JBUON 2013; 18(4): 908-914
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

# Lentivirus-mediated shRNA interference targeting cyclooxygenase-2 inhibits growth of human non-small cell lung cancer

Tianrong Li[1], Jie Lu[2], Yunhua Zhong[1]

[1]*Cadre Wards,* [2]*Department of Hematology, the First People's Hospital of Yunnan Province, Kunming, Yunnan, China*

## *Summary*

***Purpose:*** *Cyclooxygenase-2 (COX-2), one isoform of cyclooxygenase proinflammatory enzymes, plays an important role in tumor development and progression. Researches of human cancers have revealed high expression levels of COX-2 in a variety of cancers including lung cancer. The mechanism of COX-2 in the pathogenesis of non-small cell lung cancer (NSCLC) cells is not well understood.*

***Methods:*** *We constructed a lentivirus vector mediated RNA interference (RNAi) targeting COX-2 for the treatment of human NSCLC cells. RNAi technology was used to knockdown the expression of COX-2 in NSCLC cell lines. The efficiency and specificity was validated by quantitative real-time PCR and western blotting. The cell growth and cell cycle were determined by MTT and flow cytometry assay, respectively. Cell cycle-regulated gene expression, including cyclin D1, p21 and survivin, whose expression was modulated by COX-2, was also examined.*

***Results:*** *LV-COX-2-silencing (si)RNA lentivirus vector was effective and its inhibitory effects on COX-2 mRNA and protein expression was efficient and specific. Gene knockdown of COX-2 by LV-COX-2-siRNA significantly inhibited the growth and induced cell cycle arrest of NSCLC cell lines. In addition, silence of COX-2 mediated by LV-COX-2-siRNA modulated the expression of cell cycle-regulated gene, upregulating p21 and downregulating cyclin D1 and survivin.*

***Conclusions:*** *Our findings imply that COX-2 and its signaling pathway may provide a novel therapeutic target for the treatment of NSCLC.*

***Key words:*** *cell cycle, cell growth, COX-2, lentivirus, NSCLC*

## Introduction

Lung cancer is the leading cause of cancer-related deaths in men and women worldwide [1]. Despite the never-ending efforts for more effective therapies including chemotherapy, radiation therapy and surgery, the 5-year overall survival has shown little improvement over the past two decades [2]. Therefore, a need emerges for new therapeutic strategies for this malignancy.

COX, the key enzyme that catalyzes the conversion of arachidonic acid into prostaglandins (PGs) and thromboxanes, is involved in the regulation of normal cell growth and aberrant cell growth [3]. COX exists in two isoforms. COX-1 is constitutively expressed in most human normal tissues and considered responsible for various physiologic functions [4]. COX-2, an inducible isoform usually expressed at low levels and stimulated by many inflammatory mediators, plays a role in inflammatory reactions and carcinogenesis [5,6]. The expression of COX-2 was detected in many tumors including osteosarcoma, gastric, breast, bladder, pancreatic and lung cancers [7-12]. Reports have shown that COX-2 is associated with tumor development, apoptosis, angiogenesis, invasion and migration [7,13-15], and therefore it has become a target for therapeutic interventions in the treatment of malignancies [2]. Researchers indicated RNAi-mediated knockdown of COX-2 inhibits the growth, invasion and migration of SaOS2 human osteosarcoma cells [7]. COX-

*Correspondence to:* Yunhua Zhong, MD. Cadre Wards, the First People's Hospital of Yunnan Province, 157 Jinbi Road, Kunming, Yunnan, 650032, China. Tel: +86 871 3638735, Fax: +86 871 3638734, E-mail: kitty7274@126.com
Received: 27/03/2013; Accepted: 01/05/2013

2 overexpression is a marker of poor prognosis for patients with NSCLC [16]. COX-2-overexpressing NSCLC cells exert significantly higher resistance to apoptosis than the parental cells [12]. COX-2 inhibitor celecoxib decreases lung cancer cells' survival by activating caspase cascades and increasing DNA fragmentation [17]. Nimesulide, another COX-2 inhibitor, has been shown to improve the efficacy of radiation treatment against NSCLC both *in vitro* and *in vivo* [2]. The function of COX-2 in the pathogenesis of NSCLC cells is not well understood.

In addition to exploring the role of the selective COX-2 knockdown by lentivirus-mediated short hairpin (sh)RNA interference in the cell cycle progression of NSCLC cells, we examined its effect on the expression of cyclin D1, p21 and survivin to assess the potential signaling pathways in two NSCLC cell lines A549 and H358.

## Methods

*Cell culture*

The human NSCLC cell lines, A549 and H358, and the human embryonic kidney cell line 293T, were obtained from the American Type Culture Collection (ATCC). The cells were routinely maintained in Ham's F-12K, RPMI or DMEM (Gibco,USA), supplemented with 10% fetal bovine serum (FBS;Sigma,USA), 1% penicillin/streptomycin and 1% L-glutamine (Gibco,USA) in a 95% humidified environment containing 5% $CO_2$ at 37 $^{o}$C.

Cells were cultured at $2\times10^5$ cells per well into 6-well plates, and were infected with LV-COX-2-siRNA when cells were grown to reach 70% confluence. The virus titers produced were approximately $10^9$ transducing u/ml medium. Gene silencing effects were evaluated by quantitative PCR and western blotting analysis.

*Construction of silencing RNAs (siRNAs)*

The target sequence for the COX-2-siRNA has been described by Tong et al. [5] and the coding regions (5'-AACTGCTCAACACCGGAATTTTT-3') corresponding to targeting human COX-2 are at the 291-313 position in the sequence (Gene Bank Accession: NM000963.1). We cloned the shRNA (chemically synthesized by Sangon Biotech, Shanghai, China) into a lentivirus vector. This vector includes a CMV-driven GFP reporter and a U6 reporter upstream of the cloning restriction sites. The control lentivirus vector has a CMV-driven GFP reporter and an U6 reporter upstream without shRNA control and the recombinant lentivirus vectors were produced by co-transfecting with the lentivirus packaging and expression plasmids in 293T cells using lipofectamine 2000 reagent (Invitrogen, USA). Forty-eight hours post-transfection, the infectious lentivirus vectors were attained by filtering through 0.45 μm cellulose acetate filters and centrifugation. The shRNA-COX-2 lentivirus vector we constructed was named LV-COX-2-siRNA.

*Quantitative real-time PCR*

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, USA) for reverse transcription. Aliquots (2 μg) of total RNA were reverse-transcribed into cDNA using a Prime Script™ RT Reagent Kit (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) assays were performed using SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) and RT-PCR amplification equipment.

The following specific primers were used: COX-1, sense strand 5'-CTGTTCGGTGTCCAGTTCCA-3', antisense strand 5'-GCCTCAACCCCATAGTCCAC-3', COX-2, sense strand 5'-GTTCCACCCGCAGTACAGAA-3', antisense strand 5'-AGGGCTTCAGCATAAAGCGT-3', Cyclin D1, sense strand 5'- CACACGGACTACAGGGGAGT -3', antisense strand 5'- GATGGTTTCCACTTCGCAGC -3', p21, sense strand 5'- CTCAGAGGAGGCGCCATGT -3', antisense strand 5'- GGAAGGTAGAGCTTGGGCA -3', Survivin, sense strand 5'-AGGACCACCGCATCTCTACA -3', antisense strand 5'-TTTCCTTTGCATGGGGTCGT -3', β-actin, sense strand 5'-GCGAGCACAGAGCCTCGCCTTTG-3', antisense strand 5'-GATGCCGTGCTCGATGGGGTAC-3'. β-actin was used as an internal control. The expression of genes (cyclin D1, p21 and survivin) was determined by normalization of the threshold cycle of these genes to that of the control β-actin.

*Western blotting*

Cells were washed and lysed in ice-cold lysis buffer (150 mM NaCl, 100 mM Tris-HCL, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 Mm phenylmethylsulfonyl fluoride). Cells were sonicated on ice for 15 sec and centrifuged at 10,000 g for 10 min at 4 $^{o}$C. Equal amount of protein (30 μg) was separated on a 12% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Millipore, USA). Membranes were probed with anti-COX-2 (Cell Signaling, #4842, 1 μg/ml), Survivin (Cell Signaling, #2802, 1 μg/ml), cyclin D1 (Cell Signaling, #2922, 1 μg/ml), p21 (Cell Signaling, #2947, 1 μg/ml), anti-β-actin (Cell Signaling, #4970, 1 μg/ml) antibodies overnight, were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA) and visualized with the enhanced chemiluminescence system (Beyotime Biotechnology, China). Measurement and detection of proteins were determined by normalization of the integrated optical densities of cyclin D1, p21 and survivin genes to that of the control of β-actin with Image-Pro Plus 5.0 software.

*Cell proliferation assay*

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner

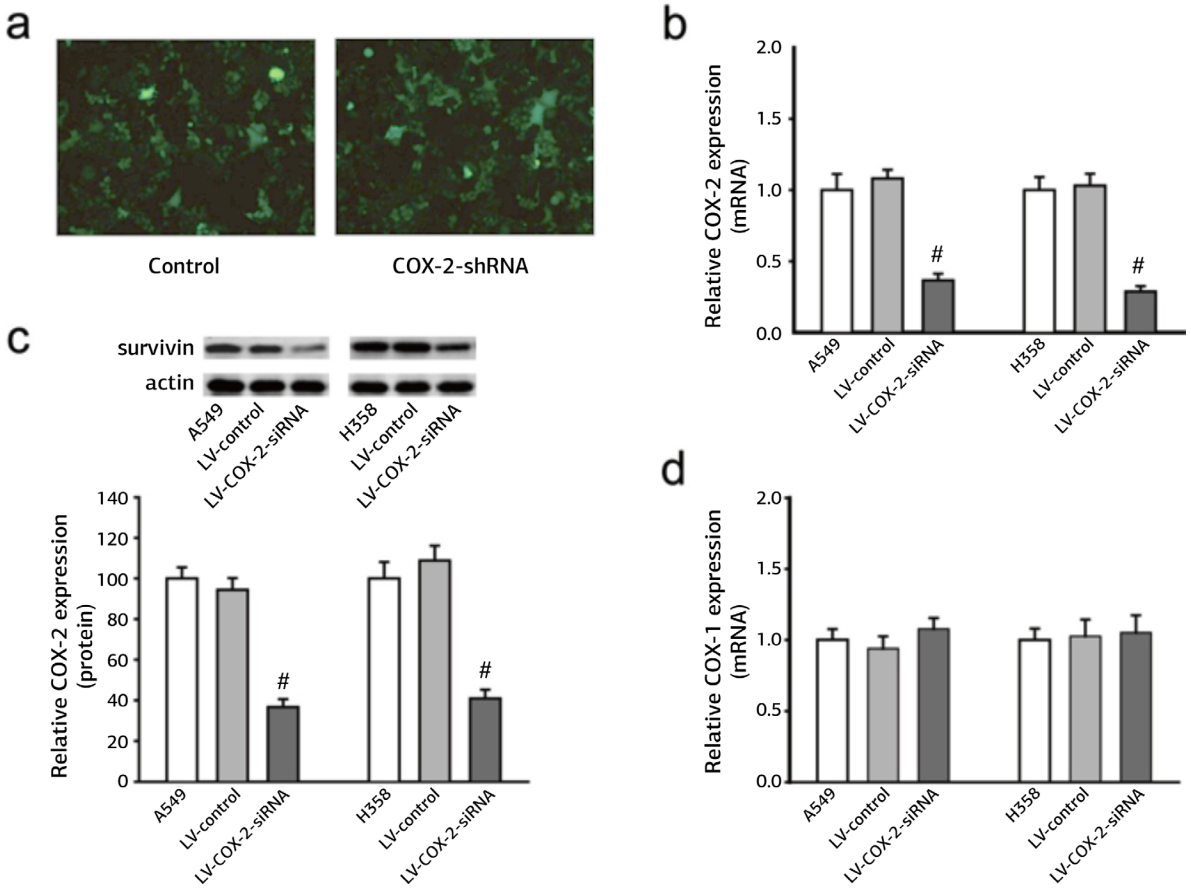


**Figure 1.** Expression of COX-2 inhibited by LV-COX-2-siRNAi in NSCLC cell lines. **(a)** GFP expression under a fluorescent microscope was taken 48 h after transfection in 293T cells (magnification 100 ×). Expression of COX-2 mRNA **(b)** and protein levels **(c)**, but not COX-1 mRNA **(d)**, were significantly suppressed in A549 and H358 cells infected with LV-COX-2-siRNAi. Data are presented as mean ± standard error of the mean. # p <0.01 compared with LV-Control and parental cell group.

salt (MTS) assay was performed to determine the antiproliferative effect of LV-COX-2siRNA on lung cancer cells. Cells ($4 \times 10^3$ cells per well) were plated in 96-well plates in triplicate wells for the parental, LV-Control and LV-COX-2siRNA and allowed to attach overnight. After 24, 48, 72 and 96 h, cells were stained with 20 µl MTS (317 µg/ml) (Sigma, St Louis, MO, USA) at 37 °C for 4 h. The absorbance of the solution was measured at 490 nm using spectrophotometer (BioRad, USA). Each experiment was performed in triplicate and repeated for three times and cell viability was compared with the control and untreated wells.

*Cell cycle analysis*

Cells were harvested and fixed in PBS containing 70% ethanol, and then were kept at 4 °C for 30 min. Before analysis, cells were centrifuged and resuspended in PBS containing 0.1% Tween 20, 0.05% BSA, 200 µg/ml RNase A and 50 µg/ml propidium iodide, then were kept at 37 °C for 30 min in the dark. Cells were analyzed on a Becton-Dickinson FACScan by flow cytometry analysis (Becton Dickinson FACScan).

*Statistics*

All values were expressed as mean ± standard deviation (SD). Student's t-test was used for all statistical analyses. A p-value < 0.05 was considered statistically significant.

## Results

*RNAi-mediated knockdown of COX-2 expression in NSCLC cells*

RNAi methodology was introduced to silence the COX-2 gene and clarify the functional role of COX-2 on NSCLC cells. GFP expression was observed 48h after transfection under fluorescent microscope in 293 T cells (Figure 1a). To examine the efficiency and specificity of the COX-2-siRNA, the mRNA and protein levels of COX-1 and COX-2 in parental cells,

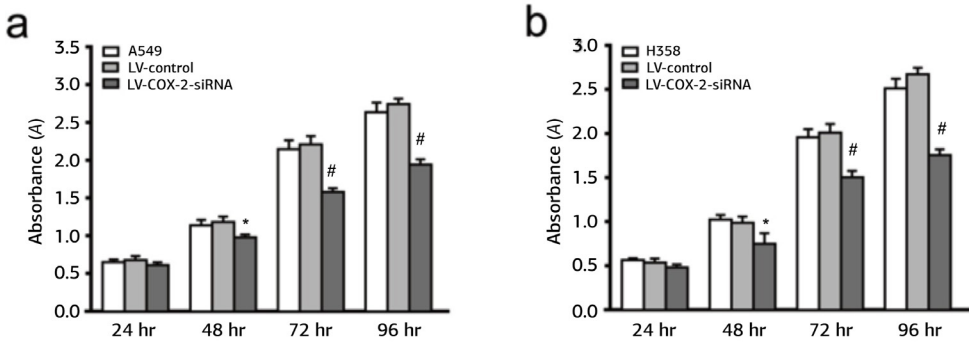


**Figure 2.** RNAi-mediated COX-2 knockdown inhibits the growth of NSCLC cell lines. Cell proliferation was detected by MTT assay. The proliferation was significantly inhibited in A549 **(a)** and H358 **(b)** cells infected with LV-COX-2-siRNAi compared with LV-Control or parental cell group, respectively. Results are representative of three independent experiments. Data are presented as mean ± standard error of the mean. * p <0.01, # p <0.001, compared with LV-Control and parental cell group.

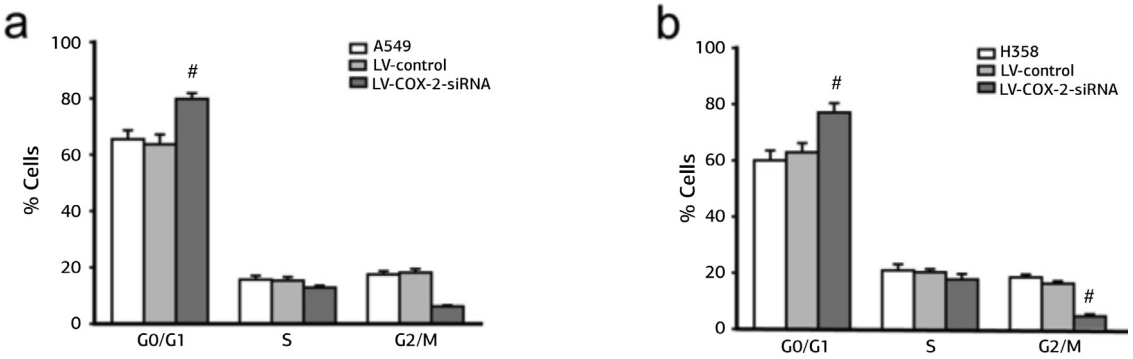


**Figure 3.** RNAi-mediated COX-2 knockdown induces cell cycle arrest in NSCLC cell lines. A549 **(a)** and H358 **(b)** cells infected with LV-COX-2-siRNAi induced an accumulation of cells in the G0/G1 phase of the cell cycle. Cell cycle distribution was determined by flow cytometry 72 h after infection of lentivirus. Data are presented as mean ± standard error of the mean. # p <0.001, compared with LV-Control and parental cell group.

LV-Control and LV-COX-2-siRNA infected cells were detected by real-time PCR and western blotting. As shown in Figure 1b and 1c, LV-COX-2-siRNA significantly decreased the COX-2 mRNA and protein levels in A549 and H538 cells compared with the parental and LV-control cells, respectively (p<0.001). LV-COX-2-siRNA had no significant effect on the mRNA (Figure 1d) and protein (data not shown) levels of COX-1 both in A549 and H538 cells. Our data demonstrated high specifically and efficiency of the RNA interference technique in the suppression of COX-2 expression in NSCLC cells.

*RNAi-mediated COX-2 knockdown induces cell growth inhibition*

To explore the effects of LV-COX-2-siRNA on the cell growth, MTT assays were carried out to determine the cell proliferation activity. Cell proliferation was detected for 96 h following the infection with lentivirus. As demonstrated in Figure 2a and 2b, the growth of both A549 and H358 cells was significantly inhibited at 48, 72 and 96 h following the infection of LV-COX-2-siRNA compared with the parental and LV-control infected cells. The results indicated that LV-COX-2-siRNA induced a reduced proliferative activity and the reduction of COX-2 expression contributed to the prominent antiproliferative effects in the NSCLC cells.

*RNAi-mediated COX-2 knockdown induces cell cycle arrest*

Due to the inhibitory effects of RNAi-mediated COX-2 knockdown on the growth of NSCLC cells, the effects of LV-COX-2-siRNA on cell cycle regulation were investigated. Seventy-two hours after the infection with LV-COX-2-siRNA, A549 and H358 cells were harvested and the G1, S and

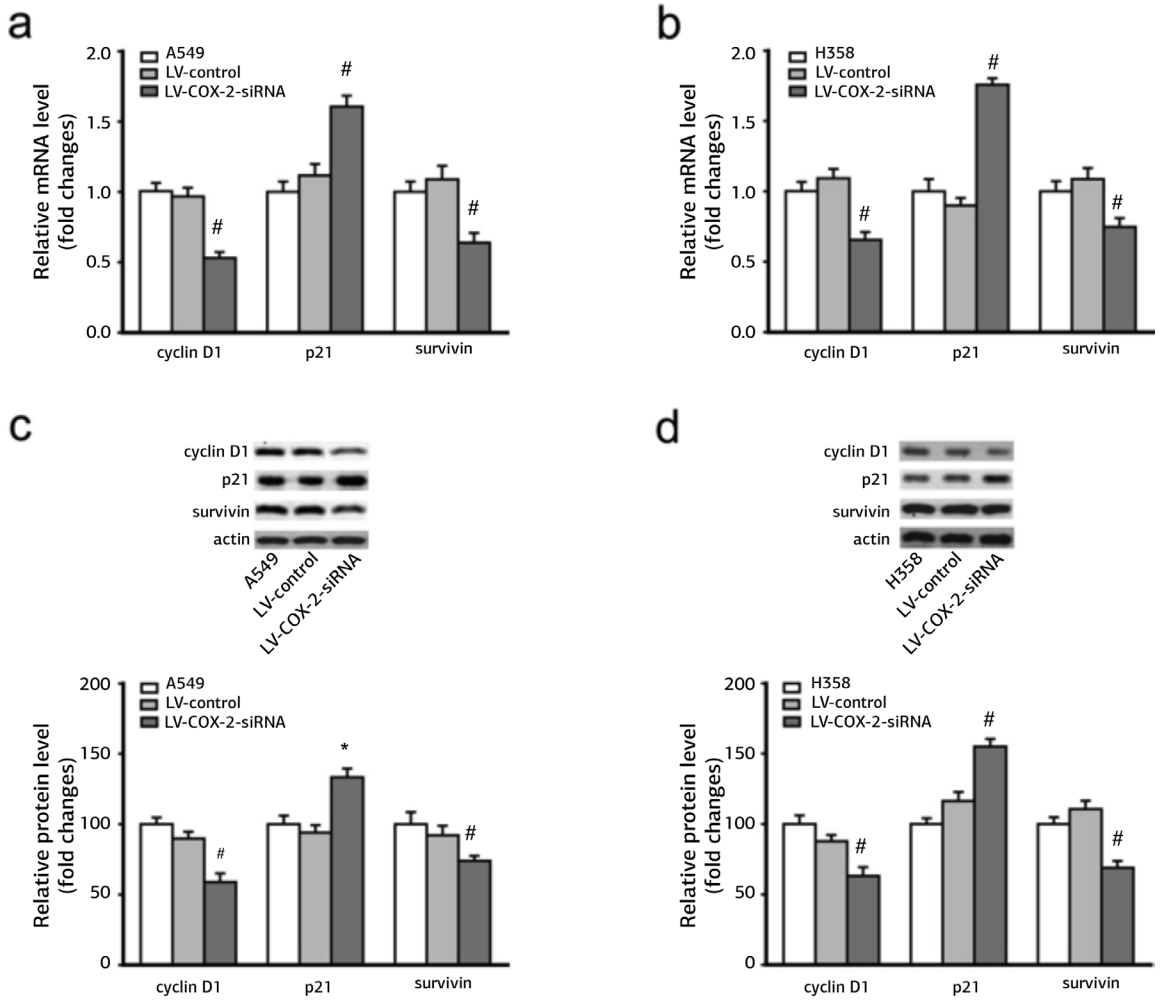


**Figure 4.** RNAi-mediated COX-2 knockdown modulates the expression of cell cycle-regulated genes in NSCLC cells. RNAi-mediated COX-2 knockdown upregulated the mRNA and protein levels of p21, and downregulated the mRNA and protein levels of cyclin D1 and survivin in A549 **(a,c)** and H358 **(b,d)** cells. Data are presented as mean ± standard error of the mean. * p <0.01, # p <0.001, compared with LV-Control and parental cell group.

G2 phase cells were detected by flow cytometric analysis. The results showed cell cycle arrest at the G0/G1 phase in both A549 and H358 cells infected with LV-COX-2-siRNA compared with the parental and LV-Control cells (p<0.001), with much less ratio of cells at the G2/M phase ( Figure 3a and 3b). Analysis was repeated with triplicate samples for each treatment. The results indicated that RNAi-mediated knockdown of COX-2 expression in NSCLC cells induced cell cycle arrest at the G0/G1 phase.

*Effects of RNAi-mediated COX-2 knockdown on the expression of cell cycle-regulated genes in NSCLC cells*

To further clarify the mechanism of RNAi-mediated COX-2 knockdown of growth inhibition and cell cycle arrest, we assessed the effects of LV-COX-2-siRNA on the expression of cell cycle-regulated genes. Three cell cycle-regulated genes were selected, including cyclin D1, p21 and survivin whose expressions were modulated by COX-2 as previously described [3,12,17-19]. Quantitative real-time PCR and western blotting analyses implied that the mRNA and protein expression levels of cyclin D1 (p <0.0001) and survivin (p< 0.0001) were significantly reduced in LV-COX-2-siRNA infected A549 and H358 cells, compared with the parental and LV-Control cells. Meanwhile, RNAi-mediated COX-2 knockdown increased the levels of p21 gene and protein expression in LV-COX-2-siRNA infected cancer cells (Figures 4a, 4b, 4c and 4d). The results revealed that silencing of the COX-2 gene resulted in downregulation of cyclin D1 and survivin and the activation of p21, which might induce cell growth inhibition and cell cycle arrest of NSCLC cells.

## Discussion

As previously described, many authors have reported that a number of human malignancies including NSCLC, such as in A549 and H358 cell lines experiments, express high levels of COX-2 [17,21-25]. COX-2 is believed to play a critical role in these tumor types and lung cancers [23,26-28]. Targeting COX-2 has been investigated for both cancer prevention and treatment for years [29-31]. Studies showed that selective COX-2 inhibitors inhibit proliferation and increase apoptosis of some carcinoma cells [32,33]. Other groups reported that the COX-2 inhibitors attenuate migration and invasion of cancer cells [34,35]. In addition, the COX-2 inhibitor nimesulide could suppress the proliferation of NSCLC cell lines *in vitro* in a dose-dependent manner [14]. All these data indicated that COX-2 is a considerable target for inhibiting growth, triggering apoptosis and reducing invasion.

To investigate the role of COX-2 as an available therapeutic target in NSCLC gene therapy, we employed RNA interference technology, a powerful tool for suppressing the expression of specific genes, to knockdown the endogenous COX-2 expression in NSCLC cell lines and analyzed phenotypical changes of the COX-2-downregulated NSCLC cells. As expected, gene silencing of COX-2 was achieved efficiently and specifically in lung cancer cells infected with LV-COX-2-siRNA as determined by quantitative real-time PCR and western blotting.

MTT analysis revealed that RNAi-mediated COX-2 led to a significant growth inhibition in NSCLC cell lines, while cell cycle analysis showed an increased accumulation in the G0/G1 phase of cells infected with LV-COX-2-siRNA. Taking the above results into account, our findings provided a strong support to the notion that the suppression of COX-2 expression in NSCLC cells was associated with cell growth inhibition and cell cycle arrest. Many studies have demonstrated that p21 plays an especially important role in the regulation of cell cycle in G1 arrest [36,37], and positive expression of p21 is a significant factor to predict a favorable prognosis in patients with NSCLC [38]. Previous research indicated that the COX-2-dependent survivin expression is critical for the apoptosis resistance, while cyclin D1 overexpression is associated with a high cell proliferation rate in NSCLC cells [12,39]. Our study identified that, relative to the parental and LV-Control infected NSCLC cells, the silencing of COX-2 expression by LV-COX-2-siRNA significantly upregulated the p21 and downregulated the cyclin D1 and survivin mRNA and protein expression levels in A549 and H358 cells. This may help to explain the mechanism of growth inhibition, and cell cycle arrest is determined by LV-COX-2-siRNA mediated gene knockdown in NSCLC cells.

## Acknowledgements

We are grateful to Assoc. Prof. Shude Li (Department of Biochemistry and Molecular Biology, Kunming Medical University, Yunnan, China) for experimental technology support and Prof. Ping Wang (Oncological Surgery, The First People's Hospital of Yunnan Province, Yunnan, China) for excellent guidance during the experiments.

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