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ORIGINAL ARTICLE

LRH1 as a promising prognostic biomarker and predictor of metastasis in patients with non-small cell lung cancer

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Abstract

Background: LRH1, which promotes the malignant transformation of carcinoma, has recently been documented in several types of malignancies. However, LRH1 has not been assessed as a potential clinical biomarker in any cancer.

Methods: LRH1 expression was tested in fresh-frozen tissue samples with quantitative real-time PCR and Western blot analysis. Surgically resected tumor tissues were collected from 156 non-small cell lung cancer (NSCLC) patients: 75 with adenocarcinoma and 81 with squamous cell carcinoma. Subsequently, the immunohistochemical expression of LRH1 was examined, and its clinical significance was evaluated.

Results: LRH1 overexpression was observed in NSCLC carcinoma tissues compared to adjacent normal lung tissues. LRH1 expression was correlated with poorer differentiation ($P = 0.023$), pathological tumor classification ($P < 0.001$), advanced pathological tumor node metastasis stage ($P = 0.017$), adenocarcinoma subtype ($P = 0.031$), and positive lymph node metastasis ($P < 0.001$). Multivariate analysis demonstrated that LRH1 expression status was an independent prognostic factor for overall (hazard ratio 1.372, 95% confidence interval 1.225–1.617; $P = 0.003$) and disease-free survival (hazard ratio 1.497, 95% confidence interval 1.059–2.115; $P = 0.011$) in patients who suffered from resectable NSCLC.

Conclusion: The results of our study indicate that LRH1 predicts NSCLC progression, metastasis, and a dismal prognosis, emphasizing its promising role as a novel target in NSCLC therapies.

Introduction

Lung carcinoma, a heterogeneous disease characteristic of high frequency and metastatic potential and poor clinical patient outcomes, results in more than 1.6 million deaths annually worldwide.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer diagnoses and the five-year survival rate is still under 15% after diagnosis.[2] The risk of relapse is high even after early-stage patients are treated with radical surgery.[3] Thus, substantial attempts have been made to identify emerging molecular biomarkers to predict prognosis and develop potential therapeutic targets.[4,5]

LRH1, a member of the nuclear receptor subfamily, is also known as nuclear receptor subfamily 5 group A member 2 (NR5A2).[6] LRH1 contributes to the regulation of plentiful biological processes, such as cholesterol transport, differentiation, steroidogenesis, bile acid homeostasis, and tumor progression.[7,8] A large number of studies have indicated that LRH1 is responsible for the pathogenesis of pancreatic, breast, colon, and gastric tumors.[8–11] LRH1 accelerates intestinal tumor development by modulating cell cycle and inflammation.[12] Crucially, emerging research has revealed that elevated LRH1 expression in pancreatic cancer affects epithelial-mesenchymal transition (EMT) progression, pancreatic cancer stem cell (CSC) potential and tumor metastasis.[11,13,14] In addition, LRH1 facilitates the invasion ability of breast cancer cells by either transforming the actin cytoskeleton or decreasing cyclin-dependent kinase inhibitor (CDKN1A) transcription independent of ERα and p53 status.[15,16] By targeting LRH1, the

downregulation of miR-30d is prominently associated with distant metastasis, poorer differentiation, advanced clinical tumor node metastasis (TNM) stage and lymph node metastasis, and promotes cell proliferation or invasion in colorectal carcinoma.[17] However, to date, no study has reported the significance and functions of LRH1 in regard to lung cancer. Therefore, the identification of LRH1 as a potential marker for detection would be critical in antineoplastic protocols and prediction of cancer metastasis.

Consequently, this research was designed to assess messenger RNA (mRNA) and protein expression of LRH1 in NSCLC tissue samples and revealed an association between LRH1 and clinicopathological factors of NSCLC. The results verified the significant value of LRH1 as a prognostic marker, a lymph node metastatic factor, and a promising therapeutic target for NSCLC.

Methods

Tissues and patients

In the present study, paraffin-embedded tissues (156 NSCLC, 30 normal tissues) were acquired for immunohistochemistry (IHC) from 156 NSCLC patients who underwent surgery from January 2006 to December 2009 at Harbin Medical University Cancer Hospital. In addition, fresh tissues, including 16 pairs of NSCLC tumors and corresponding adjacent normal tissues, were removed from 16 NSCLC patients between April 2016 and August 2017. The tissues were stored at −80°C immediately following resection until protein and RNA extraction was conducted. Patients were not administered radiotherapy, chemotherapy, or immunotherapy prior to surgery.

Table 1 shows the clinicopathological characteristics of all included NSCLC patients. The median age was 58 years (range: 28–82), while the average age was 57.55 ± 8.914 years (mean ± standard deviation). World Health Organization classifications were used to determine the histological subtype of lung cancer samples, and analyses of primary focal tumors were based on the American Joint Committee on Cancer (seventh edition) staging system. The Harbin Medical University Institute Research Medical Ethics Committee approved the study.

Overall survival (OS) was defined as the interval from excision to the date of death, while disease-free survival (DFS) was defined as the interval from the date of resection to recurrence or death from any cause. The five-year recurrence rate was calculated as the percentage of patients who suffered from recurrence five years after resection. All NSCLC patients were regularly followed up until death or the cutoff date of 30 August 2017.

Immunohistochemistry (IHC)

Resected paraffin-embedded tissue blocks (~4 μm) were stained with hematoxylin and eosin for tumor confirmation. To enhance immunoreactivity, antigen was retrieved using a pressure cooker with pH 6.0 citrate buffer (10 mM) for three minutes.

Subsequently, LRH1 staining was performed with anti-LRH1 (1:100 dilutions; ab189876; Abcam, Cambridge, UK) overnight at 4°C. Rabbit secondary antibody was added to the sample and left for 50 minutes at room temperature. The slides were then stained using Mayer's hematoxylin. The positive control contained positive LRH1 expression in human lung carcinoma, while the negative control was stained with rabbit serum. Positive cells were assigned rank by percentage: 0 = 0%, 1 = 1–10%, 2 = 11–50%, 3 = 51–70%, 4 = ≥ 71%. Staining was ranked by intensity: 0 = no staining, 1 = weak staining, 2 moderate staining, and 3 = intense staining. Finally, LRH1 expression was evaluated by the sum of the percentage of positive cells and the intensity of staining. After strict statistical analysis, the level of LRH1 expression was divided into two stages: low < 4 and high ≥ 4. Two pathologists blindly analyzed any discrepancies to avoid bias.

Quantitative real-time PCR

The total RNA extracted from human lung tissue samples was collected using the Total RNA kit I (Omega Bio-tek Inc., Hilden, Germany), followed by complementary DNA synthesis using a ReverTra Ace Qpcr RT Kit (Toyobo Co. Ltd., Osaka, Japan). Amplification was subsequently carried out using the 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The same reaction was performed in triplicate. Finally, quantitative real-time (qRT) PCR analyses were evaluated using the $2^{-\bigtriangledown\bigtriangledown CT}$ method.[18]

Western blot analysis

The frozen tissue homogenates or cell lysates were both centrifuged at 14 000 rpm (at 4°C for 15 minutes), and the protein concentrations were tested using the Pierce Bicinchoninic Acid Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). Equivalent quantities of proteins were then transferred onto polyvinylidene difluoride membranes, blocked, and incubated with primary monoclonal antibodies at 4°C overnight. The antibodies included mouse monoclonal antibody against β-actin (TA-09, 15V70208, Zhong Shan Golden Bridge Biological Technology Inc., Beijing, China) and rabbit antibody against LRH1 (ab153944, Abcam). The polyvinylidene difluoride membranes were incubated using appropriate horseradish

Table 1 Association between LRH1 expression level and clinicopathological characteristics of NSCLC

Variables	All patients (*n* = 156)	LRH1 expression		*P*
		High (%) (*n* = 101)	Low (%) (*n* = 55)	
Gender				
Male	104	70 (69.3)	34 (61.8)	0.343
Female	52	31 (30.7)	21 (38.2)	—
Age (year)				
< 60	96	63 (62.4)	33 (60.0)	0.616
≥ 60	60	38 (37.6)	22 (40.0)	—
Smoking status				
Yes	125	79 (72.2)	46 (62.4)	0.657
No	31	22 (19.8)	9 (62.4)	—
Differentiation				
Well	6	4 (14.4)	2 (22.8)	0.023*
Moderate	66	44 (44.2)	17 (49.6)	—
Poor	89	53 (41.4)	36 (27.6)	—
Histological cell type				
Adenocarcinoma	75	55 (53.5)	20 (38.2)	0.031*
Squamous cell carcinoma	81	46 (46.5)	35 (61.8)	—
pTNM stage				
I	11	6 (5.9)	5 (9.1)	0.017*
II	94	54 (53.5)	40 (72.7)	—
III	51	41 (40.6)	10 (18.2)	—
pT classification				
T1	48	34 (33.7)	14 (25.5)	<0.001*
T2	90	57 (56.4)	37 (67.3)	—
T3/4	14	10 (9.9)	4 (7.2)	—
Lymph node metastasis				
N0	75	38 (37.6)	37 (67.3)	<0.001*
N1	33	23 (22.8)	10 (18.2)	—
N2	48	40 (39.6)	8 (14.5)	—

*$P < 0.05$ was considered statistically significant. NSCLC, non-small cell lung cancer; pT, pathological tumor; pTNM, pathological tumor node metastasis.

peroxidase-conjugated secondary antibodies (1:10 000 dilutions at room temperature for 1 hour). The immunoblotting bands were subsequently obtained with Super Enhanced Chemiluminescence Western Blotting Detecting Reagent (HaiGene, China) and exposed to CL-Xposure film (Thermo Fisher Scientific).

Statistical analysis

All statistical analyses were processed using SPSS version 22.0 (IBM Corp., Armonk, NY USA). The χ^2 test was used to clarify distinction among categorical variables, while the Student's *t*-test was used to analyze distinction among different groups of continuous variables. The Kaplan–Meier method was used to plot the survival curves with the log-rank test. Multivariate analysis was conducted including the covariates that were significant in univariate analysis. Independent prognostic features for OS or DFS were evaluated by Cox proportional hazards method. A two-sided *P* value of < 0.05 was considered to represent statistical significance.

Results

LRH1 expression in non-small cell lung cancer (NSCLC) tissue samples

Quantitative RT–PCR, Western blot, or IHC assays were conducted to confirm protein and mRNA levels in NSCLC tissues. The LRH1 mRNA expression level was investigated using qRT–PCR in normal or tumor tissues ($P < 0.001$, Student's *t*-test) (Fig 1a). The results verified that the mean level of LRH1 mRNA expression in carcinoma tissue samples (1.6502 ± 0.1352-fold), normalized against β-actin gene expression, was distinctly more prominent than the expression in normal tissues (0.7301 ± 0.0921-fold) ($P < 0.001$, Student's *t*-test), signifying ~2.26-fold higher LRH1 mRNA expression in tumor tissues relative to normal tissues.

LRH1 protein expression in tumor tissue samples or corresponding adjacent normal tissues was also investigated using Western blot assay. LRH1 was determined as ~61-kDa. The relative bands manifesting LRH1 protein expression in NSCLC

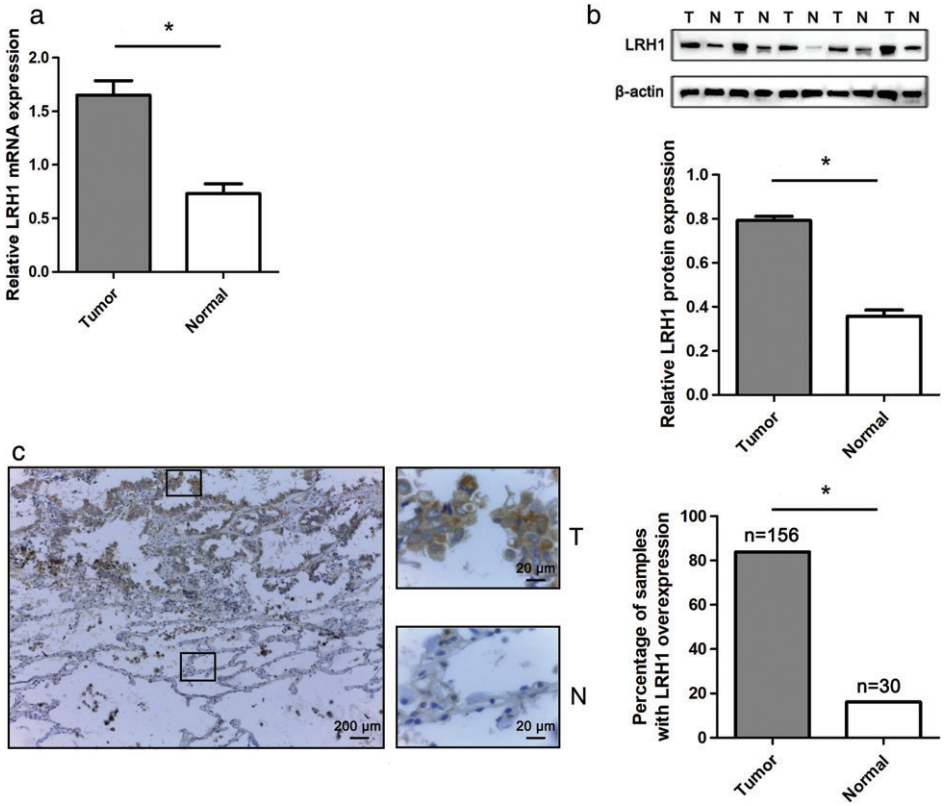

Figure 1 LRH1 expression in non-small cell lung cancer (NSCLC) tissue specimens. Representative (**a**) quantitative real-time PCR and (**b**) Western blot analysis of LRH1 expression in 16 paired NSCLC clinical tissue specimens. Histograms of LRH1 messenger RNA (mRNA) expression and pooled data in adjacent normal lung (N, n = 16) and NSCLC (T, n = 16) tissues. The relative expression of LRH1 mRNA and LRH1 were highly expressed in NSCLC tumor tissues compared to normal lung tissues. Data are expressed as mean ± standard deviation (SD). *P < 0.05. (**c**) Representative immunohistochemistry staining images of samples from an NSCLC patient (T) and adjacent normal lung (N) tissues showed LRH1 expression in NSCLC tissues. The expression of LRH1 protein significantly increased in tumor tissues compared to adjacent normal lung tissues. A histogram of pooled data (right) of NSCLC (T, n = 156) and normal lung (N, n = 30) tissues. The percentage of NSCLC tissues with high LRH1 expression significantly increased compared to that of normal lung tissues. *P < 0.05.

tissue samples (n = 16) were higher compared to those in normal samples (n = 16) (P < 0.001, Student's t-test) (Fig 1b).

According to IHC analysis, adenocarcinoma (ADC) and squamous cell carcinoma (SCC) cells from NSCLC tissues were deeply stained, demonstrating elevated LRH1 expression in the nuclear and cytoplasmic compartments of the tumor cells. Nevertheless, in normal lung tissue cells, such as alveolar and bronchial epithelial cells and stromal cells, no significant LRH1 expression was detected (Fig 1c).

Correlation between LRH1 expression and variables in NSCLC tissues

The results of IHC analysis revealed higher LRH1 expression in ADC and SCC tissue samples than in in normal lung tissue (Fig 2).

As metastasis is the primary cause of NSCLC-related death, the clinical significance of LRH1 was explored in patients with NSCLC. LRH1 expression was identified as a clinicopathological factor in NSCLC samples. As shown in Table 1, high LRH1 expression was notably correlated with poorer differentiation (P = 0.023), pathological tumor (pT) classification (P < 0.001), advanced pathological (p)TNM stage (P = 0.017), ADC subtype (P = 0.031), and positive lymph node metastasis (P < 0.001). LRH1 was highly expressed in 64.7% patients with NSCLC (101/156), including 73.3% (55/75) patients with ADC and 56.8% (46/81) patients with SCC. Notably, higher LRH1 expression was observed in ADC patients than in SCC patients (P < 0.001) (Table 1). However, LRH1 expression was rarely related to gender, age, or smoking status.

LRH1 protein expression predicts survival in NSCLC

We evaluated whether LRH1 expression was associated with independent prognostic significance for OS or DFS.

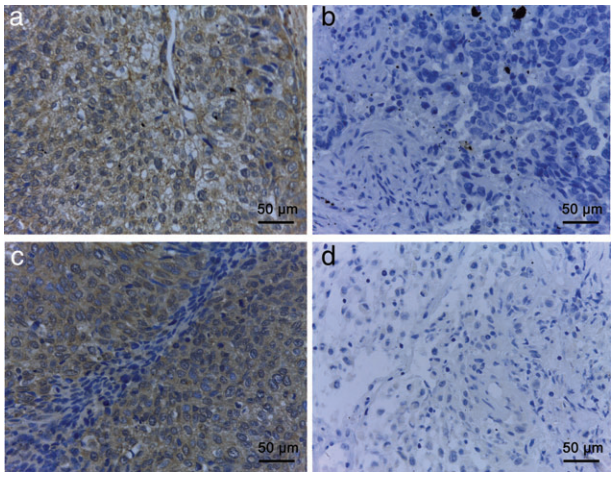

Figure 2 Representative photomicrographs of LRH1 immunohistochemical staining. (**a**) High and (**b**) low expression in adenocarcinoma (ADC). (**c**) High and (**d**) low expression in squamous cell carcinoma (SCC) (×100 original magnification).

Univariate and multivariate Cox regression analyses were estimated (Table 2). Univariate analysis showed that crucial indicators for poorer OS were poorer differentiation, ADC subtype, advanced pTNM stage, lymphoglandular metastasis, and high LRH1 expression. Multivariate analysis confirmed that advanced pTNM stage (hazard ratio [HR] 1.056, 95% confidence interval [CI] 1.146–2.933; $P = 0.016$), lymph node metastasis (HR 3.172, 95% CI 2.005–5.019; $P = 0.009$), and high LRH1 expression (HR 1.372, 95% CI 1.225–1.617; $P = 0.003$) were independent predictors for OS. In addition, the results of univariate analysis verified that crucial predictors for poorer DFS included ADC subtype, advanced pTNM stage, the presence of lymph node metastasis, and high LRH1 expression. The results of multivariate analysis indicated that poorer differentiation (HR 1.500, 95% CI 1.450–2.991; $P = 0.036$), ADC subtype (HR 0.613, 95% CI 0.410–0.918; $P = 0.018$), advanced pTNM stage (HR 1.455, 95% CI 1.307–2.674; $P = 0.013$), lymph node metastasis (HR 1.709, 95% CI 1.378–2.119; $P = 0.007$), and LRH1 overexpression (HR 1.497, 95% CI 1.059–2.115; $P = 0.011$) were independent predictors for DFS.

The patient samples were stratified according to histology (75 ADC and 81 SCC) to further evaluate the significant function of LRH1 as a predictive biomarker in NSCLC. The results revealed that high LRH1 expression was associated with poor prognosis in patients with ADC and SCC.

In NSCLC patients with elevated LRH1 expression, the median OS and DFS were 43.5 ($P < 0.001$) and 28.5 ($P < 0.001$) months compared to 51.9 ($P < 0.001$) and 47.5 ($P < 0.001$) months in patients with low LRH1 expression, respectively. The relative survival curves for NSCLC patients are illustrated in Figure 3. The five-year recurrence rates in surgically resected stage I/II/III NSCLC patients with high LRH1 expression were 50%, 66.7%, and 80.5%, and in patients with low LRH1 were 20%, 37.5%, and 60%, respectively.

Discussion

This novel study revealed the significance of LRH1 in clinical NSCLC. LRH1 expression was upregulated in mRNA or protein in tumor tissues compared to normal lung tissues. Elevated-level gene amplification or overexpression of LRH1 has been found in various types of malignant carcinomas.[13,19,20] qPCR assay showed that LRH1 mRNA expression is overexpressed in human gastric tumor tissues compared to self-paired normal controls.[19] LRH1 expression is also increased in pancreatic and osteosarcoma tumors compared to normal adjacent tissues.[13,20]

Notably, high LRH1 expression was obviously correlated with poorer differentiation, advanced pTNM stage, ADC subtype, and, most importantly, lymphatic metastasis. Consistently, LRH1 gene polymorphisms significantly contribute to the possibility of distant metastasis or regional lymph node metastasis in gastric cancer.[21] The prominent association of LRH1 protein expression with tumor grade suggests that LRH1 is a crucial indicator of tumor formation in breast cancer.[22] Elevated miR-186, which directly binds to the 3′-untranslated region of LRH1, is associated with tumor volume, advanced tumor progression, and the presence of lymph node metastasis in pancreatic ductal adenocarcinoma.[23] High LRH1 expression was notably correlated with the lung ADC subtype in our study. Previous studies have demonstrated elevated LRH1 expression in breast, pancreatic, and colon cancers, which all originated from cancerous changes to glandular cells.[11,22,24] These findings indicate that high LRH1 expression is prone to gland-derived tissues compared to squamous cell-derived tissues. Thus, LRH1 may show differential expression in tissues.

The results of our study and previous studies indicate that LRH1 might be significant as an adjunct for evaluating lymph node metastasis. Clinically, 18.6% of stage I lung cancer patients suffer nodal upstaging after operative treatment.[25] More accurate pathologic lymph node evaluation may be possible and neoadjuvant therapy administered if lymph node metastasis could be detected before surgical intervention.[26] Therefore, more research on the molecular mechanisms or signaling pathways that induce lymph node metastasis will provide new options for early diagnosis and therapeutic strategies for NSCLC.

Immunohistochemistry analysis indicated that LRH1 was critical for tumor cell proliferation and metastasis, and abundant findings have provided supporting evidence of

Table 2 Univariate and multivariate analyses of OS and DFS

	OS			DFS		
	Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
Variable	*P*	HR (95% CI)	*P*	*P*	HR (95% CI)	*P*
Age (year)						
< 60						
≥ 60	0.683	—	—	0.916	—	—
Gender						
Female						
Male	0.146	—	—	0.716	—	—
Smoking status						
Yes						
No	0.229	—	—	0.824	—	—
Differentiation						
Good	—	—	—	—	—	—
Moderate	—	—	—	—	—	—
Poor	0.034*	0.617 (0.394–0.965)	0.065	0.045*	1.500 (1.450–2.991)	0.036*
Histological cell type						
Squamous cell carcinoma	—	—	—	—	—	—
Adenocarcinoma	0.061	—	—	0.017*	0.613 (0.410–0.918)	0.018*
pTNM stage						
I	—	—	—	—	—	—
II	—	—	—	—	—	—
III	<0.001*	1.506 (1.146–2.933)	0.016*	< 0.001*	1.455 (1.307–2.674)	0.013*
Lymph node metastasis						
Absent	—	—	—	—	—	—
Present	< 0.001*	3.172 (2.005–5.019)	0.009*	< 0.001*	1.709 (1.378–2.119)	0.007*
LRH1 expression						
Low	—	—	—	—	—	—
High	< 0.001*	1.372 (1.225–1.617)	0.003*	0.004*	1.497 (1.059–2.115)	0.011*

*$P < 0.05$ was considered statistically significant. CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; NSCLC, non-small cell lung cancer; OS, overall survival; pT, pathological tumor; pTNM, pathological tumor node metastasis.

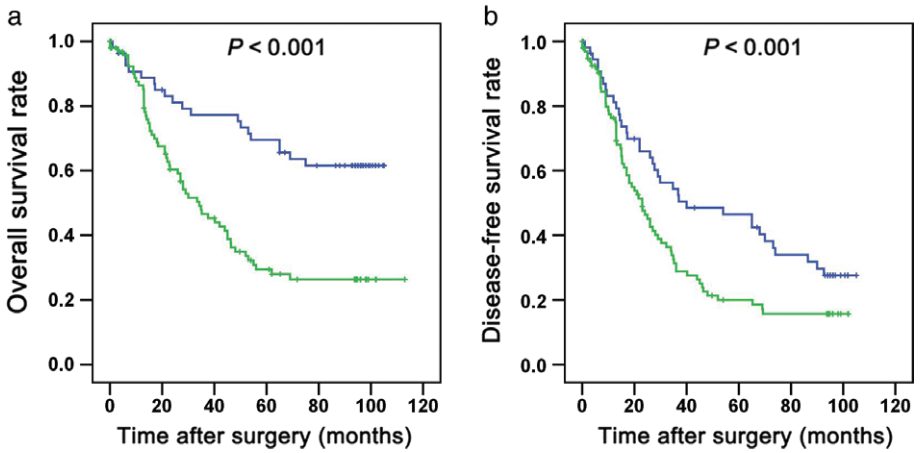

Figure 3 High LRH1 levels were associated with shorter survival in non-small cell lung cancer (NSCLC) patients. (**a**) Overall survival (OS) and (**b**) disease-free survival (DFS) were statistically shorter in patients with high LRH1 protein expression than in those with low LRH1 protein expression. Kaplan–Meier curves of OS and DFS for high and low LRH1 expression. *$P < 0.05$ was considered statistically significant.

the oncogenic roles of LRH1.[9,11,13,16,24,27–30] A study conducted in pancreatic cancer cell lines and in animal models confirmed that LRH1 overexpression accelerated unremitting cell proliferation, self-renewal, or tumor metastasis.[11] LRH1 expression is also regarded as a driving factor in pancreatic tumor growth by stimulating cyclin D1, cyclin E1, or c-Myc.[27] Additionally, LRH1 significantly prompts colon cancer progression by inhibiting CDKN1A expression in a p53-dependent condition.[24] There is an association between LRH1 and β-catenin, which subsequently induces intestinal cell proliferation and tumorigenesis in colon cancer.[28] Indeed, when cancer cells activate EMT

progression and obtain stem-cell-like potential, the likelihood of metastasis is indicated.[29–31] In breast cancer, LRH1 is involved in EMT progression, and LRH1 overexpression promotes cell motility and invasion by regulating E-cadherin and MMP9.[16] LRH1 in pancreatic CSCs promotes sphere formation and regulates CSC or EMT marker expression.[13] In gastric carcinoma, when targeting the LRH1/Wnt/β-catenin signaling pathway, miRNA-219-5p inhibits the proliferation and transfer capacities of tumor cells.[9] Collectively, these data suggest that LRH1 might be involved in the tumorigenesis of human malignant tumors. Studies of the oncogenic activity and functional mechanism of LRH1 in NSCLC cells are ongoing.

Previous studies have attempted to identify biomarkers that could improve prognostication for patients and be readily implemented into clinical practice.[32] In our study, high LRH1 expression in NSCLC was an independent factor for poorer OS and DFS. Similarly, breast cancer patients with LRH1 overexpression and amplification are reported to have a significantly poor prognosis.[33] Elevated LRH1 protein expression in tumor tissues is associated with reduced OS in patients with pancreatic carcinoma.[13] The highly refractory nature of NSCLC in response to chemotherapeutic drugs is an important factor contributing to poor prognosis[34] and LRH1-modulated chemotherapy resistance. In breast cancer, LRH1 induces chemoresistance by inhibiting DNA damage and transcriptionally activating the malignant downstream process.[8] In hepatocellular carcinoma, the suppression of LRH1 regulated by RNA interference contributes to the induction of apoptosis.[35] Determining accurate biomarkers for NSCLC is critical. Recently, circular RNAs (circRNAs), such as F-circEA, produced by the *EML4-ALK* fusion gene have been identified as "liquid biopsy" biomarkers to monitor cell migration, invasion, and tumor development, which can guide clinical targeted therapy in NSCLC.[36] Therefore, the identification of LRH1 as a novel prognostic biomarker is a dramatic breakthrough.

Despite our intriguing results, there are some limitations to this study. An investigation with a larger amount of patients is essential to evaluate the role of this novel target, as our study was retrospective and included a limited number of samples.

Based on these findings, LRH1 is not only a promising therapeutic target for drug development but also a crucial biomarker for lymph node metastasis in NSCLC patients. The function of LRH1 as an underlying molecular mechanism of NSCLC metastasis needs to be further investigated.

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Disclosure

No authors report any conflict of interest.

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