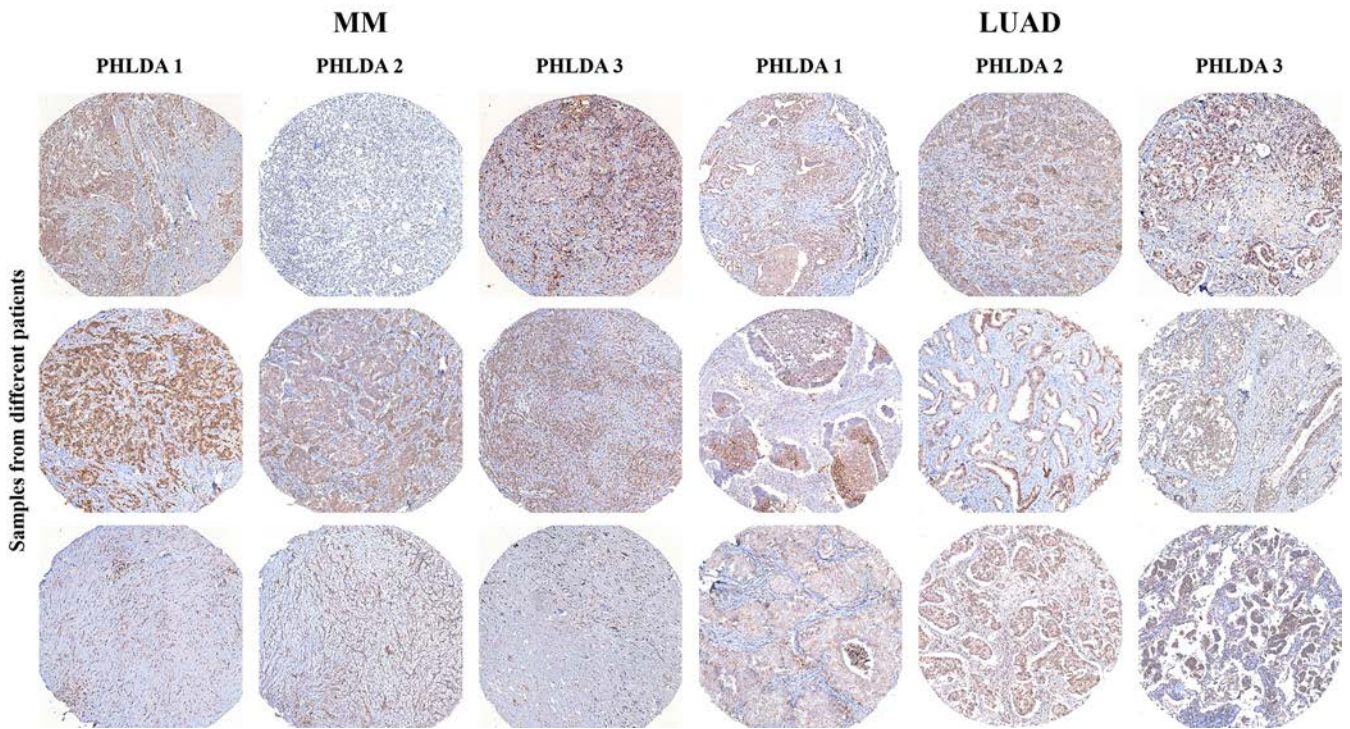


Figure S1 Expression of pleckstrin homology domain family A (PHLDA) family in malignant mesothelioma (MM) and lung adenocarcinoma (LUAD) tumors cores included in tissue microarray (TMA). Homogeneity observed between expression levels in different tumor cores. Original magnification 4 \times .

Oncomine dataset

Analysis Type by Cancer	Cancer vs. Normal	
Bladder Cancer	1	
Brain and CNS Cancer	14	
Breast Cancer		5
Cervical Cancer		2
Colorectal Cancer	22	
Esophageal Cancer		8
Gastric Cancer		
Head and Neck Cancer	1	1
Kidney Cancer	4	
Leukemia	2	1
Liver Cancer		2
Lung Cancer	1	
Lymphoma	6	4
Melanoma	5	
Myeloma	1	
Other Cancer	4	
Ovarian Cancer	1	
Pancreatic Cancer	5	
Prostate Cancer		
Sarcoma	1	2
Significant Unique Analyses	66	24
Total Unique Analyses	457	

PHLDA1

Analysis Type by Cancer	Cancer vs. Normal	
Bladder Cancer		
Brain and CNS Cancer	3	
Breast Cancer	4	
Cervical Cancer		
Colorectal Cancer	4	
Esophageal Cancer	4	
Gastric Cancer		
Head and Neck Cancer	5	
Kidney Cancer	3	
Leukemia		1
Liver Cancer		
Lung Cancer	8	
Lymphoma	10	
Melanoma		
Myeloma		
Other Cancer	4	1
Ovarian Cancer	3	
Pancreatic Cancer	5	
Prostate Cancer		
Sarcoma		1
Significant Unique Analyses	53	3
Total Unique Analyses	436	

PHLDA2

Analysis Type by Cancer	Cancer vs. Normal	
Bladder Cancer		
Brain and CNS Cancer		
Breast Cancer	1	
Cervical Cancer		
Colorectal Cancer	1	
Esophageal Cancer		2
Gastric Cancer	1	
Head and Neck Cancer		
Kidney Cancer		1
Leukemia		3
Liver Cancer		
Lung Cancer		
Lymphoma	5	
Melanoma		
Myeloma		
Other Cancer	5	
Ovarian Cancer		
Pancreatic Cancer	2	
Prostate Cancer		
Sarcoma	1	6
Significant Unique Analyses	18	8
Total Unique Analyses	360	

PHLDA3



Figure S2 Oncomine analysis of *PHLDA1*, *PHLDA2* and *PHLDA3* mRNA expression in different types of cancer, showing the differences in expression levels between tumor and normal tissue. Red cells represent overexpression of genes in tumor tissue compared to normal, whereas blue cells indicate underexpression, satisfying the thresholds $P < 0.05$; fold change ≥ 1.5 ; gene rank $\leq 10\%$. PHLDA, pleckstrin homology domain family A.

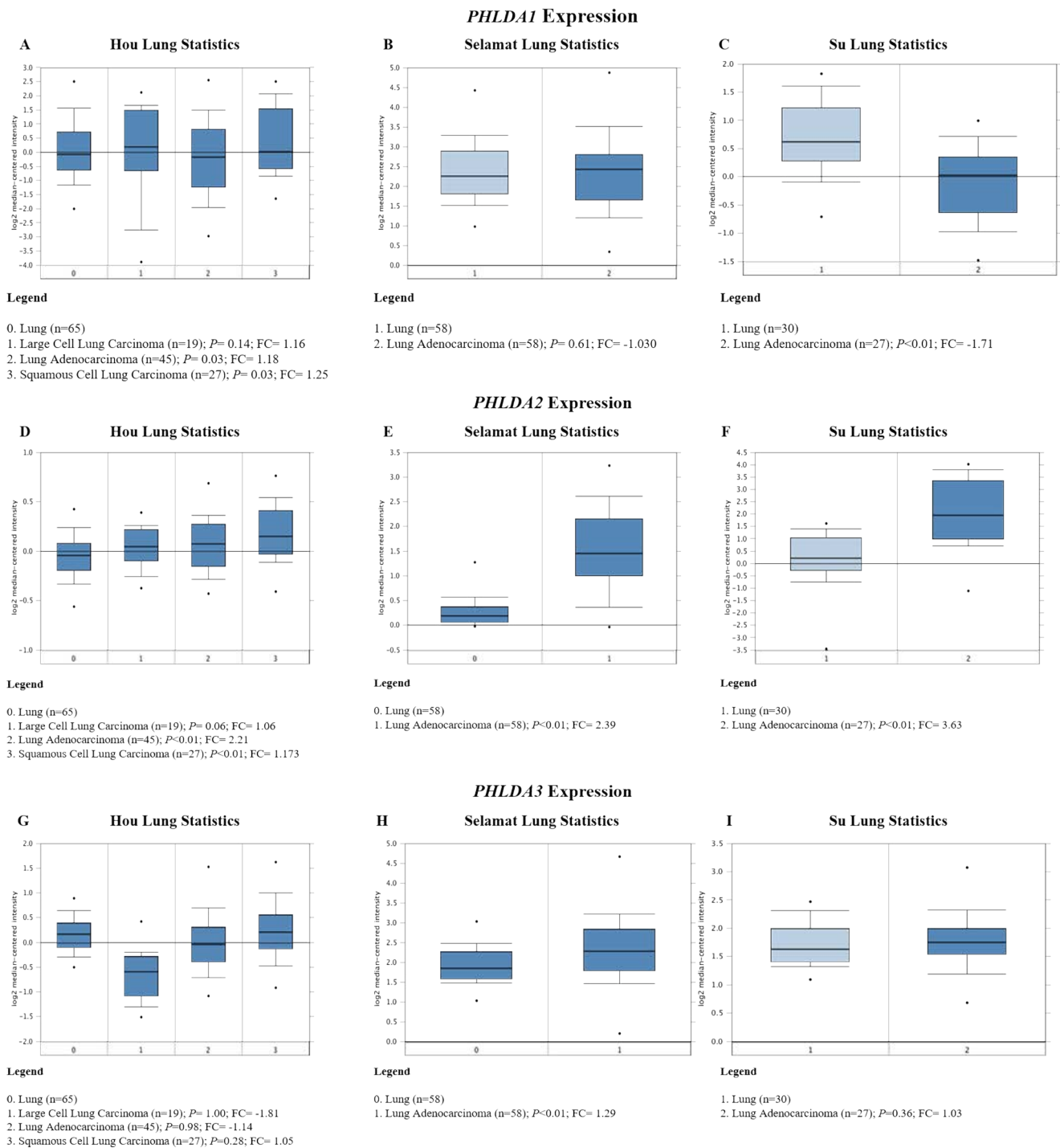


Figure S3 OncoPrint analysis of *PHLDA1*, *PHLDA2* and *PHLDA3* expression in NSCLC and their corresponding normal tissues. (A) In the Hou Lung and (B) Selamat datasets, there was no apparent change in *PHLDA1* gene expression in lung adenocarcinoma (LUAD) (fold change =1.18 and -1.03, respectively), whereas in the (C) Su dataset, there was a trend for underexpression of *PHLDA1* in LUAD tissue (fold change =-1.71). *PHLDA2* was overexpressed according to (D) Hou Lung (fold change = 2.21), (E) Selamat (fold change =2.39), and (F) Su (fold change =3.63) datasets. No changes were found in the expression levels of *PHLDA3* in LUAD according to (A) Hou Lung (fold change =-1.14), (B) Selamat (fold change =1.29) and (C) Su (fold change =1.03) datasets. *PHLDA*, pleckstrin homology domain family A.

PHLDA Expression in Gordon Mesothelioma
Grouped by Cancer Type

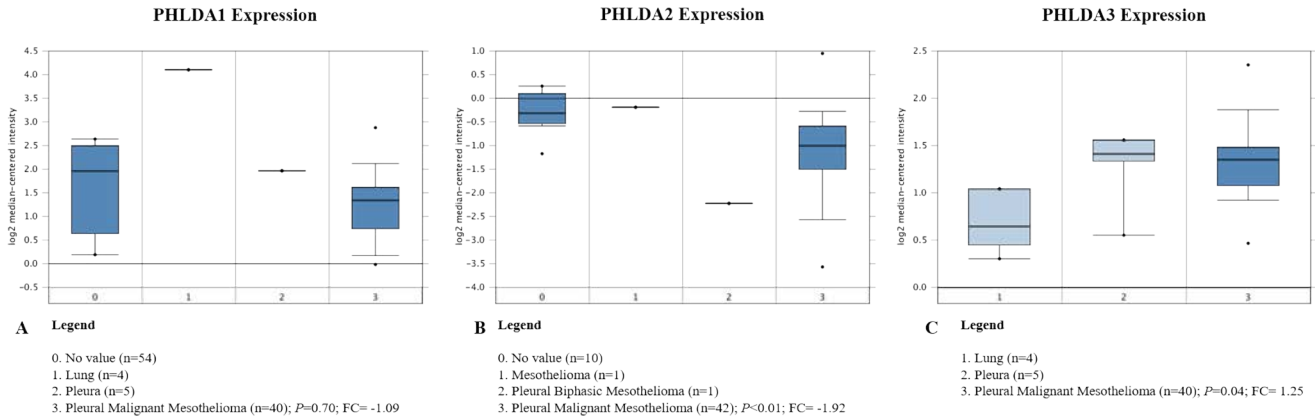


Figure S4 *PHLDA* gene expression in malignant mesothelioma (MM) according to the Gordon Mesothelioma dataset. *PHLDA1* (A) and *PHLDA3* (C) were not significantly expressed in tumors compared to normal tissue (fold change = -1.09 and 1.25, respectively), (B) *PHLDA2* was underexpressed in MM (fold change = -1.92). The X-axis of the plot represents normal vs. pleural malignant mesothelioma, the Y-axis represents mRNA expression in log₂ median-centered intensity; the line in the middle represents the median value. $P<0.05$ was considered significant. PHLDA, pleckstrin homology domain family A.

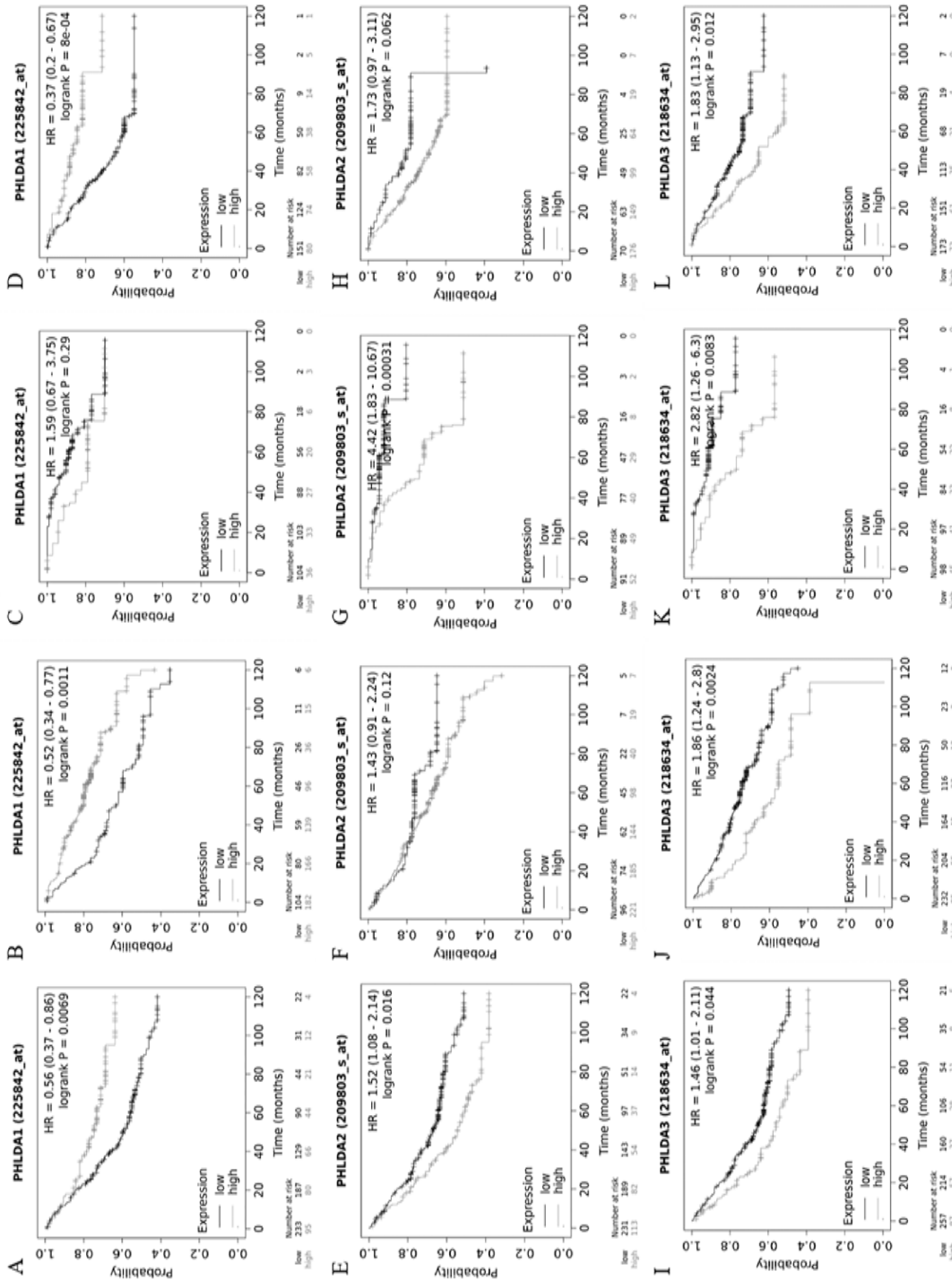


Figure S5 This Kaplan Meier survival curve shows the correlation between pleckstrin homology domain family A (PHLDA) gene expression and overall survival (OS) in lung adenocarcinoma (LUAD) patients, according to the Kaplan–Meier plotter database. Low PHLDA1 expression was significantly related to worse survival in male patients (HR = 0.56; 95% CI: 0.37–0.86, $P < 0.01$) (A), female patients (HR = 0.52; 95% CI: 0.34–0.77, $P < 0.01$) (B), and smokers (HR = 0.37; 95% CI: 0.2–0.67, $P < 0.01$) (D). (C) PHLDA1 expression was not statistically significant in never smokers. High PHLDA2 expression was significantly related to worse survival in male patients (HR = 1.52; 95% CI: 1.08–2.14, $P = 0.02$) (E), and non-smokers (HR = 4.42; 95% CI: 1.83–10.67, $P < 0.01$) (G). PHLDA2 gene expression was not statistically significant in female patients (F), and smokers (H). High PHLDA3 expression correlated with worse survival in male patients (HR = 1.46; 95% CI: 1.01–2.11, $P = 0.04$) (I), female patients (HR = 1.86; 95% CI: 1.24–2.8, $P < 0.01$) (J), non-smokers (HR = 2.82; 95% CI: 1.26–6.3, $P < 0.01$) (K), and smokers (HR = 1.83; 95% CI: 1.13–2.95, $P = 0.01$) (L).

Table S1 Variables associated with overall survival (OS) of 87 patients diagnosed with malignant mesothelioma

Variables	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	0.76 (0.3–1.8)	0.53		
Sex (Male vs. Female)	0.90 (0.5–1.6)	0.73		
TP53 (Mutated vs. Wild type)	1.04 (0.5–2.0)	0.92		
Lymph node (Positive vs. Negative)	0.91 (0.6–1.5)	0.71		
Stage		0.77		
Stage (II vs. I)	0.64 (0.3–1.5)	0.31		
Stage (III vs. I)	0.78 (0.4–1.6)	0.51		
Stage (IV vs. I)	0.71 (0.3–1.7)	0.44		
PHLDA3 (high vs. low)	1.29 (0.8–2.0)	0.27		
PHLDA1 (high vs. low)	1.80 (1.1–2.9)	0.01	1.64 (1.0–2.6)	0.04
PHLDA2 (high vs. low)	1.87 (1.2–3.0)	<0.01	1.71 (1.1–2.7)	0.03

Univariate and multivariate analysis employed a Cox proportional hazards model using the Mesothelioma TCGA Firehose Legacy database by UCSC Xena tool. HR, hazard ratio; 95% CI, 95% confidence intervals.