Background: Non-small cell lung carcinoma (NSCLC) mainly includes lung squamous cell carcinoma and adenocarcinoma. This study aimed to investigate the difference between the expression of Cbl-b in lung squamous cell carcinoma and adenocarcinoma.

Material/Methods: The clinical features and survival data of NSCLC patients and Cbl-b mRNA (FPKM) were obtained from the TCGA database. Then, lung squamous cell carcinoma and adenocarcinoma cell lines were transfected with lentivirus-mediated RNA interference vector to knockdown the expression of Cbl-b. Next, a Transwell assay was performed to study the effect of Cbl-b shRNA on migration and invasion of lung squamous cell carcinoma and adenocarcinoma cells. Finally, Western blot analysis was performed to measure the expressions of PI3K, p-PI3K, AKT, p-AKT, ERK1/2, p-ERK1/2, GSK3β, p-GSK3β, mTOR, and p-mTOR protein in lung adenocarcinoma and squamous cell carcinoma cells.

Results: The correlation of Cbl-b expression and OS was different between NSCLC adenocarcinoma and squamous carcinoma. After transfection, the expression of Cbl-b was inhibited in A549, H1975, and SW900 cells. Cbl-b shRNA promoted the migration and invasion of lung adenocarcinoma A549 and H1975 cells, but it inhibited the invasion of lung squamous cell carcinoma SW900 cells. In addition, Cbl-b regulated the expression of PI3K and ERK1/2-GSK3β pathway proteins in A549 and SW900 cells.

Conclusions: The OS of Cbl-b mRNA low expression in lung adenocarcinoma and squamous cell carcinoma was different. The difference in signal pathways may be one of the reasons for the difference in the correlation between Cbl-b expression and the survival rate of these 2 pathological types of lung cancer.

MeSH Keywords: Cell Migration Assays • Lung Neoplasms • Neoplasm Invasiveness • Neoplasm Metastasis

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Background

Lung cancer is one of the most common cancers in the world at present, with the highest mortality rate of all cancer patients [1]. According to the pathological features, lung cancer can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC). Lung adenocarcinoma and squamous cell carcinoma are 2 common types of NSCLC. Their pathogenesis may be different due to their different pathological basis, clinical characteristics, and relationship with smoking. Recent studies have shown that due to the different transcription factors (NFIC, BRCA1, and NFATC2) [2] and S100A6 [3] are associated with the development of lung squamous cell carcinoma.

Pathological type, number of bone metastases, clinical stage, ECOG score, and serum ALP are independent prognostic risk factors for bone metastasis from lung cancer [4]. Tumor metastasis is the leading cause of death in cancer patients, but is also the focus of anti-tumor therapy. At present, immunotherapy for T cell immune “checkpoint genes” is an important topic in anti-tumor therapy, involving the key transcription factors (NFIC, BRCA1, and NFATC2) [2] and S100A6 [3] are associated with the development of lung squamous cell carcinoma.

The ubiquitin ligase Cbl-b, as a member of the Cbl family, plays a significant role in post-translational regulation through ubiquitination, and regulates a variety of immune cell regulatory signaling pathways [9]. Recent studies have shown that knockdown of Cbl-b strengthens the immune activation of CD8+ T cells against tumor cells [10, 11], downregulates phosphorylated-Foxo3a in Treg cells [12], and inhibits cancer metastasis via natural killer cells [8]. Activation of T cells has been reported to be released by proteolytic elimination of Cbl-b [13]. Cbl-b is also expressed in cancer cells, but its role in tumor proliferation remains unknown. Yan et al. has found that downregulation of Cbl-b leads to stronger activation of ERK, JNK, and p38 MAPK, and a stronger coefficient action of bufalin and TRAIL in breast cancer cells [14]. Xu et al. has proved that knockdown of Cbl-b also strengthens TRAIL-induced apoptosis, EGFR translocation into lipid rafts, and EGFR pathway activation induced by TRAIL [15]. However, there is little research on the role of Cbl-b in lung cancer cell metastasis, so it is urgent to explore the biological function of Cbl-b protein in lung cancer. RNAi is widely used in cancer research, and it can stably interfere in the expression of target genes in cell lines [16]. Tim-3 interference in macrophages suppressed the optional activation of macrophages and inhibited HCC cell proliferation [17]. The knockdown TGF-β1 gene impaired the tumorigenic ability of HCC [18]. Therefore, a lentivirus-mediated RNA interference vector for Cbl-b was constructed for cytological experiments in our study.

In the present study, the clinical features and survival data of NSCLC patients and Cbl-b mRNA (FPKM) expression were obtained from The Cancer Genome Atlas (TCGA) database, investigating expression and comparison of Cbl-b in prognosis of patients with lung squamous cell carcinoma and adenocarcinoma. Then, lung squamous cell carcinoma and adenocarcinoma cell lines were transfected with lentivirus-mediated RNA interference vector to knockdown the expression of Cbl-b. Next, Transwell assay was performed to study the effect of Cbl-b shRNA on migration and invasion of NSCLC cells. Finally, Western blot analysis was performed to explore whether Cbl-b shRNA regulates the PI3K and ERK1/2 signaling pathways, and to investigate the difference in the underlying mechanism of lung squamous cell carcinoma and adenocarcinoma biological behavior.

Material and Methods

TCGA analysis

The clinical features and survival data of NSCLC patients and mRNA expression pattern of Cbl-b (FPKM) were obtained from the TCGA database (https://portal.gdc.cancer.gov/).

Cell culture

Lung adenocarcinoma cell lines (A549, H1975, H1299) and a lung squamous cell carcinoma cell line (SW900) were purchased from GeneChem (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Corning, USA) supplemented with 10% fetal bovine serum (FBS; Ausbian, Australia) at 37°C in a humidified atmosphere of 5% CO₂.

Construction of lentiviral vectors expressing Cbl-b shRNA and transfection

The RNAI target sequence was: CCT GAT GGG AGG AGT TAT A [19]. The target DNA fragment was inserted into the AgeI and transfection DNA ligase enzyme (Supplementary Figure 1).
Cells in logarithmic growth phase were digested with trypsin and seeded in 6-well plates (3–5×10^4 cells/well) until the cells were 15–30% confluent. Then, the cells were infected with viruses for 72 h. There were 8 groups: A549-negative control (NC), A549-knockdown (KD), H1975-NC, H1975-KD, H1299-NC, H1299-KD, SW900-NC, and SW900-KD. Following 72 h in culture, green fluorescent protein (GFP) expression was observed under a fluorescence microscope (IX71, Olympus, Japan), and the efficiency of cell transfection reached more than 70%.

**Cell migration**

Cells were plated in the upper insert (1×10^5 cells/well) of a 24-well chamber in serum-free medium. We added 600 μL medium containing 30% FBS to the lower insert. The cells were placed in a humidified atmosphere at 37°C for 20 h. The cells were removed from the upper surface, after which the membrane was stained with 2–3 drops of Giemsa (Sigma, USA) for 3–5 min. Migrated cells were photographed and counted by light microscopy (XDS-100, Caikon, Shanghai, China).

**Cell invasion**

The Transwell inserts were pre-coated with Matrigel matrix (Corning, USA). Cells in serum-free medium were plated in the upper insert (1×10^5 cells/well), while 750 μL medium containing 30% FBS was added to the lower insert. The cells were placed in a humidified atmosphere at 37°C for 24 h. The cells were removed from the upper surface, after which the membrane was stained with 2–3 drops Giemsa (Sigma, USA) for 3–5 min. Invasive cells were photographed and analyzed by light microscopy (XDS-100, Caikon, Shanghai, China).

**Western blot analysis**

Total proteins from cells infected with the Cbl-b shRNA lentiviral vector (KD group) or the negative control (NC group) were extracted using 2×Lysis Buffer (100 mM Tris-HCl (PH6.8), 2% mercaptoethanol, 20% glycerol, and 4% sodium dodecyl sulfate (SDS) on ice for 10–15 min. Protein concentrations were measured using a Nano-Drop instrument (ND-1000, Thermo Fisher Scientific). Total proteins (50 μg) were separated by 10% SDS-PAGE at 120 mA for 1 h, followed by transfer to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies against mTOR (#2983, 1:1000, Cell Signaling Technology, USA), p-mTOR (#2974, 1:1000, Cell Signaling Technology, USA), ERK1/2 (#9107, 1:1000, Cell Signaling Technology, USA), p-ERK1/2 (#4376, 1:1000, Cell Signaling Technology, USA), PI3K (ab32569, 1:500, Abcam, USA), p-PI3K (sc2931157, 1:1000, Santa Cruz, USA), AKT (#9272, 1:1000, Cell Signaling Technology, USA), p-AKT (#4060, 1:2000, Cell Signaling Technology, USA), GSK3β (#9325, 1:1000, Cell Signaling Technology, USA), p-GSK3β (#5538, 1:1000, Cell Signaling Technology, USA), Cbl-b (sc-376409, 1:200, Santa Cruz, USA), and GAPDH (sc-32233, 1:2000, Santa Cruz, USA) at 4°C overnight. Subsequently, the membranes were washed 4 times with TBST, and incubated with horseradish peroxidase-labeled secondary antibodies (sc-2005, 1:200, Santa Cruz, USA) at room temperature for 30 min. The bands were visualized using enhanced chemiluminescence (Beyotime Biotechnology, China), photographed, and analyzed using a gel imaging processing system (Gene Genius, Syngene, UK).

**Table 1.** Associations between clinical characteristics and overall survival in 1002 NSCLC patients from the TCGA database.

| Clinical characteristics | N(%) | Median OS (years) | HR | 95% CI          | P      |
|--------------------------|------|------------------|----|----------------|--------|
| Ages                     |      |                  |    |                |        |
| <65 years                | 418  (41.7) | 4.73             | 1.00 |                |        |
| ≥65 years                | 584  (58.3) | 3.91             | 1.32 | 1.07–1.62      | 0.009  |
| Sex                      |      |                  |    |                |        |
| Female                   | 403  (40.2) | 4.10             | 1.00 |                |        |
| Male                     | 599  (59.8) | 4.19             | 1.13 | 0.92–1.39      | 0.246  |
| Stage                    |      |                  |    |                |        |
| Stage I and II           | 801  (79.9) | 4.76             | 1.00 |                |        |
| Stage III and IV         | 201  (20.1) | 2.41             | 1.99 | 1.59–2.48      | 0.0001 |
| Histopathology           |      |                  |    |                |        |
| Adenocarcinoma           | 505  (50.4) | 4.09             | 1.00 |                |        |
| Squamous carcinoma       | 497  (49.6) | 4.54             | 1.09 | 0.89–1.33      | 0.424  |
Statistical analysis

The results are expressed as mean ±SD. Statistical analysis was performed using SAS 9.4 (SAS Inc., USA). Differences were analyzed using the t test and Fisher exact test. Kaplan Meier method and log-rank test were used to evaluate the correlation between Cbl-b expression and overall survival (OS). Survival data were evaluated by single or multivariate Cox regression analyses. * P<0.05 was considered statistically significant.

Results

Clinical analysis of Cbl-b expression in NSCLC

As shown in Table 1, data from 1002 NSCLC patients were obtained from the TCGA database. The Cbl-b high-expression group and Cbl-b low-expression group were divided by 25% or 50% of Cbl-b mRNA expression. There was no significant association between the Cbl-b mRNA expression and OS in the general population (Figure 1A, 1B), and there was no significant difference between Cbl-b mRNA expression and OS in lung adenocarcinoma (Figure 1C, 1D). In patients (age ≥65 years) with...
squamous carcinoma, the prognosis of the Cbl-b high-expression group was significantly worse than in the Cbl-b low-expression group (Figure 1E, 1F). These results may provide a clinical basis to investigate the biological behavioral differences between adenocarcinoma and squamous carcinoma in NSCLC.

Transfection efficiency

A549, H1975, H1299, and SW900 cells were transfected with lentivirus-mediated RNA interference vector. At 72 h after transfection, GFP expression was observed and photographed under a fluorescence microscope, and Western blot analysis was performed to measure the expression of Cbl-b protein. As shown in Figure 2, the expression of Cbl-b protein in the A549-KD group was markedly downregulated and the expression of Cbl-b protein in H1975-KD and SW900-KD groups was slightly downregulated, compared with their negative control (A549-NC, H1975-NC, or SW900-NC, respectively) groups. There was no significant difference in the expression of Cbl-b protein between H1299-KD and H1299-NC groups. Thus, cell migration and invasion assays were performed in A549, H1975, and SW900 cells.
Figure 1. The correlation analysis of Cbl-b mRNA expression and OS. (A) The correlation analysis of Cbl-b mRNA expression and OS in NSCLC (cutoff value: 25% of Cbl-b mRNA expression). (B) The correlation analysis of Cbl-b mRNA expression and OS in NSCLC (cutoff value: 50% of Cbl-b mRNA expression). (C) The correlation analysis of Cbl-b mRNA expression and OS in lung adenocarcinoma (cutoff value: 25% of Cbl-b mRNA expression). (D) The correlation analysis of Cbl-b mRNA expression and OS in lung adenocarcinoma (cutoff value: 50% of Cbl-b mRNA expression). (E) The correlation analysis of Cbl-b mRNA expression and OS in lung squamous cell carcinoma (cutoff value: 25% of Cbl-b mRNA expression). (F) The correlation analysis of Cbl-b mRNA expression and OS in lung squamous cell carcinoma (cutoff value: 50% of Cbl-b mRNA expression).

- a: Total; b: Age<65 years; c: Age ≥65 years; d: Stage I–II; e: Stage III–IV.
Cbl-b shRNA promotes the migration of A549 and H1975 cells

Transwell assay was performed to explore the migration of A549, H1975, and SW900 cells. There were 259±5 migrating cells in the A549-NC group and 475±10 in the A549-KD group. The migration ability of A549 cells improved by 84±4%. There were 75±1 migrating cells in the H1975-NC group and 133±9 in the H1975-KD group. The migration ability of H1975 cells improved by 77±12%. However, there were 22±1 migrating cells in both the SW900-NC and SW900-KD groups. There was no significant difference in cell migration between SW900-NC and SW900-KD groups. These results show that Cbl-b shRNA promotes the migration of A549 and H1975 cells, but not SW900 cells (Figure 3).

Cbl-b shRNA promotes the invasion of A549 and H1975 cells and inhibits the invasion of SW900 cells

Transwell assay was performed to assess the invasion of A549, H1975, and SW900 cells. There were 178±12 invading cells in the A549-NC group and 278±13 in the A549-KD group. The invasion ability of A549 cells improved by 56±4%. There were 23±2 invading cells in the H1975-NC group and 75±3 in the H1975-KD group. The invasion ability of H1975 cells improved by 323±12%. However, there were 15±1 invading cells in the SW900-NC group and 12±0 in the SW900-KD group. The invasion ability of H1975 cells declined by 24%. These results show that Cbl-b shRNA promotes the invasion of A549 and H1975 cells, but inhibits the invasion of SW900 cells (Figure 4).

Cbl-b regulates PI3K-ERK1/2 pathway in A549 and SW900 cells

To investigate the role of Cbl-b on the PI3K-ERK1/2 pathway in lung squamous cell carcinoma and adenocarcinoma, Western blot analysis was performed to measure the relative protein expression in A549 and SW900 cells transfected with Cbl-b shRNA. As shown in Figure 5, compared with the A549-NC group, the expression levels of PI3K, p-AKT, and GSK3β protein in the A549-KD group were downregulated, but the expression levels of p-PI3K, p-ERK1/2, mTOR, and p-GSK3β protein were upregulated. There was no significant difference in the expression levels of AKT, ERK1/2, and p-mTOR protein between the A549-NC and A549-KD groups. Compared with the SW900-NC group, the expression levels of PI3K and GSK3β protein in the SW900-KD group were downregulated, but the expression levels of p-PI3K, p-AKT, p-ERK1/2, mTOR, p-mTOR, and p-GSK3β protein were upregulated. There was no significant difference in the expression levels of AKT and ERK1/2 protein between the SW900-NC and SW900-KD groups.

Discussion

Lung adenocarcinoma and squamous cell carcinoma are 2 common types of NSCLC. Nakamura et al. found that the significant postoperative prognostic factors are quite different between lung adenocarcinoma and squamous cell carcinoma [20]. In the present study, TCGA analysis was performed to investigate the correlation between Cbl-b mRNA and OS in NSCLC patients. The results showed that the prognostic significance of Cbl-b mRNA low expression in lung adenocarcinoma and squamous cell carcinoma was different, suggesting that the biological functions of Cbl-b in lung adenocarcinoma and squamous cell carcinoma are different. Subsequently, a lentivirus-mediated RNA interference vector was constructed and transfected into 3 lung adenocarcinoma cell lines and 1 lung squamous carcinoma cell line. The results of Western blot analysis showed that the expression levels of Cbl-b protein were markedly downregulated in the A549-KD group, and were slightly downregulated in the H1975-KD and SW900-KD groups. Compared with the negative groups, the migration and invasion ability of A549-KD and H1975-KD groups were enhanced, while the invasion ability in the SW900-KD group was weakened. In addition, the results of Western blot analysis in A549 and SW9000 cells indicated that Cbl-b mediates cell migration and invasion via the PI3K and ERK1/2 signaling pathways differently, especially in the levels of p-AKT and p-mTOR expression.
In mammals, Cbl family proteins, consisting of Cbl, Cbl-b, and Cbl-c/Cbl-3, are highly conserved E3 ubiquitin ligases, with high structural similarity. They contain an N-terminal tyrosine kinase-binding domain, a short linker region, and a Ring finger domain [21]. The tyrosine kinase-binding domain connects with homologous phosphotyrosine protein sequences [22,23] and the Ring finger domain connects with ubiquitin conjugating enzymes (E2) [24,25]; therefore, Cbl family proteins can mediate the transfer of ubiquitin from E2 to signal molecules activated by the tyrosine kinase regulatory pathway [26–28].

Previous studies have confirmed that Cbl family proteins play an indispensable role in the degradation of activated tyrosine kinase receptors [29,30]. Cbl family proteins have been extensively studied in cancer drug resistance. Zhang Ye et al. found that Cbl-b suppressed P-gp transporter function by inhibiting its translocation into caveolae in multiple-drug-resistant gastric and breast cancers [31]. In breast cancer cells, downregulation of c-Cbl and Cbl-b led to activation of AKT and ERK, thereby promoting cell migration [32]. Gastric cancer patients with low Cbl-b expression were more likely to have tumor invasion and lymph node metastasis, and upregulation of Cbl-b inhibited cell migration in vitro and in vivo through inhibition of the EGFR-ERK/AKT-miR-200c-ZEB1 axis [33]. Another study also indicated that silencing Cbl-b expression in breast cancer cells enhanced the risk of lung metastasis in nude mice, and also found that Cbl-b can reduce RANK protein expression and

**P<0.01, ***P<0.001.

Figure 3. Effects of Cbl-b on the migration of A549, H1975, and SW900 cells. (A) There were more migrated cells in the A549-KD group than in the A549-NC group (200×). (B) There were more migrated cells in the H1975-KD group than in the H1975-NC group (200×). (C) The number of migrated cells in the SW900-KD group was similar to the number in the SW900-NC group (200×).
inhibited RANKL-induced breast cancer cells migration through negative regulation of the Src-AKT/ERK pathway [19]. In the present study, we found that Cbl-b shRNA promoted cell migration and invasion of A549 and mediated the PI3K-ERK1/2 pathways, which may help to further elucidate of the downstream signaling pathway. Cell migration and invasion of H1975 and SW900 cells were observed after transfection, showing that the invasion ability of lung adenocarcinoma cells was enhanced, but the invasion ability of lung squamous cell carcinoma was weakened. These data suggest that Cbl-b has different biological functions in lung adenocarcinoma and squamous cell carcinoma, which needs further study.

The PI3K-AKT signaling pathway plays an important role in regulating cell proliferation and cell survival. In many cancers, the PI3K/AKT-mTOR signaling pathway is overactivated, and some mTOR inhibitors have been used in clinical anticancer treatment [34,35]. Mutations, deletions, amplification, methylation, and post-translational regulation contribute to the dysregulation of this signaling pathway. Junjie Piao et al. studied the efficacy of co-treatment with the dual PI3K/mTOR inhibitor BEZ235 and histone deacetylase inhibitor Trichostatin A in NSCLC cells, which was found to inhibit cell proliferation, migration, and invasion, and promote cell apoptosis via down-regulating the expression of p-AKT and GSK-3β [36]. mTOR, an important regulator of cell proliferation, forms 2 different multiprotein complexes: mTORC1 and mTORC2 [37,38]. mTORC1 is
sensitive to rapamycin and can be activated by various stimuli, such as nutrients, growth factors, and stress signals. It is an important downstream protein of essential signaling pathways, such as PI3K and MAPK, for controlling cell proliferation and survival [39,40]. mTORC2 is resistant to rapamycin and regulates the actin cytoskeleton. A recent study showed that mTORC2 can activate insulin-like growth factor I receptor and insulin receptor through tyrosine kinase activity [41]. In human hepatocellular carcinoma, PTEN loss and overexpression of p-AKT and p-mTOR were correlated with TNM stage, vascular invasion, intrahepatic metastasis, tumor grade, and Ki-67 high expression, and PTEN loss was associated with p-AKT, p-mTOR, and MMP-9 overexpression [42]. In prostate cancer cells, knockdown of CPAN2 level suppressed cell migration and invasion ability by reducing MMP-2 and MMP-9 activation, and also repressed the protein expression of p-AKT and...
p-mTOR [43]. Cbl/Cbl-b double knockout in mammary epithelial organoids leads to activation of AKT-mTOR signaling [44]. Animal experiments also showed that c-Cbl-deficient mice had elevated ductal density and branching [45], and Cbl-c overexpression delayed breast ductal dilatation [46]. The findings indicate that deficiency of the Cbl family protein may affect mammary ductal epithelial growth. Chandrani et al. found that Cbl triple-deficiency blocked DNA synthesis, inducing cell death in mouse mammary epithelial cells, and showed that Cbl family proteins are indispensable for cell proliferation. They also found that phosphorylation-EGFR endured up to 30 min after EGF incentive in Cbl triple-deficient mammary epithelial cells, but reached its peak at 5 min after stimulation and decreased quickly to the WT cell level; the mild upregulation of subsequent signaling molecules, such as ERK and AKT, suggests that these biochemical actions can be disconnected from cell proliferation [47]. In the present study, lentiviral-mediated RNAi promoted the invasion and metastasis of A549 cells, and upregulated the expression of p-ERK and mTOR, which may agree with the conclusions reported by Chandrani et al. The present study shows that the regulation of p-ERK and mTOR might be independent of the classic MAPK signaling pathway activation that induced cell proliferation. Hence, Cbl-b may play different biological functions in breast cancer cells and lung cancer cell lines.

Many studies have shown that the PI3K pathway is involved in regulating cancer cell migration. Wenjun Wang et al. discovered that LMO4 overexpression promoted the migration of A549 cells and markedly upregulated the expression of p-PI3K and p-AKT; AKT inhibitor LY294002 could reverse the enhancement of migration of p-AKT and upregulation of p-PI3K expression induced by LMO4 [48]. Lin et al. found that aeroallergen Der p 2 promoted the migration and invasion of human NSCLC cells via the FAK and MAPK pathways, activated PI3K, AKT, ERK1/2, p38, and JNK, and upregulated the expression of MMP-2 [49]. GSK3β, as a tumor-suppressor gene in skin cancer and breast cancer, is involved in the transfer of cancer cells, and it can play an critical role in EMT by regulating Wnt and Snail pathways, and inhibition of GSK3β can promote the development of EMT [50]. GSK3β, as an oncogene, is highly expressed in ovarian cancer, colorectal cancer, and pancreatic cancer, and inhibition of GSK3β activity can inhibit cell proliferation and survival [51]. In summary, there are increases in A549 cell migration ability and activation of the molecules (p-PI3K, p-ERK1/2, and GSK3β), which were similar to the results of the present study.

Conclusions

The OS of Cbl-b mRNA low expression in lung adenocarcinoma and squamous cell carcinoma was shown to be different by TCGA analysis. The difference in signal pathways may be one of the reasons for the difference in the correlation between Cbl-b expression and the survival rate of these 2 pathological types of lung cancer.

Conflict of interest

None.

Supplementary Figure

Supplementary Figure 1. The map of plasmid vector for DNA transfection.
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