Down-regulation of lipocalin 2 suppresses the growth of human lung adenocarcinoma through oxidative stress involving Nrf2/HO-1 signaling

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Abstract
Lipocalin 2 (LCN2), a multifunctional secretory protein known as neutrophil gelatinase-associated lipocalin (NGAL), is expressed in a variety of cancers. However, little is known about the biological functions of NGAL in the development of lung adenocarcinoma. In the present study, we primarily found that NGAL expression was up-regulated in human lung adenocarcinoma tissues. Additionally, depletion of NGAL expression decreased the ability of cell proliferation and induced cell apoptosis. Furthermore, with the addition of N-acetylcysteine, a scavenger of reactive oxygen species (ROS), it was found that NGAL depletion was sufficient to cause apoptosis of lung adenocarcinoma cells by generating ROS through the inhibition of the nuclear factor E2-related factor 2/heme oxygenase-1 anti-oxidant pathway. Finally, the effect of NGAL down-regulation on the growth of human lung adenocarcinoma was determined in BALB/c nude mice. These findings demonstrate that NGAL may be a potential therapy target for patients with lung adenocarcinoma.

Key words: neutrophil gelatinase-associated lipocalin, lung adenocarcinoma, apoptosis, reactive oxygen species

Introduction
Lung cancer is the most common non-cutaneous cancer in the world and the number one cause of cancer death in males and the second in females. Approximately 80% of lung cancer patients are diagnosed with non-small-cell lung cancer [1,2]. Resistance to apoptosis enables tumor cells to escape the effects of anticancer drugs and natural effectors, rendering the effective treatment to be more difficult. The high mortality of lung cancer is attributed to its anti-apoptosis, especially for lung adenocarcinoma [3,4]. Therefore, further insight into the biological and molecular mechanisms of lung adenocarcinoma growth is significantly important and may facilitate the identification of new molecular targets for the treatment of the disease.

Lipocalin 2 (LCN2), known as neutrophil gelatinase-associated lipocalin (NGAL), is a 25-kDa secretory glycoprotein that belongs to the lipocalin family [5]. Soon after the discovery of LCN2 as a constituent of neutrophil-specific granules, LCN2 was found to be highly up-regulated in epithelial cells at sites of inflammation and to be highly expressed in acute ischemic renal injury [6–8]. Physiological functions of NGAL have been shown to involve in the modulation of immune response, regulation of cell growth and metabolism, fatty acid and iron transportation, and prostaglandin synthesis [9]. Growing evidence has demonstrated that elevated NGAL expression was detected in a variety of malignancies and involved in the invasion and progression of tumors, including breast cancer, thyroid carcinoma, hepatocellular carcinomas, etc. [10–13]. Thus, the elucidation of NGAL function will help to identify new strategies for the treatment of cancer.

It has been reported that NGAL acts as a cytoprotective factor against cellular stresses such as H2O2 and cisplatin toxicity, while it...
has been reported that NGAL might protect cells from reactive oxygen species (ROS) [14,15]. Recently, nuclear factor E2-related factor 2 (Nrf2) was found to be a critical transcription factor that could bind to anti-oxidant response elements present in the promoter region of a number of genes encoding anti-oxidant and phase II enzymes. Nrf2 was confirmed to protect cells in normal tissues from harmful stimuli such as inflammation, trauma, ischemia, hemorrhage, and cancer [16,17]. Heme oxygenase (HO)-1, an important Nrf2 target gene, catalyzes heme metabolism to yield iron, carbon monoxide, and bilirubin. Likewise, HO-1 was recognized as a protective gene in the kidney and involved in degradation of pro-oxidant heme, resulting in the production of anti-inflammatory, anti-oxidant, and anti-apoptotic metabolites [18–20]. However, the role and molecular mechanisms of NGAL in lung adenocarcinoma stress and proliferation still remain to be elucidated.

In the present study, we explored the role of NGAL in lung adenocarcinoma tumorigenesis and progression. It was shown that the NGAL expression was elevated in the majority of the human lung adenocarcinoma tissues. Specific knockdown of NGAL by RNA interference remarkably inhibited lung adenocarcinoma cell proliferation and induced cell apoptosis. Furthermore, in a mouse xenograft model of human lung adenocarcinoma, down-regulation of NGAL was demonstrated to reduce tumor growth and promote extensive cell death in vivo. Our results suggest that NGAL may be a potential therapy target for patients with lung adenocarcinoma.

Materials and Methods

Tissue sample collection

Human normal lung tissue samples and lung adenocarcinoma tissue samples were provided by the Department of Thoracic Surgery, The First Affiliated Hospital of Nanchang University (Nanchang, China). All experiments were approved by the ethics committee of Nanchang University and informed consents were obtained from all patients prior to specimen collection.

Immunohistochemistry

Immunohistochemical staining was done according to the manufacturer’s instructions. The sections were fixed in 10% neutral buffered formalin, paraffin-embedded, rehydrated routinely, and cut into 4 mm serial sections. Then, the sections were soaked in 3% H2O2–methanol for 5 min, washed three times in phosphate-buffered saline (PBS) (each 3 min). The sections were then blocked with 1% bovine serum albumin in PBS containing 10% normal serum for 10 min at 37°C. Slides were incubated with primary antibody against NGAL (at the dilution of 1:100, Abcam, Cambridge, UK) overnight at 4°C, and washed three times with PBS (each 5 min). The sections were then incubated with secondary antibodies for 30 min at room temperature and washed three times with PBS (each 10 min). For visualization, slides were incubated in the 3,3′-diaminobenzidine solution until desired staining was approached. Finally, the sections were stained by hematoxylin and observed under a microscope and the images were taken by a digital camera.

Cell culture

The human lung adenocarcinoma cell lines A549 and PC9 were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium (Gibco, Carlsbad, USA) containing 10% fetal bovine serum (FBS, HyClone, Logan, USA), 100 U/ml penicillin-streptomycin, at 37°C in a humidified atmosphere of 5% CO2.

Construction and infection of lentivirus vectors

The lentivirus vector corresponding to NGAL gene (NM_005564) was designed as follows: short hairpin RNA (shRNA) targeting NGAL is sequenced as 5′-CAATTTCTCAAGAGAAGAACAAGCTGGA GCCTTTGTTCCTCTGAGAATTTG-3′; an unrelated shRNA sequence with no homology to any human genes was used as a nonspecific shRNA control (shControl) is 5′-GATCCGTTCTCCGAAC GGTTGACGTGTCAAGAACGTGACGTGATT TTTGAAAA-3′. Then, these oligonucleotides were inserted into the pSilencer-U6-RFP vector. These lentiviral vectors expressing shRNA were all confirmed by DNA sequencing before being transfected into 293FT cells together with virus-packaging-related vectors psPAX2 and pMD2.G plasmids. Lentivirus was subsequently harvested at 72 h post-transfection, centrifuged to get supernatants, and then filtered before purification using ultracentrifugation. A549 and PC9 cells (5 × 104 cells/well) were seeded in 6-well plates and infected with NGAL–shRNA lentivirus or shControl lentivirus. After selection by puromycin, cells were collected and evaluated for the efficiency of infection.

Cell proliferation assay

Cell proliferation activity was assessed by using the cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). After infection for 72 h, cells (2 × 104 per well) were seeded in 200 μl culture medium into 96-well plates. Cell proliferation was assessed at indicated time points (0, 12, 24, 48, and 72 h) using the CCK-8. Briefly, 20 μl of CCK-8 was added into each well. After incubation for 1 h at 37°C, the absorbance of each plate was measured at 450 nm using a microplate reader.

 Colony formation assay

Approximately 200 cells were plated in 60 mm2 culture dishes. After 14 days, cells were washed with PBS, fixed in methanol and stained with 1% crystal violet for 30 min. Excess stain was removed with PBS. Stained colonies made up of more than 50 cells were scored as colony-forming units and were counted.

Cell apoptosis detection by flow cytometry

Lung cancer cells were cultured in the RPMI 1640 medium supplemented with 2% FBS, which favored apoptosis. Cells were cultured for 72 h and collected, then washed twice with PBS. The cells were resuspended in 500 μl binding buffer containing 5 μl Annexin V and 5 μl propidium iodide for 30 min at room temperature in the dark. After incubation, at least 1 × 104 cells were measured by a flow cytometer (BD Bio-science, San Diego, USA).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using Trizol® Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. cDNA was synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech China Co., Ltd., Beijing, China). Real-time polymerase chain reaction (qRT–PCR) was carried out with an ABI 7500 qRT–PCR system using SYBR Green mix (ABI, Foster City, USA). The qRT–PCR program was as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 60 s, and the dissociation curve was added. The fold change of gene expression
relative to the control was calculated by 2−ΔΔCt. The experiments were repeated three times and every sample was performed in triplicate. Primers used in the experiments are listed in Table 1.

Western blot analysis
Cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with phenylmethylsulfonyl fluoride (Invitrogen). Protein (30 μg) samples were then resolved by SDS–PAGE. Proteins were subsequently transferred to polyvinylidene fluoride membranes for immunoblotting. The primary antibodies used in this study including anti-Lipocalin (1:1000; Abcam), anti-Bax (1:1000; Cell Signaling, Beverly, USA), anti-Bcl-2 (1:500; Cell Signaling), anti-NRF2 (1:1000; Cell Signaling), anti-HO-1 (1:1000; Abcam). β-Actin (1:2000; Abcam) was used as a loading control and blotted protein bands were visualized by ECL detection reagent (ECL, Thermo Scientific, Waltham, USA).

Caspase-3 activity assay
Caspase-3 activity in each group was measured using the colorimetric activity assay (Beyotime Institute of Biotechnology) following the manufacturer’s instructions. In brief, cells were lysed on ice, the lysates were centrifuged at 16,000 g at 4°C for 15 min. Caspase-3 activity assays were performed in 96-well microtiter plates by incubating 10 μl of cell lysates in 80 μl of reaction buffer with 10 μl of caspase-3 substrate (Ac-DEVD-pNA) at 37°C for 2 h prior to colorimetric measurement of p-nitroanilide product at 405 nm.

ROS determination
ROS level was determined using Reactive Oxygen Species Assay Kit (DCFH-DA) (Beyotime Institute of Biotechnology). The cells (1 × 10⁶) were directly treated with 10 μM DCFH-DA at 37°C for 20 min. Cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan) and quantified by a BD FACS Aria flow cytometer.

Determination of superoxide dismutase-like activity
The levels of superoxide dismutase (SOD)-like activity in the extracts were measured using the SOD Assay Kit-WST (Dojindo) according to the manufacturer’s protocol. Briefly, in a 96-well microplate, 20 μl of sample solution was mixed with 200 μl of WST working solution. For blank well, 20 μl of dilution buffer was added. Then, 20 μl of enzyme working solution was added to each sample well and blank well. The plate was incubated at 37°C for 20 min and the OD was determined at 450 nm using a microplate reader.

Tumor formation in BALB/c nude mice
The athymic BALB/c nude mice (5–7 weeks old) were purchased from the Institute of Laboratory Animals, Chinese Academy of Medical Sciences (Beijing, China). Animal studies were approved by the IACUC of the Institute of Hematology & Hospital of Blood Diseases, PUMC. A549 cells, A549-shControl cells or A549-NGAL-shRNA cells (1 × 10⁷ cells) were suspended in 100 μl of PBS and injected into either flank of mice, respectively (n = 5). Tumor volumes were monitored every 5 days with vernier caliper and calculated as length x width²/2. The mice were killed on the 35th day after the inoculation.

Triphosphosphate nick end labeling staining
The DNA fragmentation was detected in situ using a terminal transferase-mediated biotinylated deoxyuridine triphosphosphate nick end labeling (TUNEL) kit (Roche, Penzberg, Germany) according to the manufacturer’s instructions. Briefly, sections were first dewaxed and dehydrated. Then, the sections were treated with proteinase K (Sigma) at 37°C for 8 min. After being rinsed in PBS three times at room temperature for 5 min, the sections were incubated with TUNEL reaction mixture at 37°C in the humidity chamber for 60 min. After three times rinse in PBS at room temperature for 5 min, the sections were placed in 50 μl FITC (Roche) and then incubated at 37°C for 40 min. After three times rinse in PBS for 5 min, the sections were dipped in DAB (Roche) at RT for 3 min, and the reaction was observed under a microscope. The reaction was terminated with distilled water and the nuclei were counterstained with hematoxylin buffer.

Table 1. Primer sequences of the target genes

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<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>NGAL</td>
<td>5′−CAAGGAGCTGACTCGGAAC-3′</td>
<td>5′−TCACATGGTCGATTGGAGACA-3′</td>
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<tr>
<td>BAX</td>
<td>5′−GACATTGGTTTTCTGACGCACAC-3′</td>
<td>5′−AAAGTCATGGTCGACTTGGC-3′</td>
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<tr>
<td>BCL-2</td>
<td>5′−TTCTTTGATGGTGGGCTG-3′</td>
<td>5′−TGCAATTGGTTTTGAGGGC-3′</td>
</tr>
<tr>
<td>NRF2</td>
<td>5′−TCAGCATGCGATCTCCCGAC-3′</td>
<td>5′−GCACTGCTGGTGGTAGAAGCC-3′</td>
</tr>
<tr>
<td>HO-1</td>
<td>5′−CGAGGGCAGAGAATGCTGATTTG-3′</td>
<td>5′−TGCAAGCTTTCTGAGGAGTACG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′−CGAGATTTGCGTATGTGGGC-3′</td>
<td>5′−CTTCCGGTCTAAGCGCTT-3′</td>
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Statistical analysis
All data are presented as mean ± SD. The differences between samples were analyzed using the two-tailed paired or unpaired t-test or one-way analysis of variance in GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). P < 0.05 was considered as statistically significant.

Results
NGAL was up-regulated in human lung adenocarcinoma
To investigate the potential role of NGAL in lung adenocarcinoma, we first examined the mRNA expression of NGAL in human primary lung adenocarcinoma tissues and normal lung tissues by qRT–PCR (n = 35). It was found that the lung adenocarcinoma tissues exhibited an up-regulated NGAL than normal lung tissues (Fig. 1A). IHC analysis demonstrated that the lung adenocarcinoma tissues are positive for NGAL staining. The NGAL protein appeared to be expressed in the cytoplasm and membrane as well as in the surrounding regions (Fig. 1B). Meanwhile, the normal lung tissues exhibited negative or low NGAL staining (Fig. 1B). These results indicated that both mRNA and protein expression of NGAL were up-regulated in lung adenocarcinoma tissues compared with those in normal lung tissues.
NGAL expression was down-regulated by shRNA in A549 and PC9 cells
To evaluate the effect of NGAL on the growth of lung adenocarcinoma in vitro, we constructed the stable NGAL-knockdown cells. A specific lentivirus-shRNA system (Lv-shNGAL) was designed and used to infect A549 and PC9 cells, respectively. In addition, to avoid the nonspecific gene-silencing effect of the lentivirus alone, an shControl was constructed and used to infect both cell lines. Then, we measured the NGAL expression at the mRNA and protein levels in the control, shControl, and NGAL-shRNA groups. The mRNA and protein levels of NGAL were greatly repressed in the NGAL-shRNA group (Fig. 2A,B). These data demonstrate the successful construction of stable NGAL-knockdown lung adenocarcinoma cells.

Knockdown of NGAL inhibited cell proliferation and induced apoptosis of lung adenocarcinoma cells in vitro
Next, to assess the potential effect of NGAL knockdown on the proliferation of lung adenocarcinoma cells, CCK-8 assay was performed. The results showed that the NGAL–shRNA group exhibited a remarkable inhibition of cell growth in both A549 and PC9 cells compared with the control and the shControl groups (Fig. 3A). Meanwhile, the CFU assay also revealed that the growth of A549 and PC9 cells was significantly inhibited in the NGAL–shRNA group (Fig. 3B). To further characterize the effect of down-regulation NGAL on the cells, we analyzed the cell apoptosis by flow cytometry. The results showed that the NGAL–shRNA-transfected A549 and PC9 cells had significant apoptosis compared with the control group and shControl group, whereas the apoptosis level was similar between the control and the shControl groups (Fig. 3C). Meanwhile, we found that knockdown of NGAL expression enhanced the expression of pro-apoptotic Bax and suppressed the expression of anti-apoptotic Bcl-2 (Fig. 3D) by western blot analysis. To test whether the apoptosis was associated with the activation of caspase-3, caspase-3 activity was tested. We found that the activated caspase-3 was markedly increased in NGAL–shRNA group compared with those in the control and the shControl groups (Fig. 3E). These results suggest that knockdown of NGAL suppresses cell proliferation and induces cell apoptosis in vitro.

Down-regulation of NGAL increased the activity of ROS via Nrf2/HO-1 signaling
To elucidate the effectors involved in regulating NGAL depletion-induced apoptosis, we focused on the ROS and SOD production. As shown in Fig. 4A,B, cells transfected with NGAL–shRNA showed a significantly increase in ROS generation, meanwhile SOD activity was dramatically decreased, indicating that NGAL is important in preventing oxidative stress in lung adenocarcinoma cells. Furthermore, to explore its possible mechanisms of this action, we focused on Nrf2 and its target gene HO-1. It was found that knockdown of NGAL in A549 and PC9 cells resulted in a marked reduction of Nrf2 and HO-1 mRNA and protein levels (Fig. 4C). There was no difference between the control group and the shControl group. These results demonstrate that the Nrf2/HO-1 anti-oxidant signaling, as a down-stream target of NGAL, is partially responsible for the induction of ROS.

Anti-oxidants N-acetylcysteine blocked knockdown of NGAL-induced ROS generation and apoptosis in vitro
To determine whether ROS plays an essential role in the apoptosis induced by depletion of NGAL in vitro, we measured the effect of anti-oxidants N-acetylcysteine (NAC) (Beyotime Institute of Biotechnology) on NGAL-mediated ROS generation and apoptosis. After incubation with 5 mM NAC for 24 h, the NGAL-knockdown-induced ROS increase was reversed (Fig. 5A). Meanwhile, pretreatment with NAC (5 mM) could completely block the depletion of NGAL-induced apoptosis (Fig. 5B). These results indicate that ROS plays a critical role in the induction of cell apoptosis by down-regulation of NGAL.

Depletion of NGAL inhibited tumor formation in vivo
To confirm the tumor suppressor role of NGAL, we established a BALB/c nude mouse xenograft model using A549 cells (Fig. 6A). The stable NGAL–shRNA-transfected A549, shControl-transfected A549 and A549 cells (1 × 10⁷ per mouse) were injected into female BALB/c athymic nude mice (n = 5). Meanwhile, we also investigated whether NGAL could be down-regulated in the NGAL–shRNA tumor tissues. Western blot analyses revealed that the expression of NGAL in the NGAL–shRNA tumor tissues was indeed down-regulated compared with those in the A549 group and shControl group (Fig. 6B). The tumor volume was measured every 5 days until day 35. Tumor growth of NGAL–shRNA group was greatly restrained when compared with A549 group and shControl group (Fig. 6C). When the tumors were harvested on the 35th day, the size of implanted tumors (A549 transfected with NGAL–shRNA) was reduced in comparison with control and shControl groups (Fig. 6D). The average weight of tumors derived from cells transfected with NGAL–
Figure 2. NGAL mRNA and protein expression was determined by qRT-PCR and western blot analyses (A) NGAL expression was analyzed in A549, A549-shControl, and A549-NGAL-shRNA cells. (B) NGAL expression was analyzed in PC9, PC9-shControl, and PC9-NGAL-shRNA cells. *P < 0.05.

Figure 3. NGAL-knockdown-suppressed tumor cell growth and induced apoptosis in vitro (A) Proliferation of cancer cells was measured by CCK-8 assay. (B) Effects of NGAL on colony formation of tumor cells. (C) The cell apoptosis was determined by flow cytometry. (D) Bax and Bcl-2 were assessed by qRT-PCR and western blot analysis. (E) The activity of caspase-3 was measured using the substrate Ac-DEVD-pNA. *P < 0.05, **P < 0.01.
Figure 4. NGAL-knockdown-induced apoptosis was associated with ROS generation involving Nrf2/HO-1 signaling

(A) ROS production was analyzed by laser-scanning confocal microscopy and FACS analysis of DCF-DA-stained cells. (B) SOD activity was detected using a SOD Assay Kit-WST. (C) Levels of Nrf2/HO-1 mRNA and protein were determined by qRT-PCR and western blot analyses. *P<0.05.
Figure 5. Effects of anti-oxidants NAC treatment on ROS levels and cell apoptosis. (A) Effect of NAC treatment on ROS levels was determined with DCFH-DA. (B) The cell apoptosis was assessed by flow cytometry. *P < 0.05.
shRNA was also lower than those from control and shControl groups (Fig. 6E).

To further substantiate the anticancer effect in vivo, TUNEL assay was used to measure the apoptosis in tumor issues. The proportion of apoptotic-positive cells in the NGAL–shRNA tumor tissues was significantly higher than that in the A549 group. Meanwhile, the proportion of apoptotic-positive cells in the A549 group and in the shControl group was not significantly different (Fig. 6F). This result indicates that depletion of NGAL significantly inhibits the tumorigenicity of A549 cells in the nude mouse xenograft model by inducing apoptosis.

**Discussion**

NGAL, a member of the lipocalin family, is up-regulated in a number of pathological conditions, including multiple cancer types [21]. However, the role of NGAL in lung adenocarcinoma development has not been well studied. Here, we detected the significantly high expression levels of NGAL in human lung adenocarcinoma tissues. Further, we found that down-regulation NGAL inhibited cell proliferation and induced apoptosis by increasing of ROS generation partially via Nrf2/HO-1 pathway. Our results suggest that NGAL plays an important role in the tumorigenesis of lung adenocarcinoma.

Growing evidence has identified NGAL as a mediator of the nuclear factor kappa B oncogenic activity in cancers [22], while its anti-tumor effect is related to the inhibition of the pro-neoplastic factor hypoxia inducible factor-1 alpha (HIF-1α), the HIF-1α-dependent vascular endothelial growth factor and focal adhesion kinase [23]. Oxygen density is considered to be the most important factor in the process of cancer development. It is known that ROS-induced oxidative stress could affect the function of multiple redox-sensitive transcription factors and lead to the up-regulation of anti-oxidant genes. ROS is widely generated in biological systems that are defended by anti-oxidant systems through limitation of ROS production as a promising therapeutic approach [24,25]. Nrf2 regulates the cellular cytoprotective defense against oxidative stress by transactivation of detoxification enzymes and anti-oxidant proteins such as NAD(P)H quinone oxidoreductase-1, glutathione S-transferases, and HO-1, which contributes to cellular protection by removing ROS including...
superoxide anions, hydrogen peroxide, and hydroxyl radicals [26]. Nrf2 silencing in HT29 cells could significantly alter the intracellular redox state by up-regulating the ROS production that can highly sensitize the HT29 cells to AGP-induced cell death [27]. HO-1, an important Nrf2 target gene, plays an essential role in both anti-oxidative stress and anti-inflammation following cellular injury and oxidative stress [28]. Therefore, ROS activation may involve the modulation of the NAGL in the anti-tumor effect of lung adenocarcinoma via Nrf2/HO-1 signaling.

In this study, we first investigated the involvement of NAGL in the human lung adenocarcinoma. We found that NAGL was significantly up-regulated in lung adenocarcinoma tissues compared with that in normal lung tissues. Subsequently, we used shRNA targeting NAGL to inhibit the NAGL expression in A549 cells and PC9 cells. The results demonstrated that down-regulation of NAGL decreased the proliferation and induced apoptosis in A549 and PC9 cell lines in vitro. Based on our previous data, we hypothesized that the ROS activation might play an important role in the effects of NAGL. In the subsequent experiments, we found the elevated ROS production, decreased SOD activation in NAGL down-regulated lung adenocarcinoma cells. We also investigated the role of ROS in apoptosis induced by down-regulation of NAGL. These apoptosis induced by NAGL-silencing were blocked by anti-oxidants NAC, indicating the important role of ROS in the depletion of NAGA-induced apoptosis. Furthermore, our findings also demonstrated that down-regulation of NAGL significantly inhibited the expression level of Nrf2 and HO-1, which suggested that silencing NAGL could induce oxidative stress targeting Nrf2/HO-1 signaling pathway. Finally, we confirmed that the growth of tumor was significantly inhibited in NAGL-knockdown group compared with other groups in vivo.

Taken together, our study proved that NAGL expression was increased in clinical lung adenocarcinoma tissues, the knockdown of NAGL inhibited cell proliferation and induced cell apoptosis through inducing the production of intracellular ROS levels, which might be partly correlated with the decreased expression of Nrf2 and subsequently HO-1. These results suggested that NAGL might be a potential target for the treatment of human lung adenocarcinoma. However, further studies are needed to verify the specific mechanism of NAGL knockdown involving the Nrf2/HO-1 pathway.

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