The deubiquitinase OTUD1 inhibits non-small cell lung cancer progression by deubiquitinating and stabilizing KLF4

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Abstract

Background: Lung cancer results in the highest mortality associated with cancer worldwide. Non-small cell cancer (NSCLC) is the leading subtype of lung cancer. Ovarian tumor protease (OTU) domain-containing protein 1 (OTUD1) is a member of the OTU subfamily of DUBs, and its function in NSCLC remains unclear.

Methods: GEPIA database was employed to reveal the expression level of OTUD1 in addition to Krüppel-like factor 4 (KLF4) in NSCLC tissue samples and prove the correlation between OTUD1 and KLF4. The protein level was estimated using western blot. Cell counting kit-8 (CCK-8) assay was used to detect cell viability and transwell assay was utilized to observe cell migration and invasion. Cycloheximide (CHX) was introduced to measure half-lives of KLF4 and deubiquitination assay was used to detect deubiquitination ability of OTUD1.

Results: OTUD1 expression was downregulated in NSCLC tissues and cells. Overexpression of OTUD1 inhibited NSCLC cell progression and it was promoted by knockdown of OTUD1. OTUD1 was positively correlated with KLF4 and stabilized KLF4 at protein level by deubiquitinating KLF4. Overexpressing KLF4 dramatically eliminated the effects of OTUD1 on the development of NSCLC cells.

Conclusions: Our study revealed that OTUD1 suppresses NSCLC progression by mediating KLF4 stabilization, which suggests a potential gene target for the future treatment of NSCLC.

Keywords: deubiquitinating, KLF4, non-small cell lung cancer, OTUD1

INTRODUCTION

Lung cancer is identified as the leading cancer contributing to the highest mortality related to cancer worldwide, with approximately 85% of these cases being non-small-cell lung cancer (NSCLC). The high death rate may due to diagnosis at a late stage and absence of effective treatment for patients with advanced lung cancer. The 5-year survival rate for patients with early lung cancer has been reported to be about 70% while it is no more than 20% for those diagnosed with advanced lung cancer. The development of NSCLC is affected by living conditions, and genetic factors in addition to smoking and gene mediation have been proven to be involved in the occurrence of NSCLC. Therefore, the discovery of novel target molecules is vital for exploring effective NSCLC treatments.

Ubiquitination is a universal and reversible biological process in which ubiquitin covalently binds to the target molecule by introducing the following three enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) as well as ubiquitin ligases (E3s). Ubiquitination is involved in a variety of diseases including diverse cancers and is regarded as a potential therapeutic target. The ubiquitination of proteins is changed and reversed by members of the deubiquitinase (DUB) family. Previous studies have found that about 100 DUBs are...
transcribed and translated from the human genome. DUBs are commonly classified into seven subtypes and play essential functions in diverse biological activities by mediating various substrates. Ovarian tumor protease (OUT) family as a crucial subfamily of DUBs is divided into four OTU subtypes including OTUB, OTUD, OTULIN and A20-like subtypes, which exert important roles in regulating signaling cascades. For example, A20 and OTUD7B in addition to OTULIN have been revealed to be associated with the NF-κB signaling pathway. OTUD2 and VCPIP modulate the p97-related pathway.

OTU domain containing protein 1 (OTUD1) is a member of the OTUD subfamily. It is composed of 481 amino acids and is associated with various physiological functions which influence different molecules. Zhang et al. revealed that OTUD1 inhibited breast cancer progression by deubiquitinating SMAD7 and then repressed TGF-β-induced tumorigenesis. Grattarola et al. found that reducing OTUD1 expression enhanced the sensitivity of pancreatic ductal adenocarcinoma to chemotherapy drugs by down-regulating the content of Nrf2 and YAP. Carneiro et al. proved that OTUD1 was differentially expressed in thyroid and might be the promising therapeutic target for treatment of thyroid cancer. Additionally, OTUD1 has been discovered to be related with improved prognosis of patients with NSCLC. Nevertheless, whether and how OTUD1 affects the development of NSCLC is at present unclear.

Krüppel -like factor 4 (KLF4) is a vital member of the KLF-like factor family containing a zinc finger domain and exerts diverse effects in normal progression as well as carcinogenesis. KLF4 has been investigated and found to be both an oncogene and tumor suppressor in different cancers. In lung cancer, recent studies have confirmed that KLF4 suppressed lung tumorigenesis by mediating the cell cycle. It has been reported that KLF4 is always modulated through post-translational modifications (PTMs) including ubiquitination. Despite the fact that multiple E3 ligases have been reported to be involved in KLF4 polyubiquitination, more information on DUBs related with KLF4 is required.

In our study, we reveal that OTUD1 was downregulated in NSCLC and inhibited the progression of NSCLC cells. Additionally, OTUD1 was discovered to deubiquitinate and stabilize KLF4. Thus, for the first time, we expounded that OUTD1 suppressed NSCLC development by stabilizing KLF4 proteins, which provides a promising therapeutic target for the future treatment of NSCLC patients.

METHODS

NSCLC and paracarcinoma tissues

The NSCLC patients were diagnosed in Weifang People’s Hospital. All patients were in agreement that their tissue samples could be used in this study and signed the informed consent form. The tissues obtained from patients were immediately frozen in liquid nitrogen for further analysis. This research was approved by the Ethical Committee of Weifang People’s Hospital.

Cell culture

The human bronchial epithelial cell line (HBE) and NSCLC cell lines (A549, PC9, H1299, Calu-3) were purchased from Sunnecell and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (11875101, Gibco) containing 10% fetal bovine serum (FBS, 10091155, Gibco) and 1% penicillin, as well as streptomycin (15140163, Gibco). They were maintained in a 37°C atmosphere containing 5% CO2.

Cell counting kit-8 (CCK-8) assay

PC9 or A549 cells transfected with specific vectors were cultured in 96-well plates (1 × 104 cells/well) for 0, 1, 2, 3 and 4 days respectively. Then, 10 μl CCK-8 reagent (G100001, GipBio, USA) was added into the relevant wells and incubated with cells for 1 h at 37°C. Finally, the absorbance value at 450 nm was recorded using a SpectraMax iD5-multifunctional microplate reader (China).

Transwell assay

The migrated and invasive abilities of NSCLC cells were detected as described in a previous study. Briefly, for migration assay, PC9 or A549 cells (6 × 104) transfected with indicated vectors were seeded in the upper section of the chamber (CLS3396-2EA, Merck) with noncoated membrane. The lower section was prepared with 500 μl RPMI-1640 medium containing 20% FBS. The chamber was incubated at 37°C for 48 h and the upper membrane was gently removed. Then, 0.1% crystal violet was used to stain the cells transferred into the lower chamber. For the invasion assay, the upper chamber was precoated with matrigel components and the other steps are the same as those used to detect cell migration. The procedure followed was the same as that from a previous study.

Plasmids and shRNA

Full-length human OTUD1 cDNA sequence was cloned into pcDNA3.1-Myc vector (#31698, Addgene), full-length human KLF4 cDNA was cloned into pcDNA-3 × Flag vector (#24484, Addgene) and ubiquitination (Ub) was cloned into pCMV4-HA vector (#27553, Addgene). Sh-OTUD1 (sh-1#: GCGAGATCTGCTGAATGTGAATAT, sh-2#: GCAGATGCTGAATGTGAATAT, sh-3#: GCAGATGCTGAATGTGAATAT, sh-4#: GCAGATGCTGAATGTGAATAT) was cloned into pLKO.1 vector (#13425, Addgene). All these vectors were transfected into cells by using lipofectamine 3000 (L3000075, Invitrogen) according to the manufacturer’s instructions.
Real-time RT-PCR (qRT-PCR)

QRT-PCR analysis was conducted as described in a previous study. Briefly, total RNAs were collected using TRIzol reagent (15596018, Invitrogen). Then, 2 μg RNA was employed to be reverse-transcribed into cDNA with Rt-pcr first strand cDNA synthesis kit (11483188001, Roche). RT-PCR was performed using PowerUp SYBR Green premix (A25742, Applied Biosystems) and ABI 7500 quantitative PCR instrument (Applied Biosystems). The expression level of all genes were normalized with GAPDH. The following primers were used in this section: KLF4-sense-5'-CGGA CCACCTCGCCCTACA-3', KLF4-antisense-CTGGGCTCC TTCCCTCATCG-3'; β-actin-sense-5'-CACCTTCTACAAATG AGCTGCCGTGTG-3', β-actin-antisense-5'-ATAGCACAG CCTGGATAGCAACGTAC-3'.

Western blot analysis

Total proteins were isolated from cells transfected with the indicated vectors by using RIPA buffer containing protease inhibitor cocktail (P8340-1ML, Sigma). A BCA protein assay kit (71285-3, Millipore) was employed to measure the protein concentration. Immunoblot analysis was conducted with primary antibodies anti-OTUD1 (ab122481, Abcam, 1:1000), anti-Flag (F3165, MilliporeSigma, 1:1000), anti-KLF4 (4038, Cell Signaling Technology, 1:1000), anti-Myc (sc-40, Santa Cruz Biotechnology, 1:1000) or anti-ubiquitin (sc-8017, Santa Cruz Biotechnology. 1:1000). After washing using phosphate buffer saline (PBS), the membranes were incubated with Rabbit anti-Mouse IgG (H+L) secondary antibody, HRP (31 450, Invitrogen, 1:5000) or anti-ubiquitin (sc-8017, Santa Cruz Biotechnology. 1:1000). After washing using phosphate buffer saline (PBS), the membranes were incubated with Rabbit anti-Mouse IgG (H + L) secondary antibody, HRP (31 450, Invitrogen, 1:5000) or Goat anti-Rabbit IgG (H + L) secondary antibody, HRP (31 460, Invitrogen, 1:5000). Finally, the signals of western blot analysis were observed using Immobilon ECL Ultra Western HRP Substrate (WBULS0100, Millipore).32

Cycloheximide (CHX) chase assay

Cells transfected with indicated vectors were seeded in 6-well plates (1 × 10⁵ cells/well) and pretreated with CHX (100 μg/ml) for 0–9 h. The cells were then harvested at the indicated time for subsequent western blot analysis as described above.

Immunoprecipitation analysis

Immunoprecipitation analysis was conducted as described in a previous study. Cells transfected with specific vectors using Nonidet P-40 (NP-40) lysis buffer containing protease inhibitors cocktail (P8340-1ML, Sigma). The lysates were centrifuged and supplemented with primary antibodies anti-Flag (F3165, MilliporeSigma, 1:500) or anti-Myc (sc-40, Santa Cruz Biotechnology, 1:500) and

Ubiquitination assay

Cells were harvested using 100 μl 1% SDS lysis buffer and heat-denatured for 10 min. Then, cell lysates were diluted in 900 μl NP-40 lysis buffer containing protease inhibitors cocktail (P8340-1ML, Sigma) and 20 mM N-ethylmaleimide (E3876-5G, Sigma). The mixture was then sonicated and centrifuged, followed by immunoprecipitation and western blot analysis as described above. The detailed protocols were in accordance with those reported in a previous study.34

Statistical analysis

All data are presented as mean ± standard deviation and were analyzed by employing an unpaired Student’s t-test. p-values < 0.01 were considered statistically significant. GraphPad Prism 6 was used to illustrate the graphs in this study.
RESULTS

OTUD1 expression is downregulated in NSCLC tissues and cell lines

First, we analyzed the data from GEPIA database and found that OTUD1 expression was obviously lower in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) samples (Figure 1a), which was further proven by detecting the OTUD1 protein level expressed in NSCLC tissues and paracarcinoma tissues of six patients (Figure 1b). Moreover, the protein level of OTUD1 in human bronchial epithelial cell line (HBE) and NSCLC cell lines including A549, PC9, H1299 plus Calu-3 was evaluated. The results dramatically illustrated lower OTUD1 expression in NSCLC cell lines compared with the normal cell line (Figure 1c). We then chose PC9 and A549 cells to perform the following exploration. Collectively, the findings demonstrated that OTUD1 expression was lower in NSCLC tissues and cell lines than in the normal cell line.

OTUD1 overexpression inhibits NSCLC cell proliferation, migration and invasion

To explore the roles of OTUD1 in NSCLC cells, the vectors overexpressing OTUD1 were transfected into PC9 and A549 cells. Figure 2a shows the successful establishment of overexpressed OTUD1 in both PC9 and A549 cell lines (Figure 2a). A CCK-8 assay was performed to prove that overexpression of OTUD1 significantly suppressed cell growth ($p < 0.01$, Figure 2b). Additionally, the data from the
transwell assay further verified that overexpressed OTUD1 remarkably inhibited the migration as well as invasion of PC9 and A549 cells ($p < 0.01$, Figure 2c,d). Taken together, these results suggested that overexpression OTUD1 notably inhibits NSCLC cell progression including proliferation, migration and invasion.

**OTUD1 knockdown promotes NSCLC cell proliferation, migration and invasion**

Two sh-RNAs were subsequently employed to silence OTUD1 in PC9 and A549 cells, and sh-RNA efficiency was measured by western blot analysis (Figure 3a). Additionally, sh-OTUD1-2# exhibited better efficiency in both PC9 and A549 cells, so we determined PC9-sh-OTUD1-2# cell line and A549-sh-OTUD1-2# cell line to conduct the next assay. CCK-8 assay showed that OTUD1 knockdown dramatically upregulated the cell viability of PC9 and A549 cells ($p < 0.01$, Figure 3b). Transwell assay demonstrated that silencing OTUD1 significantly accelerated PC9 and A549 cell migration and invasion ($p < 0.01$, Figure 3c,d). All results proved that knockdown of OTUD1 obviously facilitated the development of NSCLC cells.

**OTUD1 is positively correlated with KLF4 and stabilizes the KLF4 protein**

In this investigation, we screened related genes of OTUD1 involved in NSCLC samples using the GEPIA database and KLF4 attracted our attention. Additionally, we found an obvious positive correlation between OTUD1 and KLF4 in NSCLC samples (Figure 4a). The expression profile from the GEPIA database also showed that KLF4 expression was significantly inhibited in LUAD and LUSC tissues.
We then employed PC9-OTUD1 cells and PC9-sh-OTUD1-2# cells to prove the relationship between OTUD1 and KLF4. The results illustrated that OTUD1 overexpression increased KLF4 protein levels while OTUD1 knockdown remarkably decreased the protein load of KLF4. However, OTUD1 did not significantly affect KLF4 mRNA expression in PC9 cells (Figure 4c,d), implying that OTUD1 might mediate the post-translational modification of KLF4. Taken together, these data demonstrated that OTUD1 clearly stabilized KLF4 at protein level.

OTUD1 stabilizes KLF4 by deubiquitinating KLF4

To explore how OTUD1 stabilizes the KLF4 protein, we overexpressed or silenced OTUD1 in NSCLC cells to detect the half-lives of KLF4 protein with cycloheximide (CHX). As expected, overexpression of OTUD1 significantly extended KLF4 half-lives compared with control group (Figure 5a,b). Nevertheless, knockdown of OTUD1 dramatically reduced the KLF4 half-lives (Figure 5c,d), which suggested that OTUD1 reduced the degradation of KLF4. To further determine the detailed mechanisms by which OTUD1 stabilizes KLF4, we explored the effects of OTUD1 on KLF4 ubiquitination. We transfected hemagglutinin (HA)-Ub, OTUD1-Myc and KLF4-3×flag into 293T cells, followed by introduction of the proteasome inhibitor MG132 to treat cells for preventing protein degradation. As shown in Figure 5e, OTUD1 overexpression notably repressed the polyubiquitination of KLF4 protein (Figure 5e). These findings suggest that OTUD1 deubiquitinated KLF4, thereby inhibiting KLF4 protein degradation.

KLF4 overexpression blocks sh-OTUD1-increased NSCLC cell progression

To further confirm whether OTUD1 exerts its inhibitory roles in NSCLC cells by stabilizing KLF4, we
detected proliferation, migration and invasion ability of PC9 cells transfected with sh-OTUD1-2# and KLF4 was overexpressed. We successfully established that the PC9 cell line stably expressed KLF4 (Figure 6a). A CCK-8 assay was employed to illustrate that overexpression of KLF4 effectively reduced cell viability increased by knockdown of OTUD1 ($p < 0.01$, Figure 6b). Furthermore, the results obtained from the transwell assay proved that KLF4 overexpression obviously counteracted the upregulated effects of silencing OTUD1 on migration and invasion ability of PC9 cells ($p < 0.01$, Figure 6c,d).

Thus, these investigations verified that OTUD1 affected NSCLC cell progression, at least in part by modulating KLF4.

**DISCUSSION**

DUBs have recently been shown to be involved in various cancers including prostate cancer, ovarian cancer, glioblastoma and NSCLC. In our study, we verified that deubiquitinating enzyme OTUD1 was downregulated in NSCLC tissues and cells. Overexpressing OTUD1 significantly inhibited NSCLC cell proliferation, migration and invasion, whereas silencing OTUD1 dramatically promoted the progression of NSCLC cells. Moreover, OTUD1 has been shown to be the potent DUB of KLF4 and stabilizes KLF4 at protein level by deubiquitinating KLF4. Furthermore, overexpression of KLF4 effectively eliminated the effect of OTUD1 knockdown on NSCLC cell progression as expected.
OTUD1 as a type of cysteine protease containing OTU domain is capable of hydrolyzing the cleavage of polyubiquitin chains. Previous studies have revealed that OTUD1 exerted vital roles in a variety of biological activities including enhancing host antitumor immunity, inhibiting innate immune response and regulating cell death. Moreover, it has been proven that OTUD1 is closely with various cancers as a DUB of different substrates. However, the detailed functions and mechanisms of OTUD1 on NSCLC are still unclear. In this investigation, the GEPIA database illustrated low OTUD1 expression in LUAD and LUSC tissues compared with normal tissues. We then detected protein level of OTUD1 in NSCLC tissues and paracarcinoma tissues collected from 15 patients with NSCLC and the results confirmed the above described statistics, consistent with the findings in a recent study. Additionally, the gain- and loss-function experiments verified that OTUD1 was able to inhibit NSCLC cell progression such as cell growth, migration and invasion. It is the first study to expound the detailed effects of OTUD1 on the development of NSCLC cells.

For exploring how OTUD1 affect NSCLC cells, we screened a gene set highly related to OTUD1 in NSCLC samples using the GEPIA database and pinpointed a common tumor suppressor, KLF4. KLF4 is regarded as a transcription factor involved in mediating cell proliferation, differentiation and other cellular processes. Further studies have revealed that KLF4 could inhibit tumor progression in lymphoma, cervical cancer and neuroblastoma. In NSCLC, it is reported that KLF4 overexpression represses NSCLC development. We consistently discovered that KLF4 was downregulated in NSCLC tissues. A previous study has identified USP10 as the DUB of KLF4 in NSCLC while other potent DUBs of KLF4 participating in regulating NSCLC process remain undiscovered. Our present study elucidated that OTUD1 was positively correlated with KLF4 in NSCLC tissues. Although the data from the database suggested that OTUD1 may also affect KLF4 mRNA levels, we found that overexpressing OTUD1 stabilized KLF4, content at protein level not at mRNA level remarkably in PC9 cells, indicating that OTUD1 might modulate the post-translational level of KLF4. Nevertheless, whether OTUD1 affects KLF4 mRNA levels in animal and clinical models needs further study. OTUD1 as a member of deubiquitination enzymes is able to remove polyubiquitins from its target molecule such as p53 and MCL1. We confirmed that OTUD1 obviously extended half-lives of KLF4 and inhibited KLF4 ubiquitination level. Furthermore, functional experiments proved that overexpression of KLF4 effectively repressed the proliferation, migration and invasion ability of PC9 cells increased by silencing OTUD1.

Some limitations exist in this study. For example, we did not verify whether OTUD1 interacts with KLF4 directly and confirm the ubiquitination site of KLF4. Moreover, more studies need to be performed to determine whether OTUD1 exerts consistent roles in mice models.

In conclusion, this investigation for the first time has elucidated that OTUD1 inhibits NSCLC cell progression including cell growth, migration and invasion by stabilizing and ubiquitinating the tumor suppressor, KLF4. Furthermore, our study provides a promising therapeutic target for treating patients diagnosed with NSCLC.

CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

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FIGURE 6 KLF4 overexpression blocks sh-OTUD1-increased NSCLC cell progression. (a) PC9 cells were transfected with pcDNA3.1 vector or vector containing KLF4. Then the expression level of KLF4 was measured by western blot assay. (b) PC9 cells were transfected with pcDNA3.1 vector or vector containing KLF4. Cell proliferation was then measured by CCK-8 assay (p < 0.01). (c) PC9 cells were transfected with pcDNA3.1 vector or vector containing KLF4. Then the migration ability of PC9 cells was measured by transwell assay (p < 0.01). (d) PC9 cells were transfected with pcDNA3.1 vector or vector containing KLF4. Then the invasion ability of PC9 cells was measured by transwell assay (p < 0.01).
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