Original Paper

Limb-Bud and Heart Attenuates Growth and Invasion of Human Lung Adenocarcinoma Cells and Predicts Survival Outcome

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Key Words

Lung adenocarcinoma • LBH • Prognostic biomarker • Proliferation • Metastasis

Abstract

Background/Aims: The transcription cofactor limb-bud and heart (LBH) is involved in embryonic development. However, its role in human lung cancer, especially lung adenocarcinoma (LUAD), remains unclear. **Methods:** A public database and tissue microarray (TMA) were used to compare differences in LBH expression and its relationship with clinical characteristics. Tissue from an additional 70 LUAD patients with follow-up records was used to explore the correlation of LBH expression with prognosis. Cellular and molecular studies validated the role of LBH in LUAD growth and invasion. **Results:** LBH was significantly down-regulated in lung cancer tissue samples and was correlated with the prognosis and clinical characteristics of lung cancer patients based on a public database and TMA. Survival analysis revealed that LBH-negative expression was associated with poor overall survival of LUAD patients ($P = 0.021$). Cox regression analysis showed that LBH expression status was a favorable independent prognostic factor (hazard ratio = 0.120, 95% confidence interval = 0.016–0.894, $P = 0.039$). LBH knockdown accelerated LUAD cell proliferation, migration, and invasion. Furthermore, bioinformatics analysis indicated that LBH was significantly related to the cell adhesion pathway. Western blot analysis confirmed that LBH could regulate the expression of integrin family members (integrin- $\alpha 1$, integrin- $\alpha 2$, integrin- $\alpha 4$, integrin- αv , and integrin- $\beta 4$). **Conclusion:** Our data suggest that LBH plays an important role in lung cancer. Importantly, LBH is an independent prognostic factor in LUAD and can attenuate cell growth and invasion. LBH may be a potential prognostic biomarker in LUAD patients.

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Introduction

Lung cancer is one of the leading causes of cancer-related death [1]. There are two main histological types of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer. NSCLC accounts for 85% of lung cancers, of which lung adenocarcinoma (LUAD) is the most common subtype [2]. Despite improvements in lung cancer diagnosis and treatment, the 5-year overall survival of patients remains low [3], which is largely due to patients being diagnosed with advanced lung cancer, and is adversely affected by local invasion, regional lymph node involvement, and distant metastasis [4]. Therefore, further exploration of prognostic markers for lung cancer and potential drug targets to provide new treatments is expected to greatly enhance patient survival.

There is growing evidence that embryonic development and tumorigenesis have similar molecular mechanisms [5, 6]. In particular, developmental signaling pathways and abnormal reactivation of transcription factors in tumor cells are closely related to advanced and aggressive cancers [7, 8]. Limb-bud and heart (LBH) is an important transcription cofactor involved in embryonic development [9]. In the early stage of vertebrate embryogenesis, LBH regulates the formation of the limbs and heart. Moreover, Rieger et al. reported that the WNT signaling pathway can directly target LBH to participate in the occurrence and development of breast cancer [10]. Liu et al. showed that nasopharyngeal epithelial cells may initiate malignant transformation due to the Epstein-Barr virus-mediated downregulation of LBH [11]. However, the role of LBH in lung cancer has not been elucidated.

This study is the first to report the aberrant expression and clinical significance of LBH in lung cancer, especially LUAD. Furthermore, knockdown of LBH accelerated LUAD cell proliferation, migration, and invasion. In addition, LBH could regulate the expression of several members of the integrin family. Taken together, these findings indicate that LBH plays an important role in lung cancer, especially LUAD, and could be a potential prognostic biomarker and therapeutic target of LUAD patients.

Materials and Methods

Bioinformatics analysis

The Cancer Genome Atlas (TCGA) datasets TCGA_LUNG_exp_HiSeqV2-2015-02-24 and TCGA_LUAD_exp_HiSeqV2-2015-02-24 were downloaded from the website of the UCSC cancer browser (<https://genome-cancer.ucsc.edu>) [12]. GSE19188 was downloaded from the Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo>) [13]. Kaplan-Meier Plotter (<https://www.kmplot.com/analysis>) was used to draw Kaplan-Meier survival curves of lung cancer patients with different expression levels of LBH [14].

A list of 319 genes with the highest co-expression correlation (Spearman score > 0.5) with LBH in the TCGA LUAD dataset was submitted to DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov>) for Gene Ontology (GO) pathway enrichment analysis [15, 16]. In order to investigate the pathways that LBH may regulate, Gene Set Enrichment Analysis (GSEA) was carried out using GSEA Java software, by comparing the expression of genes in the LBH-high/low groups divided by the median expression level of LBH [17]. Kyoto Encyclopedia of Genes and Genomes signaling pathways were used as a reference in this step to evaluate the pathways that LBH may modulate.

Patients and tissue samples

This study was approved by the Human Ethics Review Committee of the First Hospital of China Medical University. Seventy primary LUAD tissue samples and another 41 cases with matched nontumorous and tumorous tissue samples were enrolled in the study. Demographic and clinical characteristics, such as age, sex, age at initial diagnosis, and stage at diagnosis (tumor, node, metastasis [TNM] classification), were obtained from medical records and pathology reports.

Immunohistochemistry (IHC) analysis

IHC staining was performed as described previously [18]. An anti-human LBH rabbit antibody was used at a dilution of 1:100 (ab122223; Abcam, Cambridge, UK); phosphate-buffered saline was used as

a negative control. Each section was evaluated and scored independently by two pathologists. A semi-quantitative scoring system was used in this assay. The percentage of positively stained cells was scored as 0, <10%; 1, 10–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. Intensity was scored as “–” (negative), “+” (weak), “++” (moderate), and “+++” (strong). The percentage score was multiplied by the staining intensity score to generate the IHC score. Positive expression of LBH was defined as detectable immunoreactions in the nucleus with an IHC score ≥ 4 .

Cell lines and culture

The human LUAD cell lines HCC827, A549, and PC9 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy (Shanghai, China). All cell lines were cultured in RPMI 1640 with 10% fetal bovine serum and placed in an incubator at 37 °C with 5% CO₂.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed according to our previously reported method [19]. The relative expression of mRNA was calculated via the comparative cycle threshold method, and the expression of 18S was used as reference. The following primer sequences were used: LBH, forward 5'-CCTGAGGAGTTCCTGGTCC-3' and reverse 5'-CAGATGCTGGCTGGTATGAC-3'; and 18S, forward 5'-GGTGAAGGTCGGAGTCAACGG-3' and reverse 5'-GAGGTCAATGAAGGGGTCATTG-3'.

Western blot analysis

Protein was extracted from the cells and quantified as described previously [18, 19]. Equal amounts of protein were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in TTBS (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5% Tween-20) for 40 min at room temperature and incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: anti-LBH, anti-ZEB1, anti-ZEB2, anti-SNAIL, and anti-SLUG (Abcam); anti-integrin- α 1, anti-integrin- α 2, anti-integrin- α 4, anti-integrin- α v, anti-integrin- β 1, anti-integrin- β 3, anti-integrin- β 4, anti-integrin- β 5, anti-E-cadherin, anti-vimentin, and anti-actin (Cell Signaling Technology, Boston, MA, USA). The membranes were washed in TTBS and incubated with secondary antibodies for 40 min. After extensive washing, the membranes were visualized using the enhanced chemiluminescence reagent. Final images were analyzed using ImageJ software.

Cell transfection

Transfection was performed according to the small interfering RNA (siRNA) sequences transfection protocol for Lipofectamine 2000 (Invitrogen, Shanghai, China). The sequence of the LBH-specific siRNA was 5'-CGGCCAAGATGACTGAGGTGATGAT-3'. Nonsense RNAi (5'-GUACCUUGACAGUACCGAUdTdT-3') was used as a negative control (NC). At 48 h after transfection, the transfection efficiency of LBH was identified by qRT-PCR and western blotting analyses.

Cell viability assays

Cells (2000 cells/well) were seeded in a 96-well plate overnight. Then, they were transfected with either LBH-siRNA or NC-siRNA. Before transfection, absorbance was determined to confirm an identical number of cells within the groups. At 1–4 days after transfection, cell viability was evaluated using a 1-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenyltetrazoliumbromide (MTT) assay. Specifically, 20 μ L MTT solution (5 mg/mL) was added to each well and the cells were incubated for another 4 h at 37 °C. The supernatant was removed and 200 μ L DMSO was added to each well to dissolve the precipitate. Absorbance was measured spectrophotometrically at 570 nm.

Colony formation assay

Transfected A549 cells were harvested and seeded at a density of 500 cells per well in 12-well plates and incubated at 37 °C and 5% CO₂ in a humidified incubator for 2 weeks. The number of colonies was counted in each well.

Cell migration and invasion assay

For the migration assay, 200 μ L serum-free medium with cells (2.0×10^4 cells/chamber) was seeded in an upper Transwell chamber (Corning Inc. Corning, NY, USA). The cell invasion experiment was almost identical to the cell migration assay, except the Transwell upper chamber was coated with Matrigel (BD Biosciences). For each assay, RPMI-1640 containing 2% fetal bovine serum was added to the lower chamber. Non-migrated or non-invaded cells on the upper membrane were removed after incubation for 24 h at 37 °C. The migrated or invaded cells were stained using the Wright-Giemsa method and photographed under a light microscope at $\times 200$.

Wound healing assay

The cells (2.0×10^5 cells/well) were seeded into 6-well plates and incubated overnight. They were then transfected with LBH-siRNA or NC-siRNA for 48 h. When the cells grew to nearly 100% confluence, the monolayer of cells was scratched with a 200- μ L pipette tip. Five scratched fields were selected randomly. Images were captured using a bright-field microscope.

Flow cytometry

Transfected cells were collected and incubated with 5 μ L annexin V and 10 μ L propidium iodide for 15 min in the dark. The samples were evaluated by flow cytometry and analyzed using CellQuest software (Becton-Dickinson, USA).

Statistical analysis

Statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean $\pm$ standard deviation. Student's t-test was used to compare means between groups. Cox regression methods and log-rank test were used to compare survival outcome between the two LBH expression groups. The chi-square test was applied to analyze the relationship between LBH expression and clinical-pathological parameters. $P < 0.05$ was considered statistically significant.

Results

LBH is significantly down-regulated in NSCLC and correlates with clinical characteristics in public datasets

To investigate the clinical values of LBH in lung cancer, 1016 lung cancer samples and 109 normal lung samples were extracted from TCGA. LBH expression was significantly down-regulated in lung cancer tissue compared with normal lung tissue (Fig. 1A). In addition, an NSCLC dataset (GSE19188) confirmed this expression pattern (Fig. 1B). To explore the clinical significance of LBH, we investigated the association between LBH expression and prognosis in NSCLC with the Kaplan-Meier Plotter software program (<https://www.kmplot.com/analysis>). Using the TCGA dataset, 739 NSCLC patients with complete clinical information were selected for further analysis. The patients were divided into two groups: LBH-low expression group ($n = 369$) and LBH-high expression group ($n = 370$) by median expression value. Compared with the LBH-high expression group, the LBH-low

Table 1. Correlation between LBH expression and clinical characteristics in TCGA lung cancer dataset ($n = 739$). *Significant correlation

Clinical pathological parameter	Number	LBH expression		P-value
		High ($n = 370$)	Low ($n = 369$)	
Age (years)				0.002*
<60	171	68	103	
≥ 60	568	302	266	
Sex				0.035*
Male	465	219	246	
Female	274	151	123	
Depth of invasion				<0.001*
T1	188	119	69	
T2	431	195	236	
T3+T4	120	56	64	
Lymph node status				0.274
N0	465	240	225	
N1-3	274	130	144	
Metastasis status				0.053
M0	711	361	350	
M1	28	9	19	
TNM stage				0.043*
I	365	199	166	
II	213	101	112	
III+IV	161	70	91

Fig. 1. LBH is significantly down-regulated in NSCLC and correlates with prognosis in public datasets. (A and B) Differences in LBH expression between normal and lung cancer tissue samples from TCGA ($P < 0.001$) and GSE19188 ($P < 0.001$) datasets. (C) Kaplan-Meier survival analysis from Kaplan-Meier Plotter indicates that lower LBH expression is associated with a worse overall survival in lung cancer patients (HR = 0.62, $P < 0.001$).

Fig. 2. Decreased LBH protein expression in NSCLC patients. (A) IHC scores of LBH were significantly reduced in cancer tissue samples compared to adjacent normal controls ($P < 0.001$). (B) Subgroup analysis based on histological type revealed that LBH was decreased in LUAD ($P < 0.001$) and LUSC ($P < 0.001$). The IHC scores of LBH were higher in LUAD patients than in LUSC patients ($P = 0.0437$). (C) Representative images of LBH staining in human LUAD tissue samples and their adjacent normal tissue samples: “-” (negative staining intensity), “+” (weak staining intensity), “++” (moderate staining intensity), and “+++” (strong staining intensity). Scale bar: 300 μ m. (D) Representative images of LBH staining in human LUSC tissue samples and their adjacent normal tissue samples. Scale bar: 300 μ m.

expression group displayed poor overall survival (hazard ratio [HR] = 0.62, $P < 0.001$). A chi-square test indicated that low LBH expression was significantly correlated with younger age ($P = 0.002$), male gender ($P = 0.035$), increased depth of invasion ($P < 0.001$), and higher TNM stage ($P = 0.043$) (Table 1).

Decreased LBH protein expression in NSCLC patients

To confirm the clinical significance of LBH in NSCLC

patients, we used a TMA containing 41 NSCLC tissue samples and paired non-tumor tissue samples. LBH was significantly down-regulated in NSCLC tissue samples compared with non-tumor tissue samples (Fig. 2A). Subgroup analysis based on histological type revealed that LBH was decreased in LUAD and lung squamous carcinoma (LUSC) (Fig. 2B). In addition, LBH expression levels based on the IHC score were higher in LUAD patients than in LUSC patients (3.519 ± 0.5213 , $n = 27$ vs. 1.857 ± 0.455 , $n = 14$, respectively, $P = 0.044$). Representative images of LBH expression in LUAD patients are shown in Fig. 2C. IHC images of LUSC patients are shown in Fig. 2D. The rate of LBH-positive lung cancer tissues was 11/41 (26.83%), while the rate of LBH-positive normal tissues was 38/41 (92.68%). Chi-square test indicated that a higher level of LBH in lung cancer was significantly associated with lower TNM stage ($p = 0.014$).

LBH protein level can indicate prognosis in LUAD patients

We performed IHC analysis of 70 LUAD tissue samples with long-term follow-up records. Indicative examples of each level of staining are shown in Fig. 3A. On the basis of the IHC results, the staining was quantified and classified into two groups: positive and negative. A chi-square test showed that low LBH expression was significantly correlated with increased depth of invasion ($P = 0.048$), lymph node metastasis ($P = 0.031$), and higher TNM stage ($P = 0.007$) (Table 2). LBH-positive patients presented with a longer survival time than LBH-negative patients ($HR = 0.095$, $P = 0.021$) (Fig. 3B).

A Cox proportional hazards model was used to analyze the impact of clinical and pathological parameters on the prognosis of patients. Univariate analysis showed that T stage ($HR = 1.748$, 95% confidence interval [CI] = 1.173–2.713, $P = 0.007$), N stage ($HR = 7.620$, 95% CI = 3.110–18.668, 5.325, 95% CI = 2.568–11.040, $P < 0.001$), and LBH expression level ($HR = 0.095$, 95% CI = 0.013–0.698, $P = 0.021$) were risk factors for prognosis. Furthermore, multivariate analysis showed that N stage ($HR = 4.592$, 95%

Table 2. Correlation between LBH expression and clinical characteristics in LUAD patients ($n = 70$). *Significant correlation

Clinical pathological parameter	Number	LBH expression		P-value
		Positive ($n = 14$)	Negative ($n = 56$)	
Age (years)				0.467
<60	41	7	34	
≥60	29	7	22	
Sex				0.719
Male	33	6	27	
Female	37	8	29	
Differentiation				0.618
Well/moderate	54	12	42	
Poor	16	2	14	
Depth of invasion				0.048*
T1	45	13	48	
T2	15	2	13	
T3+T4	10	0	10	
Lymph node status				0.031*
N0	37	11	26	
N1–3	33	3	30	
TNM stage				0.007*
I	34	12	22	
II	12	0	12	
III+IV	24	2	22	

Table 3. Cox regression analysis of overall survival in LUAD patients. Features with a P-value < 0.05 in univariate analysis were taken into multivariate analysis. *Significant correlation

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (years)						
(≤60 vs. >60)	0.851	0.593–1.222	0.383			
Gender						
(male vs. female)	0.868	0.434–1.737	0.690			
Tumor differentiation	1.509	0.698–3.263	0.295			
pT stage	1.784	1.173–2.713	0.007*	0.841	0.518–1.364	0.483
pN stage	7.620	3.110–18.668	0.001*	4.592	1.480–14.249	0.008*
pTNM stage	5.325	2.568–11.040	0.001*	2.083	0.808–5.375	0.129
LBH expression	0.095	0.013–0.698	0.021*	0.120	0.016–0.894	0.039*

Fig. 3. High level of LBH protein can indicate a good prognosis in LUAD patients. (A) Representative images of LBH staining in human LUAD tissue samples: “-” (negative staining intensity), “+” (weak staining intensity), “++” (moderate staining intensity), and “+++” (strong staining intensity). Scale bar:300µm (B) Univariate Cox regression survival analysis and log-rank test indicates that positive LBH expression is associated with good overall survival (HR = 0.095, P = 0.021).

Fig. 4. Knockdown of LBH increases LUAD cell growth, migration, and invasion. (A) LBH mRNA and protein expression in three LUAD cell lines. (B) A549 cells were transfected with either LBH-siRNA or NC-siRNA. LBH expression levels were detected by qRT-PCR and western blotting analyses. For further investigation, A549 cells were chosen as an appropriate cellular model to test the effects of LBH knockdown. (C) Cell viability assays showed LBH knockdown increases A549 cell proliferation. (D) Number of colonies formed by A549 cells transfected with NC-siRNA was less than those transfected with LBH-siRNA. (E) Wound healing assay showed that knockdown of LBH could increase the migration ability of A549 cells. (F and G) The number of cells on Transwell membranes was monitored at 24 h after LBH-siRNA transfection for 48 h (upper). Migration and invasion abilities were further quantified (lower). (H) No significant difference in apoptosis was observed between the LBH-siRNA and NC-siRNA transfected groups in A549 cells. *P<0.05, **P<0.01, ***P<0.001, ns: not significant.

CI = 1.480–14.249, $P = 0.008$) and LBH expression (HR = 0.120, 95% CI = 0.016–0.894, $P = 0.039$) were independent risk factors for prognosis (Table 3).

LBH knockdown increases LUAD cell proliferation, migration, and invasion

We first compared the expression level of LBH in LUAD cells (HCC827, PC9, and A549 cell lines) (Fig. 4A). The A549 cell line was chosen for further investigation because of its high level of LBH expression. Knockdown of LBH expression was performed using siRNA. Transfection efficiency was measured with qRT-PCR and western blot analyses (Fig. 4B). LBH knockdown markedly increased the proliferation of A549 cells (Fig. 4C). Moreover, the LBH-siRNA transfected group formed more colonies than the NC-siRNA transfected group (Fig. 4D). A wound healing assay indicated that LBH knockdown could increase the migration ability of A549 cells (Fig. 4E). Transwell assays showed that the migration and invasion abilities of LBH-siRNA transfected groups were significantly enhanced (Fig. 4F). The effects of LBH on apoptosis were assessed by flow cytometry analysis; however, there was no significant difference in apoptosis between the LBH-siRNA and NC-siRNA transfected groups (Fig. 4G).

LBH influences key proteins to regulate cell adhesion

To explore how LBH exerts its function, we used GO analysis. Most of the correlated genes were enriched in the cell adhesion pathway (Fig. 5A). To investigate further the pathways that LBH may regulate, gene set enrichment analysis was used. Pathways related to “cell adhesion,” “ECM receptor interaction,” and “focal adhesion” were identified as significantly altered along with aberrant LBH expression (Fig. 5B).

Integrins are transmembrane receptors that facilitate cell–extracellular matrix (ECM) adhesion, and the integrin family plays an important role in cell adhesion [20]. The expression levels of integrin- α 1, integrin- α 2, integrin- α 4, integrin- α v, and integrin- β 4 were increased

Fig. 5. LBH influences key proteins to regulate cell adhesion. (A) Genes co-expressed with LBH are enriched in the “cell adhesion” pathway using GO enrichment analysis. (B) GSEA results showed that “cell adhesion,” “focal adhesion,” and “ECM receptor interaction” are significantly associated with LBH. (C) Western blot analysis showed that the expression levels of integrin- α 1, integrin- α 2, integrin- α 4, integrin- α v, and integrin- β 4 were increased to different degrees after LBH-siRNA transfection, while there was no significant alteration in integrin- β 1, integrin- β 3, and integrin- β 5 expression. (D) Western blot analysis also showed that there was no significant difference in the expression levels of E-cadherin, vimentin, ZEB1, ZEB2, SNAIL, and SLUG between the LBH-siRNA and NC-siRNA transfected groups.

to different degrees after the cells were transfected with LBH-siRNA (Fig. 5C). In contrast, there was no significant difference in integrin- β 1, integrin- β 3, and integrin- β 5 expression. It has been reported that integrins can promote the transformation of tumor cells from an epithelial phenotype to a mesenchymal phenotype, that is, epithelial to mesenchymal transition (EMT), through the regulation of EMT-related proteins [21]. We first examined the expression levels of EMT-related proteins (E-cadherin and vimentin) and some transcription factors (ZEB1, ZEB2, SNAIL, and SLUG). There was no significant difference in the expression of these proteins between the LBH-siRNA and NC-siRNA transfected groups (Fig. 5D).

Discussion

Lung cancer is a worldwide problem that causes a large number of deaths each year. Exploring new prognostic markers and new drug targets to enhance patient survival is essential to improve overall survival. LBH was identified originally as a transcriptional coactivator involved in the molecular signaling pathways of heart and limb development [9, 22]. Other studies have confirmed that LBH plays an important role in embryonic development [23, 24]. Over the years, researchers have found it to be associated with several autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis [25-30]. Embryonic development, immune system disorders, and tumorigenesis are known to have similar molecular mechanisms [31]. Thus, it is suggested that LBH may play an important role in tumorigenesis and development.

Reports show that LBH is a Wnt pathway target gene that is overexpressed in basal type breast cancer and may contribute to Wnt pathway-induced tumorigenesis [10]. A study on nasopharyngeal carcinoma showed that nasopharyngeal epithelial cells may undergo malignant transformation due to the Epstein-Barr virus-mediated downregulation of LBH, and the expression level of LBH is correlated with prognosis [11]. However, its role in lung cancer has not been elucidated. In the present study, based on public datasets and our IHC results, we are the first to propose that LBH expression levels are widely down-regulated in NSCLC tissue samples and correlate with patient prognosis and clinicopathological parameters. We then explored the prognostic value of LBH in LUAD. Kaplan-Meier analysis showed that patients with higher LBH expression had longer survival, while those with lower LBH expression had a poorer prognosis. Univariate and multivariate Cox regression analyses indicated that LBH expression was an independent prognostic factor in LUAD patients. On the basis of our experimental results, we are the first to report that the expression of LBH in LUAD and its relationship with clinical characteristics and prognosis in LUAD can be of therapeutic value.

Sustained proliferation and high metastatic potential are the major hallmarks of cancer cells [32]. One study confirmed that LBH overexpression inhibits the proliferation and tumor growth of nasopharyngeal carcinoma cells *in vivo* and *in vitro* [11]. In the present study, we examined the role of LBH expression in LUAD cells by knocking down its expression. Cell viability, colony formation, wound healing assay, and Transwell assays showed that knocking down LBH expression in LUAD A549 cells promoted cell proliferation, migration, and invasion. These results support the hypothesis that LBH is a potential tumor suppressor gene in LUAD. Subsequently, we explored the molecular mechanism by which LBH inhibits the proliferation and metastasis of LUAD cells. It has been reported that LBH can inhibit the proliferation of nasopharyngeal carcinoma cells by down-regulating the transcriptional activity of NF- κ B, and can also regulate cell cycle-related proteins to induce G1/S arrest in these cells [11]. We predict that LBH may inhibit the growth and metastasis of LUAD by regulating some key pathways in tumor progression. A similar result was obtained in this study with GO analysis and GSEA: the cell adhesion pathway could potentially be associated with LBH expression. It is well known that cell adhesion molecules are closely related to tumor progression and metastasis [33]. Among them, the integrin family plays an important role in cell adhesion and even tumor progression as the major cell surface receptors for adhesion to the ECM

[20]. Similarly, EMT is closely related to cell adhesion, and it has been reported that integrin family proteins could regulate EMT-related proteins [21]. We found that the expression levels of integrin- α 1, integrin- α 2, integrin- α 4, integrin- α v, and integrin- β 4 were up-regulated to some extent by siRNA-mediated LBH knockdown; however, the expression of EMT-related proteins did not change significantly. These results suggest the potential mechanism of LBH in repressing proliferation and invasion in LUAD by down-regulating integrin proteins.

There are some limitations to this study. The specific mechanism by which LBH regulates integrin family protein expression as a transcriptional cofactor has not been elucidated fully, and needs further study *in vitro* and *in vivo*. In addition, the samples involved in this study were acquired retrospectively, the clinical application of LBH as a potential biomarker will need further comprehensive and in-depth analyses.

On the basis of the current research results, we confirmed that LBH plays a significant role in lung cancer. Importantly, LBH, as an independent prognostic factor for LUAD, attenuates the growth and invasion of LUAD cells, and may serve as a potential prognostic biomarker and therapeutic target.

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Disclosure Statement

The authors declare to have no conflict of interests.

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