Overexpression of CHD1L is positively associated with metastasis of lung adenocarcinoma and predicts patients poor survival

Li-Ru He1,2, Ning-Fang Ma4, Jie-Wei Chen1,3, Bin-Kui Li1, Xin-Yuan Guan1,5, Meng-Zhong Liu1,2, Dan Xie1,3

1The State Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, Collaborative Innovation Center for Cancer Medicine, Guangzhou, China
2Department of Radiation Oncology, Sun Yat-Sen University Cancer Center, Guangzhou, China
3Department of Pathology, Sun Yat-Sen University Cancer Center, Guangzhou, China
4Department of Histology and Embryology, State Key Laboratory of Respiratory Disease, Guangzhou Medical University, Guangzhou, China
5Departments of Clinical Oncology, The University of Hong Kong, Hong Kong, China

Correspondence to:
Dan Xie, e-mail: xiedan@sysucc.org.cn

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ABSTRACT

CHD1L (chromodomain helicase/ATPase DNA binding protein 1-like gene) has been demonstrated as an oncogene in hepatocellular carcinoma (HCC), however, the role of CHD1L in non-small-cell lung cancer (NSCLC) tumorigenesis hasn’t been elucidated. In this study, the expression and amplification status of CHD1L were examined by immunohistochemistry and fluorescence in situ hybridization respectively in 248 surgically resected NSCLCs. The associations between CHD1L expression and clinicopathologic features and the prognostic value of CHD1L were analyzed. Overexpression and amplification of CHD1L was found in 42.1% and 17.7% of NSCLCs, respectively. The frequency of CHD1L overexpression (53.2% vs. 28.1%, $P = 0.002$) and amplification (25.2% vs. 8.2%, $P = 0.020$) in adenocarcinoma (ADC), was much higher than that in squamous cell carcinoma (SCC). CHD1L overexpression was associated closely with ascending pN status ($P < 0.001$), advanced clinical stage ($P = 0.001$) and tumor distant metastasis ($P = 0.001$) in ADCs, but not in SCCs. For the whole cohort and ADC patients, univariate survival analysis demonstrated a significant association of CHD1L overexpression with shortened survival; and in multivariate analysis, CHD1L overexpression was evaluated as a independent predictor for overall survival and distant metastasis free survival. These results suggested that overexpression of CHD1L is positively associated with tumor metastasis of lung ADC, and might serve as a novel prognostic biomarker and potential therapeutic target for lung ADC patients.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide [1]. Non-small-cell lung cancer (NSCLC), which accounts for almost 80% of such death, is a very heterogeneous group of malignancies [2]. Even for earlier stages patients, a significant proportion of them will suffer from local recurrence and/or distant metastasis after radical surgery [3]. However, the international staging system is still inadequate to reliably predict patients’ prognosis. Since chromosomal aberrations are believed to play an important role in tumor progression [4], it will be of great value to search the specific gene alterations in NSCLC which
can provide additional staging information to optimize individual therapy.

Chromodomain helicase/ATPase DNA binding protein 1-like gene (CHD1L) is a newly identified oncogene that we previously isolated from a frequently amplified region at chromosome 1q of human hepatocellular carcinoma (HCC) [5]. A series of our further studies demonstrated that CHDIL contributes to HCC cell migration, invasion and metastasis, and is positively associated with tumor progression in HCC patients [5–7]. Recently, CHD1L has also been reported as a novel biomarker for patients’ prognosis in several types of solid tumor, including breast cancer [8], gastric cancer [9], colorectal cancer [10], bladder cancer [11] and ovarian cancer [12]. To date, however, the abnormalities of CHD1L and its oncogenic role in NSCLC have not been studied. Since amplification of 1q, in which the CHDIL gene is located, was frequently examined in NSCLC and correlated closely with tumor recurrence and metastasis [13, 14], we conducted this study to examine the expression and amplification dynamics of CHDIL in a large cohort of NSCLC patients, and its clinicopathologic and prognostic significance was further evaluated.

RESULTS

Expression of CHD1L in NSCLCs

The expression of CHD1L could be informatively examined by IHC in 233/248 (93.9%) of the NSCLCs and 27/30 (90.0%) of normal lung tissues. The non-informative samples included unrepresentative samples, samples with too few tumor cells (<300 cells per case) and lost samples, which we didn’t use in our data compilation. Since the expression scores of CHD1L in normal lung tissues varied from 0 to 2 (intensity 0–2, proportion 0–1), overexpression of CHD1L was designated when the score was equal or more than 3. Using this criteria, CHD1L overexpression was observed in 98/233 (42.1%) of the NSCLCs, 58/109 (53.2%) of the adenocarcinomas (ADCs) and 25/89 (28.1%) of the squamous cell carcinomas (SCCs), respectively (Fig. 1).

Association between CHD1L expression and clinic-pathological variables in NSCLCs

The associations between CHD1L expression and several clinico-pathological variables in NSCLC patients are assessed and displayed in Table 1. Overexpression of CHDIL in NSCLCs was significantly associated with tumor histology (P = 0.002), advanced pN status (P < 0.001) and advanced stage (P < 0.001). There was no significant association between CHD1L expression and other clinic-pathological features, such as patients’ age, gender, tumor grade, pT status, surgical procedure, adjuvant chemotherapy and adjuvant radiotherapy. We further evaluated the associations in ADC and SCC patients, respectively, and found that the significant associations between CHD1L overexpression and advanced pN status/ advanced stage were only seen in ADC patients, but not in SCC cases (Table 1).

Association between CHD1L expression and post-surgical failure patterns

During the median observation period of 46.1 months (range 3.6–199.3 months) for all patients, 67 experienced local-regional failure, 109 experienced distant metastasis, and 173 had cancer-specific death. The failure patterns of the NSCLC patients are presented in Table 2. The incidence of distant metastasis was much higher in patients with CHD1L overexpression than those with CHD1L normal expression for all patients (56.9% vs. 43.1%, P < 0.001); however, in subgroup analysis, this significant association was only seen in ADC patients (P < 0.001) and not in SCC patients (P = 0.162). No significant incidence difference was found in local-regional recurrence between groups with CHD1L normal/overexpression for all patients, SCC patients or ADC patients (P > 0.05, Table 2).

The impact of CHD1L expression on NSCLC patients’ survival

In univariate analysis, CHD1L overexpression was evaluated to correlate closely with shorten overall survival (OS), shorten local-regional failure free survival (LRFFS) and shorten distant metastasis free survival (DMFS) for the whole cohort and the ADC patients, but not for the SCC patients (Fig. 2). Besides CHD1L overexpression, the impact value of age, gender, tumor grade, histology, stage, surgical procedure, adjuvant chemotherapy and adjuvant radiotherapy have also been tested in univariate analysis for OS, LRFFS and DMFS, respectively. Variables that showed a significant impact on patients’ survival for the whole cohort and the ADC patients in univariate analysis were listed in Table 3, which were further tested in multivariate analysis. Other variables, including tumor grade, surgical procedure, adjuvant chemotherapy and adjuvant radiotherapy, were not evaluated as significant prognostic factors in univariate analysis (Data not shown). Our multivariate analysis results showed that CHD1L overexpression was evaluated as an independent predictor of poor OS and poor DMFS for the whole cohort and ADC patients (Table 3).

Amplification of CHD1L in NSCLCs

The FISH analysis was informative in 50.0% (15/30) of the normal lung tissues and 52.4% (130/248)
of the NCSLCs. The main reasons for most of the non-informative cases were samples without FISH signal and samples with weak target signals or those with a strong signal background. Amplification of CHD1L was not detected in any of the normal lung tissues, but was observed in 17.7% (23/130) of the informative NSCLCs. In univariate analysis by Cox regression models, CHD1L amplification was evaluated as a significant predictor for distant metastasis (hazard ratios (HR) = 3.255, 95% confidence intervals (CI) = 1.984–5.339, \( P < 0.001 \)), recurrence (HR = 2.795, 95% CI = 1.426–5.481, \( P = 0.003 \)) and OS (HR = 3.185, 95% CI = 2.039–4.975, \( P < 0.001 \)).

In our study, 117 cases were shown to be simultaneously informative by both IHC and FISH tests. Amplification of CHD1L gene was significantly associated with CHD1L protein overexpression (\( P < 0.001 \)) and tumor histology (\( P = 0.020 \), Table 4). The frequency of CHD1L amplification in ADC (25.5%) was much higher than that in SCC (8.2%). Overexpression of CHD1L was observed in the majority (16/19, 84.2%) of NSCLCs with CHD1L amplification; whereas, CHD1L overexpression can also be detected in 35.7% (35/98) of the NSCLCs without CHD1L gene amplification.

**DISCUSSION**

In this study, our results showed that the expression of CHD1L in all of the normal lung tissue specimens was absent or at low levels; in contrast, overexpression of CHD1L was frequently examined in many of the NSCLC specimens. Moreover, the frequency of CHD1L overexpression increased with an ascending pN status and advancing clinical stage in NSCLC, especially in

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**Figure 1: Immunohistochemical stainings of CHD1L and FISH assay of CHD1L gene in NSCLC tissues.**

A. A normal lung tissue (case 19) showed a negative staining of CHD1L in all the epithelium cells (×100).

B. A squamous carcinoma of lung (case 115) showed normal expression of CHD1L with a negative staining of CHD1L in all tumor cells (×100).

C. Overexpression of CHD1L was detected in a squamous carcinoma of lung (case 216), in which about 50% of tumor cells were observed moderate positive staining of CHD1L (×100).

D. Overexpression of CHD1L was detected in an adenocarcinoma of lung (case 57), in which about 40% of tumor cells were observed strong positive staining of CHD1L (×100).

E. Overexpression of CHD1L was examined in an adenocarcinoma of lung (case 113), in which about 60% of tumor cells showed strong positive staining of CHD1L (×100).

F. Amplification of CHD1L gene was examined by FISH in the same case of lung adenocarcinoma (case 113), in which CHD1L gene signals (red) were detected at least 3 times more than chromosome 1 centromere signals (green) (×1000).
Table 1: CHD1L expression and clinic-pathological variables

| Characteristics       | Total cases | Informative cases | CHD1L protein Overexpression (%) | P*  |
|-----------------------|-------------|-------------------|----------------------------------|-----|
| Age (years)           |             |                   |                                  |     |
| ≤59†                  | 130         | 119               | 49 (41.2)                        | 0.780 |
| >59                   | 118         | 114               | 49 (43.0)                        |     |
| Gender                |             |                   |                                  | 0.190 |
| Male                  | 181         | 172               | 68 (39.5)                        |     |
| Female                | 67          | 61                | 30 (49.2)                        |     |
| Tumor grade           |             |                   |                                  | 0.521 |
| G1                    | 24          | 22                | 8 (36.4)                         |     |
| G2                    | 173         | 164               | 67 (40.9)                        |     |
| G3                    | 51          | 47                | 23 (48.9)                        |     |
| Histology             |             |                   |                                  | 0.002 |
| SCC                   | 94          | 89                | 25 (28.1)                        |     |
| ADC                   | 113         | 109               | 58 (53.2)                        |     |
| Others‡               | 41          | 35                | 15 (42.9)                        |     |
| pT status             |             |                   |                                  | 0.139 |
| pT1–2                 | 147         | 139               | 53 (38.1)                        |     |
| pT3–4                 | 101         | 94                | 45 (47.9)                        |     |
| pN status             |             |                   |                                  | <0.001 |
| pN0                   | 120         | 113               | 31 (27.4)                        |     |
| pN1                   | 49          | 46                | 20 (43.5)                        |     |
| pN2                   | 79          | 74                | 47 (63.5)                        |     |
| Stage                 |             |                   |                                  | <0.001 |
| I                     | 82          | 80                | 22 (27.5)                        |     |
| II                    | 59          | 57                | 20 (35.1)                        |     |
| III                   | 107         | 96                | 56 (58.3)                        |     |
| Surgical procedure    |             |                   |                                  | 0.405 |
| Lobectomy             | 223         | 209               | 86 (41.1)                        |     |
| Pneumonectomy         | 25          | 24                | 12 (50.0)                        |     |
| Adjuvant chemotherapy |             |                   |                                  | 0.126 |
| Yes                   | 89          | 82                | 40 (48.8)                        |     |
| No                    | 159         | 151               | 58 (38.4)                        |     |
| Adjuvant radiotherapy |             |                   |                                  | 0.670 |
| Yes                   | 35          | 33                | 15 (45.5)                        |     |
| No                    | 213         | 200               | 83 (41.5)                        |     |

(Continued)
lungs. Similar results were also observed in other
types of human cancer [6, 8, 9]. In HCC, overexpression
of CHD1L was associated closely with microsatellite
formation and venous invasion [6]. In gastric cancer,
up-regulated expression of CHD1L was reported to be
correlated with tumor depth, nodal involvement and
distant metastasis [9]. While in breast cancer, presence
of CHD1L expression was associated with higher Ki-67
index and HER2 amplification [8]. These findings suggest
that increased expression of CHD1L is associated with
aggressive tumor biology in certain types of human solid
tumors, including lung ADC.

Interestingly, we found that the expression level
of CHDIL in NSCLCs was significantly associated with
tumor histology. On the basis of cell morphology, ADC
and SCC are the two most common types of NSCLC.
Given the difference in biological and clinical behavior
of the two types of NSCLC, the patterns of chromosomal
aberrations in ADC and SCC are thought to be different
to some extent [15]. One of the differences between the
2 types of NSCLC is that 1q amplification was more
common in ADC as compared to that in SCC [14, 16,
17]. Since CHD1L is an oncogene locates at a frequently
amplified region of 1q [5], our results which showed that
the frequency of CHDIL overexpression and the frequency
of CHDIL amplification in ADC were much higher than
those in SCC, is in agreement with the previous reports.

Most importantly, overexpression of CHDIL was
significantly associated with distant metastasis, and was evaluated as an independent adverse predictor of OS and DMFS for lung ADC patients. As we know, lung ADC is a highly malignant disease which tends to disseminate hematogenously at an early stage. Even after a curative resection, the majority patients still die within 5 years because of tumor metastasis [1], thus, effective adjuvant treatment are actually needed. However, the benefit of adjuvant chemotherapy is still limited [18, 19], and the available target therapy agents

| Characteristics | Total cases | Informative | CHDIL protein |
|-----------------|-------------|-------------|---------------|
|                 |             |             | Overexpression (%) | P*  |
| SCC subgroup    |             |             |                |     |
| pN status       |             |             |                |     |
| pN0             | 49          | 48          | 12 (25.0)      | 0.753|
| pN1             | 18          | 17          | 5 (29.4)       |     |
| pN2             | 27          | 24          | 8 (33.3)       |     |
| Stage           |             |             |                |     |
| I               | 33          | 32          | 8 (25.0)       | 0.568|
| II              | 25          | 22          | 5 (22.7)       |     |
| III             | 36          | 35          | 12 (34.3)      |     |
| ADC subgroup    |             |             |                |     |
| pN status       |             |             |                |     |
| pN0             | 53          | 49          | 15 (30.6)      | <0.001|
| pN1             | 22          | 22          | 12 (54.5)      |     |
| pN2             | 38          | 38          | 31 (81.6)      |     |
| Stage           |             |             |                |     |
| I               | 38          | 37          | 12 (32.4)      | 0.001|
| II              | 27          | 25          | 12 (48.0)      |     |
| III             | 48          | 47          | 34 (72.3)      |     |

*Chi-square test; †Mean age; ‡Others include adenosquamous cell carcinoma, anaplastic large-cell carcinoma, sarcoma, adenoid cystic carcinoma, mucoepidermoid carcinoma and carcinoid tumor.

Abbreviation: CHD1L, chromodomain helicase/ATPase DNA binding protein 1-like gene; G, grade; SCC, squamous cell carcinoma; ADC, adenocarcinoma; pT, pathological tumor; pN, pathological node.
are quite rare. Thus, identification of more potential therapeutic targets is still highly desirable. When compared the chromosomal imbalances between primary and metastatic lung ADC, Goeze et al [14] reported that DNA overrepresentation on 1q was the most common alteration. And the overrepresentations on 1q21–q25 were evaluated to be associated with the metastatic phenotype of lung ADC [14]. Our results, together with these findings, suggest that up-regulated expression of CHD1L, a gene locates at 1q21, may provide a selective advantage in metastasis process of lung ADC; and CHD1L might be served as a potential therapeutic target for lung ADC patients.

With regard to the mechanism of up-regulated protein expression of CHD1L in NSCLCs, it is well established that overexpression of an oncogene is often caused by DNA amplification. Amplification of CHDIL has been detected in 50.6% of HCCs [5] and 18.3% of colorectal cancer samples [10]. However, none of the previous studies has evaluated the association of CHD1L gene amplification and CHD1L protein overexpression in any types of human cancers. To determine whether or not overexpression of CHD1L in NSCLCs was caused by gene amplification, we further examined the amplification status of CHDIL by FISH and tested its association with CHD1L expression. We observed that overexpression of CHDIL protein was associated, but not always coincided with CHDIL gene amplification in NSCLC. This result indicates that gene amplification might be one of the mechanisms that produce an excess of CHDIL protein; however, the modification of CHDIL expression could also be regulated by other mechanisms, such as post-translational modification.

When come to the gene function of CHD1L, it has not been well clarified in human cancers except for HCC. Our previous studies showed that increased expression of CHD1L promoted tumor cell migration and metastasis by increasing cell motility and inducing epithelial-mesenchymal transition (EMT) in HCC [6].

### Table 2: Comparison of the failure patterns between NSCLC patients with CHD1L overexpression and normal expression

|                      | CHD1L protein |          |          |          |  |
|----------------------|---------------|----------|----------|----------|---|
|                      |               | Normal   | Overexpression |     |
|                      |               | expression (%) | (%)     |          |  |
|                      |               |     |          | P*       |  |
| All patients         |               |          |          |          |  |
| Local-regional       |               |          |          |          |  |
| recurrence           | No            | 166      | 102 (61.4) | 64 (38.6) | 0.088 |
|                      | Yes           | 67       | 33 (49.3)  | 34 (50.7) |          |
| Distant metastasis   | No            | 124      | 88 (71.0)  | 36 (29.0) | <0.001 |
|                      | Yes           | 109      | 47 (43.1)  | 62 (56.9) |          |
| SCC                  |               |          |          |          |  |
| Local-regional       | No            | 58       | 45 (77.6)  | 13 (22.4) | 0.103 |
| recurrence           | Yes           | 31       | 19 (61.3)  | 12 (38.7) |          |
| Distant metastasis   | No            | 63       | 48 (76.2)  | 15 (23.8) | 0.162 |
|                      | Yes           | 26       | 16 (61.5)  | 10 (38.5) |          |
| ADC                  |               |          |          |          |  |
| Local-regional       | No            | 81       | 40 (49.4)  | 41 (50.6) | 0.356 |
| recurrence           | Yes           | 28       | 11 (39.3)  | 17 (60.7) |          |
| Distant metastasis   | No            | 48       | 31 (64.6)  | 17 (35.4) | 0.001 |
|                      | Yes           | 61       | 20 (32.8)  | 41 (67.2) |          |

*Chi-square test.
Abbreviation: CHDIL, chromodomain helicase/ATPase DNA binding protein 1-like gene; NSCLC, non-small-cell lung carcinoma; SCC, squamous cell carcinoma; ADC, adenocarcinoma.
Figure 2: Survival curves according to CHDIL expression level for NSCLC patients. A, B, and C. overall survival for the whole cohort, SCC and ADC patients; D, E, and F. local-regional failure free survival for the whole cohort, SCC and ADC patients; and G, H, and I. distant metastasis free survival for the whole cohort, SCC and ADC patients. CHDIL normal expression groups are shown as solid lines; CHDIL overexpression groups are shown as dashed lines.

Table 3: Multivariate Cox regression analysis for OS, LRFFS and DRFS

| Variables                  | OS            |         | LRFFS        |         | DRFS         |         |
|----------------------------|---------------|---------|--------------|---------|--------------|---------|
|                             | HR (95%CI)    | P       | HR (95%CI)   | P       | HR (95%CI)   | P       |
| ALL patients                |               |         |              |         |              |         |
| Age†                       | 1.406 (1.042–1.897) | 0.026   | –            | –       | 1.379 (0.945–2.011) | 0.095   |
| Histology‡                  | 1.269 (0.924–1.744) | 0.142   | –            | –       | 2.154 (1.375–3.372) | 0.001   |
| Stage§                     | 1.745 (1.436–2.121) | <0.001  | 3.365 (2.284–4.958) | <0.001  | 1.764 (1.374–2.264) | <0.001  |
| CHD1L expression¶          | 1.526 (1.120–2.079) | 0.007   | 1.432 (0.881–2.328) | 0.148   | 1.755 (1.185–2.599) | 0.005   |
| ADE patients                |               |         |              |         |              |         |
| Age†                       | 1.295 (0.836–2.005) | 0.247   | –            | –       | –            | –       |
| Stage§                     | 1.994 (1.477–2.691) | <0.001  | 5.800 (2.779–12.107) | <0.001  | 2.102 (1.480–2.984) | <0.001  |
| CHD1L expression¶          | 1.720 (1.091–2.712) | 0.020   | 1.478 (0.681–3.208) | 0.323   | 2.019 (1.168–3.491) | 0.012   |

†Age > 59 yrs vs. Age ≤ 59 yrs; ‡Non squamous cell carcinoma vs Squamous cell carcinoma; §Stage III vs. Stage II vs. Stage I; ¶Overexpression vs. Normal expression.
Abbreviation: CHD1L, chromodomain helicase/ATPase DNA binding protein 1-like gene; OS, overall survival; LRFFS, local-regional failure free survival; DMFS, distant metastasis free survival.
More recently, we have also found that CHD1L promoted HCC cell invasiveness and metastasis by activating kazal-like domains proteoglycan 1 (SPOCK1)-AKt signaling pathway [20]. Collectively, both CHD1L-ARHGEF9-Cdc42-EMT pathway and CHD1L-SPOCK1-AKt pathway might be involved in HCC progression and metastasis. However, the specific underling mechanisms of CHD1L in lung ADC metastasis have not been investigated. Clearly, further functional studies are needed.

Our study did have some limitations. It is a retrospective study, we didn’t include all the clinical information because of the unavailability of some data. And there was a high heterogeneity in the regimens and cycles of adjuvant chemotherapy that we could not further analyzed. Thus, further studies are needed to confirm these results.

In summary, our results provide some evidences for the concept that (1) overexpression of CHD1L might provide a selective advantage for distant metastasis of lung ADC; and (2) CHD1L plays an adverse role in the prognosis of lung ADC, and might serve as a novel prognostic marker and potential therapeutic target for lung ADC patients.

### Patients and Methods

**Patients and tissue specimens**

In this study, paraffin-embedded tissue samples from 248 NSCLC patients were obtained from the Pathology Department of Cancer Center, Sun Yat-sen University, Guangzhou, China, between February 1994 and January 1998. All the selected patients had received complete resection (223 had lobectomy and 25 underwent pneumonectomy) and with adequate follow-up data. Patients who had preoperative treatment or those who had a second malignant disease were excluded.

Data regarding stage was according to the pathology Tumor-Node-Metastasis (pTNM) system (AJCC/UICC 2007). Tumor differentiation and histotype were according to the World Health Organization classification for NSCLC. Local/regional failure was defined as the recurrence of the primary tumor and regional lymph nodes, while distant failure was defined as the metastasis to any site beyond the primary tumor and regional lymph nodes. The study was approved by the medical ethics committee of Cancer Center, Sun Yat-Sen University and was performed in accordance with the Declaration of Helsinki.

### Construction of tissue microarrays (TMA)

The TMA was constructed according to a method described previously [21]. Briefly, the formalin-fixed, paraffin-embedded tissue blocks and the corresponding histological H&E stained slides were overlaid for tissue TMA sampling. The tissues (248 NSCLC and 30 normal lung tissues taken from regions that were not affected of the same patients) were sampled using a tissue arraying instrument (Beecher Instruments, Silver Spring, MD); a 0.6-mm-diameter cylinder of tissue was removed. Subsequently, we re-embedded the tissue cylinder into a predetermined position in a recipient paraffin block. Three cores of sample were selected from each primary NSCLC and normal lung tissue, and multiple sections (5 µm thick) were cut from the TMA block and mounted on microscope slides.

### Immunohistochemistry (IHC)

The IHC study of CHD1L was performed using a standard streptavidin-peroxidase method as previously described [12]. The TMA sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked with 3% H2O2 for 10 minutes. For the antigen retrieval, slides were immersed in 10 mM citrate buffer (pH 6.0) and boiled in a microwave oven for 15 minutes. Non-specific binding was blocked by 5% normal goat serum for 10 minutes. The slides were

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**Table 4: Association of CHD1L expression and amplification in NSCLC**

| CHD1L protein   | No amplification | Amplification | P*  |
|-----------------|------------------|---------------|-----|
| Normal expression | 66               | 63 (95.5)     | 3 (4.5) |
|                 |                  | 3 (1.2)       | 2 (3.1) |
| Overexpression   | 51               | 35 (68.6)     | 16 (31.4) |
|                 |                  | 4 (1.2)       | 3 (5.8) |
| Histology       | 0.020            |               |     |
| SCC             | 49               | 45 (91.8)     | 4 (8.2) |
| ADC             | 55               | 41 (74.5)     | 14 (25.5) |

*Chi-square test.

Abbreviation: CHD1L, chromodomain helicase/ATPase DNA binding protein 1-like gene; NSCLC, non-small-cell lung carcinoma; SCC, squamous cell carcinoma; ADC, adenocarcinoma.
incubated with a 1:100 dilution of monoclonal antibody against CHD1L (Abcam) at 4°C overnight in a moist chamber. The slides were sequentially incubated with biotinylated goat anti-mouse IgG (1:100 dilution; Santa Cruz Biotechnology) and then streptavidin-peroxidase conjugate, each for 30 minutes at room temperature. Isotope-matched human IgG was used in each case as a negative control. Finally, the 3',5-diaminobenzidine (DAB) Substrate Kit (Dako) was used for color development followed by Mayer hematoxylin counterstaining.

Positive expression of CHD1L in normal and malignant lung tissues was primarily a nuclear pattern (Fig. 1). For the evaluation of CHD1L staining, a semi-quantitative scoring criterion was used, in which both staining intensity and positive cells percentage were recorded [9, 12]. A staining index (with values from 0 to 12) was obtained as the intensity of CHD1L staining (0 = negative, 1 = weakly positive, 2 = moderate positive, 3 = strongly positive) times the proportion of immunopositive tumor cells (0% = 0, <10% = 1, 10% to <50% = 2, 50% to <75% = 3, ≥75% = 4). A minimum of 300 epithelial cells was counted for each case. Two independent pathologists (Dr. Xie D and Chen JW) blinded to the clinicopathologic information performed the scorings. The inter-observer disagreements (about 6% of the total informative cases) were reviewed a second time, followed by a conclusive judgment by both pathologists.

**Fluorescence in situ hybridization (FISH)**

Two-color FISH was applied to the sections of TMA. The BAC clone (RP11–337C18) at 1q21 containing the CHD1L gene probe and the chromosome 1 centromere probe were labeled with Spectrum-red and Spectrum-green (Vysis, Downers Grove, IL), respectively. The FISH reaction was performed as described previously with slight modification. Briefly, the deparaffinized TMA section was treated with proteinase K (400 μg/ml) at 37°C for 45 min, followed by denaturing in 70% formamide, 2 × SSC at 75°C for 6 min. Fifty nanograms of each probe were mixed in a 20μl hybridization mixture (containing 55% formamide, 2 × SSC, and 2 μg human Cot1 DNA), denatured at 75°C for 5 minutes and then hybridized to the denatured TMA section at 37°C for 24 hours. After washing, the TMA section was counterstained with 1 μg/ml DAPI in an anti-fade solution and examined with a Zeiss Axiophot microscope equipped with a triple-band pass filter. A minimum of 300 tumor cells was evaluated per specimen. Amplification of CHD1L gene were defined as the presence (in ≥20% of tumor cells) of either 6 (or more) gene signals or more than 3 times as many gene signals than reference chromosome 1 centromere signals (Fig. 1F) [22–24]. All samples not meeting the criteria for gain or amplification were considered normal. Control hybridizations to normal fibroblasts and to normal epithelial cells were performed to confirm that the hybridization efficiency of the test and reference probes was similar. The slides were assessed by the two independent pathologists (Dr. Xie D and Chen JW) mentioned above. The inter-observer disagreements (about 3% of the total informative cases) were reviewed a second time, followed by a conclusive judgment by both pathologists.

**Statistical analysis**

Statistical analysis was performed with the SPSS software (SPSS Standard version 13.0, SPSS Inc. Chicago, IL). The association of CHD1L protein expression with NSCLC patient’s clinicopathological features and its correlation with CHD1L gene amplification were assessed by the Chi-square test. DMFS, LRFFS and OS were defined as the time from the date of diagnosis to tumor metastasis, local-regional tumor recurrence and death, respectively. Survival curves were assessed by the Kaplan-Meier method and compared by the log-rank test. Cox regression analysis was carried out to assess the significance of variables for survival. Two-sided, \( P < 0.05 \) was considered statistically significant.

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**CONFLICTS OF INTEREST**

The authors declare that there is no conflict of interest.

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