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COX-2 silencing inhibits cell proliferation in A549 cell

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Abstract ***Objective:*** The aim of this study was to explore the effects on malignant proliferation of A549 cell by silencing cyclooxygenase (COX)-2. ***Methods:*** In the present study, we constructed three siRNA vectors producing small interference RNA. The siRNA vectors and the vacant vectors were transfected into A549 cell with lipofectamine respectively and the transfected cell strains were constructed. The change of COX-2 expression levels was examined by Western blot and RT-PCR. The effects on the proliferation of lung cancer cells were studied by cell growth curve, clonogenic assay and xenograft assays. ***Results:*** The siRNA expression vectors produced marked effects in A549 cell but the inhibited effects were different. The effect of psi-10 was best and the mRNA and protein levels of COX-2 reduced 61.2% and 56.2% respectively in A549-si10 cell in contrast to the control. The growth of A549 cell slowed and the colony formation rate reduced after silencing COX-2. In xenograft assays, the growth speeds of tumor became slow and the numbers of tumor reduced after silencing COX-2. ***Conclusion:*** The si10 target of COX-2 has the best silencing effect in A549 cell and the best inhibition effect on malignant proliferation of A549 cell *in vivo* and *in vitro*.

Key words cyclooxygenase (COX)-2; A549 cell; malignant proliferation

Cyclooxygenase (COX) is a rate-limiting enzyme in the conversion of arachidonic acid into prostaglandin (PG) and other eicosanoids including PGD2, PGE2, PGF2, PGI2 and thromboxane A2 [1]. Prostaglandin plays an important physiological role but also pathophysiological role in the occurrence and development of some diseases，so the rate-limiting enzyme of prostaglandin biosynthesis becomes a research focus. So far, COX is found to have at least three isoenzymes: COX-1, COX-2 and COX-3. COX-2 does not express in most of the normal body tissues and organs and is activated by cytokines, growth factors, phorbol esters, oncogenes, and chemical carcinogens [2].

Recent studies have found that COX-2 is responsible for producing large amounts of PGE2 in tumor tissues [3–6] and plays an important role in the development of many tumors except for involved in inflammation [7–13]. These molecules are thought to play a critical role in tumor growth, because they reduce apoptotic cell death, stimulate angiogenesis and invasiveness [14, 15]. The enzyme inhibitor has produced encouraging results in the tumor treatment [16–18].

In this study, we selected COX-2 as the subject and explored whether three RNAi targets could inhibit COX-2 gene expression by the RNAi technique and silencing COX-2 could affect the malignant proliferation of A549 cell. Our data showed the si10 target of COX-2 had the best silencing effect in A549 cell and the best inhibition effect on malignant proliferation of A549 cell *in vivo* and *in vitro*.

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Materials and methods

Cell culture

A549 was purchased from the Cell Center of Peking Union Medical College Beijing and is a well-characterized human lung adenocarcinoma cell line. The cells were routinely grown in RPMI1640 (Gibco, USA) supplied with 10% fetal bovine serum (Gibco, USA), 100 U/ml of penicillin and 100 U/ml of streptomycin in a humidified 37 ℃ incubator with 5% CO_2.

Construction of siRNA vectors

According to the designed principle of siRNA, three 19-nucleotide sequences were selected from COX-2 mRNA. U6 snRNA and U6-sense-harpin-antisense-transcriptonal end signal were acquired by PCR and cloned into pEGFP plasmid respectively. The positive clones were confirmed by PCR, restriction endonucleases and sequencing. The resulting plasmids were named as pU6, psi3, psi7 and psi10.

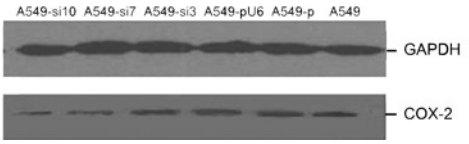

Fig. 1 The COX-2 expression of A549, A549-p, A549-pU6, A549-si3, A549-si7, A549-si10 cells

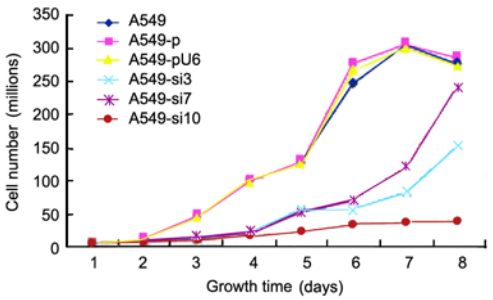

Fig. 2 The growth state of six groups cells

The cell strains of transfection

A total of 3×10^5 cells were seeded into 35mm culture plate. The next day (when the cells were 70-80% confluent), A549 cells were transfected respectively with the plasmids (pEGFP, pU6, psi3, psi7 and psi10) by using lipofectamine reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. 10% RPMI1640 with G418 (the end concentration was 800 μg/mL) was used after the transfection had finished. The cells were not cultured until the clones against G418 appeared. The A549 cell strains transfected were named as A549-p, A549-pU6, A549-si3, A549-si7 and A549-si10.

Western blotting

The cell strains were harvested and lysed in mammalian cell lysis buffer, and then western blot analysis was performed with the use of conventional protocols. In brief, the protein concentration was determined with a bicinchoninic acid kit with bovine serums album as a standard (Pierce, USA). Equal amounts of total protein were then separated on 12% polyacrylamide gels by using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques, then transferred to nitrocellulose membranes. Mouse anti-COX-2 monoclonal antibodies (Zhongshan Company, China) were used, followed by peroxidase-conjugated goat anti-mouse immunoglobulin for immunoblotting. GAPDH was used as an internal control. Enhanced chemiluminescence (Pierce, USA) was adopted for detection.

Cell growth curve assay

The cells were seeded at a density of 8×10^4/bottle in triplicate respectively. The cell numbers were quantified every day and recorded for seven days in all. Then the cell growth curve was drawn.

Colony formation assay

The cells were seeded for colony formation in 35 mm dishes and 300 cells were seeded in every dish. After 14-d incubation, the colonies were stained with crystal violet and manually counted. Colonies containing more than 50 cells were scored. Every group was in triplicate.

Tumor growth in nude mice

The cells were re-suspended at a density of 1.5×10^7/mL. Three 4-week-old male nude mice of every group were given bilateral subcutaneous injections. These mice were kept in pathogen-free environments. The date at which a palpable tumor first arose was recorded and the tumors were weighed.

Immunohistochemistry assay

The tumor tissue samples of nude mice were fixed in 10% neutral-buffered formalin and then embedded in paraffin. The 4-μm-thick sections were immersed in 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, microwaved in citrate phosphate

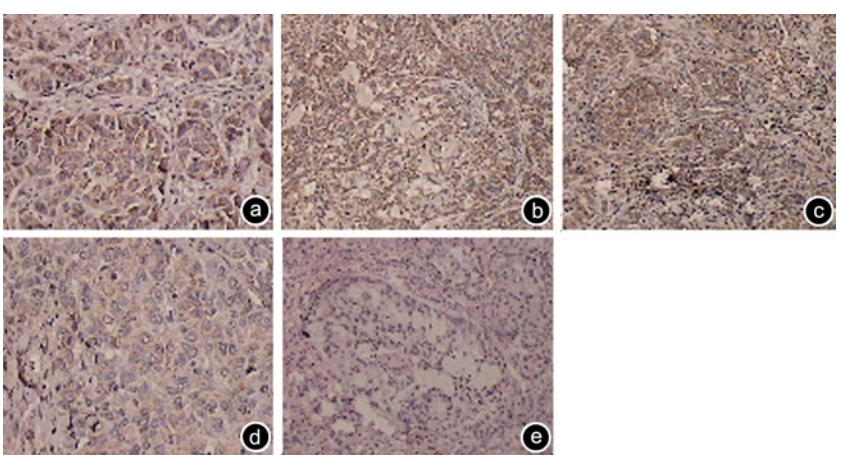

Fig. 3 The results of COX-2 expression of xenograft assays on nude mice. (a) COX-2 expression of A549 group; (b) COX-2 expression of A549-pU6 group; (c) COX-2 expression of A549-si3 group; (d) COX-2 expression of A549-si7 group; (e) COX-2 expression of A549-si10 group

Table 1 The colony formation assay of six groups

Group	Colony number			Colony formation rate (%)
	No. 1	No. 2	No. 3	
A549	132	128	75	35.9
A549-p	158	83	123	40.1
A549-pU6	110	101	97	35.1
A549-si3	100	98	103	32.6
A549-si7	106	108	114	36.5
A549-si10	68	32	28	14.8

$P = 0.003$ (A549-si10 vs A549); $P = 0.001$ (A549-si10 vs A549-pEGFP); $P = 0.006$ (A549-si10 vs A549-pU6); $P = 0.008$ (A549-si10 vs A549-si3); $P = 0.003$ (A549-si10 vs A549-si7)

buffer (pH6.0) for antigen retrieval, and incubated with 10% normal goat serum for 30 min to block nonspecific binding. Mouse monoclonal antibody for human COX-2 (Zhongshan Company, China) were applied as the primary antibody at 4 ℃ overnight, followed by a standard staining procedure using the ABC kit (Zhongshan Company, China).

Statistical analysis

SPSS 13.0 software was used to analyze the data and plot curves. One way ANOVA was used to compare the statistical significance of the differences in data from the more than two groups. Differences were considered to be significant at $P \leq 0.05$. The protein expression was analyzed with a Gel EDAS analysis system and Gel-Pro Analyzer 3.1 software.

Results

Suppression of COX-2 gene in A549 cells by RNAi

The COX-2 levels were corresponding in A549, A549-p, A549-pU6 three control groups and decreased in other three RNAi groups (A549-si3, A549-si7 and A549-si10; Fig. 1). The COX-2 levels of A549-si3, A549-si7, and A549-si10 reduced 26.7%, 44.7% and 56.2% respectively in contrast A549 cell.

Silencing COX-2 significantly altered the growth rate of A549 cell

The growth state of six group cells was recorded (Fig. 2). The growth speeds of three controls were corresponding and those of three RNAi groups became slow, above all A549-si10 cell. The results of statistical analysis showed the A549-si10 group was significant difference ($P < 0.05$) and the A549-si3 and A549-si7 groups were not ($P > 0.05$) in contrast to the controls (A549, A549-p and A549-pU6).

Silencing COX-2 inhibit the colony formation of A549 cell

The colony formation assays showed the A549-si10 group had a significant decrease in colony formation ($P < 0.05$) and the A549-si3 and A549-si7 groups were not in contrast to the control groups ($P > 0.05$; Table 1).

Silencing COX-2 reduces tumor growth in nude mice

To address the potential effects of RNAi *in vivo* on inhibiting the growth of lung cancer cells, equal numbers of five groups (A549, A549-pU6, A549-si3, A549-si7 and A549-si10) were injected into male nude mice. At 3 weeks after injection of these cells, the tumors appeared in five groups. Six locations appeared tumors in A549 group, four locations in A549-pU6 group, two locations in A549-si3, four locations in A549-si7 group, one location in A549-si10 group. At 4 weeks, five locations appeared tumors in A549-pU6 group, four locations in A549-si3 group, six locations in A549-si7 group, and three locations in A549-si10 group. At 45 days, five locations grew tumors in A549-si3 group and four locations in A549-si10 group. There was not change in the other groups. The mice were killed and the tumors were weighted (Table 2). A549-si10 cell grew slowly from growth time, growth speed and tumor numbers and there was significant difference in contrast the control groups ($P < 0.05$). A549-si3 and A549-si7 groups were not significant difference in contrast the controls ($P > 0.05$).

The expression of COX-2 in tumor tissues

We firstly examined the expression of COX-2 of nude mice tumor tissues. The results were as follows (Fig. 3). The COX-2 expression of A549, A549-pU6 was positive and scored ++. The staining area of A549-si3 was not as wide as the controls (A549, A549-pU6), but the difference was not big and it was scored ++. The staining inten-

Table 2 The growth state of tumors in five groups

Group	Tumor weight (g)						Average tumor weight (g)
A549	0.55	0.30	0.30	0.10	0.55	0.40	0.37 ± 0.17
A549-pU6	0.35	0.60	0.20	0.15	0.15	0.35	0.30 ± 0.17
A549-si3	0.35	0.30	0.10	0.10	0.05	0.35	0.21 ± 0.14
A549-si7	0.23	0.30	0.20	0.20	0.23	0.10	0.21 ± 0.07
A549-si10	0.20	0.30	0.05	0.05	0.20	0	0.13 ± 0.11*

* $P = 0.008$ (A549-si10 vs A549); $P = 0.049$ (A549-si10 vs A549-pU6)

sity and amount of A549-si7 was weaker than the controls and it was scored +. The staining of A549-si10 was negative. The results basically corresponded with those of western blot assays. We firmed the RNAi vectors played role in the cell strains.

Discussion

Recent many studies showed COX-2 was involved in tumorigenesis and development except for involving inflammatory. Overexpression of COX-2 is a usual phenomenon in tumor tissues. Increased COX-2 expression is also seen in human non-small cell lung cancer (NSCLC) [19–22]. In human, COX-2 expression is up-regulated in about one-third of atypical adenomatous hyperplasias and carcinoma in situ specimens obtained from lung, and in 70-90% of invasive adenocarcinomas of the lung. The proportion of adenocarcinoma cells with increased COX-2 expression is much greater in lymph node metastases than in the corresponding primary tumors. The inhibitor of COX-2 could reduce tumor cell motion, adherence and invasion *in vivo* and *in vitro* [23–25]. So COX-2 became the interested subject.

In the present study, the COX-2 expression had different degrees' inhibition after interfering in three different targets. Among them, the effect of psi10 was best, the COX-2 mRNA and protein levels reduced 61.2% and 56.2% in contrast to A549 cell. That of psi7 was slightly poor and that of psi3 was the poorest. Different targets produced different interfering effect, which could correlate with the secondary frame of mRNA.

We explored whether silencing COX-2 had an effect on the malignant proliferation of A549 cell *in vitro* and *in vivo. In vitro*, the growth of A549-si10, A549-si7, and A549-si3 cells became slow, but A549-si10 cell was significant difference and A549-si3, A549-si7 cells were not in contrast to the controls. The study that silencing COX-2 could reduce malignant phenotype of tumor cells *in vitro* has reported [26–29] and their results are corresponding with our. In addition, we carried on *in vivo. In vivo*, we examined the expression of COX-2 in tumors and the results were corresponding with the results *in vitro*, which showed psi-10 played a role when A549-si10 cell grew in nude mice. *In vivo*, as far as cell growth speed, tumor numbers and weight were concerned, the growth of A549-si10 cell became slow but that of A549-si3 and A549-si7 cells was not significantly different in contrast to the controls, which firmed further the malignant proliferation of A549 cells might correlate with COX-2. The study that silencing COX-2 can reduce malignant phenotype of A549 cell *in vivo* is not seen.

References

1. Smith WL, Langenbach R. Why are there two cyclooxygenase enzymes? J Clin Invest, 2001, 107: 1491–1495.
2. Brown JR, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. J Clin Oncol, 2005, 23: 2840–2855.
3. Denkert C, Kobel M, Berger S, *et al*. Expression of cyclooxygenase-2 in human malignant melanoma. Cancer Res, 2001, 61: 303–308.
4. Masferrer JL, Leahy KM, Koki AT, *et al*. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res, 2000, 60: 1306–1311.
5. Kulkarni S, Rader JS, Zhang F, *et al*. Cyclooxygenase-2 is overexpressed in human cervical cancer. Clin Cancer Res, 2001, 7: 429–434.
6. Kokawa A, Kondo H, Gotoda T, *et al*. Increased expression of cyclooxygenase-2 in human pancreatic neoplasms and potential for chemoprevention by cyclooxygenase inhibitors. Cancer, 2001, 91: 333–338.
7. Eberhart CE, Coffey RJ, Radhika A, *et al*. Upregulation of cyclooxygenase-2 gene in human colorectal adenomas and adenocarcinomas. Gastroenterology, 1994, 107: 1183–1188
8. Gupta S, Srivastava M, Ahmad N, *et al*. Over expression of cyclooxygenase-2 in human prostate adenocarcinoma. Prostate, 2000, 42: 73–78.
9. Kulkarni S, Rader JS, Zhang F, *et al*. Cyclooxygenase-2 is over expressed in human cervical cancer. Clin Cancer Res, 2001, 7: 429–434.
10. Sahin M, Sahin E, Gümüslü S. Cyclooxygenase-2 in cancer and angiogenesis. Angiology, 2009, 60: 242–253.
11. Fidler MJ, Argiris A, Patel JD, *et al*. The potential predictive value of cyclooxygenase-2 expression and increased risk of gastrointestinal hemorrhage in advanced non-small cell lung cancer patients treated with erlotinib and celecoxib. Clin Cancer Res, 2008, 14: 2088–2094.
12. Van Dyke AL, Cote ML, Prysak GM, *et al*. COX-2/EGFR expression and survival among women with adenocarcinoma of the lung. Carcinogenesis, 2008, 29: 1781–1787.
13. Denkert C, Kobel M, Berger S, *et al*. Expression of cyclooxygenase 2 in human malignant melanoma. Cancer Res, 2001, 61: 303–308.
14. Masferrer JL, Leahy KM, Koki AT, *et al*. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res, 2000, 60: 1306–1311.
15. Banu N, Buda A, Chell S, *et al*. Inhibition of COX-2 with NS-398 decreases colon cancer cell motility through blocking epidermal growth factor receptor transactivation: possibilities for combination therapy. Cell Prolif, 2007, 40: 768–779.
16. Leahy KM, Ornberg RL, Wang Y, *et al*. Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells *in vivo*. Cancer Res, 2002, 62: 625–631.
17. Jang MC, Liao CF, Lee PH. Aspirin inhibits matrix metalloproteinase-2 activity, increases E-cadherin production and inhibits *in vitro* invasion of tumor cells. Biochem Biophys Res Commun, 2001, 282: 671–677.
18. Tsujii M, Kawano S, Tsuji S, *et al*. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, 1998, 93: 705–716.
19. Wolff H, Saukkonen K, Anttila S, *et al*. Expression of cyclooxygenase-2 in human lung cancer. Cancer Res, 1998, 58: 4997–5001.
20. Dohadwala M, Luo J, Zhu Li, *et al*. Non-small cell lung cancer cyclooxygenase-2-dependent invasion in mediated by CD44. J Biol Chem, 2001, 276: 20809–20812.
21. Hosomi Y, Yokose T, Hirose Y, *et al*. Increased cyclooxygenase 2 (COX-2) expression occurs frequently in precursor lesions of human

adenocarcinoma of the lung. Lung Cancer, 2000, 30: 73–81.
22. Kokawa A, Kondo H, Gotoda T, *et al*. Increased expression of cyclooxygenase-2 in human pancreatic neoplasms and potential for chemoprevention by cyclooxygenase inhibitors. Cancer, 2001, 91: 333–338.
23. Li G, Yang T, Yan J. Cyclooxygenase-2 increased the angiogenic and metastatic potential of tumor cells. Biochem Biophys Res Commun, 2002, 299: 886–890.
24. Choe MS, Zhang X, Shin HJC, *et al*. Interaction between epidermal growth factor receptor and cyclooxygenase 2 mediated pathways and its implications for the chemoprevention of head and neck cancer. Mol Cancer Ther, 2005, 4: 1448–1455.
25. Charames GS, Bapat B. Cyclooxygenase-2 knockdown by RNA interference in colon cancer. Int J Oncol, 2006, 28: 543–549.
26. Strillacci A, Griffoni C, Valerii MC, *et al*. RNAi-based strategies for cyclooxygenase-2 inhibition in cancer. J Biomed Biotechnol, 2010, Epub 2010 Jun 13.
27. Chan MW, Wong CY, Cheng AS, *et al*. Targeted inhibition of COX-2 expression by RNA interference suppresses tumor growth and potentiates chemosensitivity to cisplatin in human gastric cancer cells. Oncol Rep, 2007, 18: 1557–1562.
28. Zhao Q, Wang C, Zhu J, *et al*. Do RNAi-mediated knockdown of cyclooxygenase2 inhibits the growth, invasion and migration of SaOS2 human osteosarcoma cells: a case control study. J Exp Clin Cancer Res, 2011, 30: 26.
29. Li WY, Wang H, Lai BT, *et al*. The effects of the same target on malignant proliferation of human lung cancer cells with different expression levels of COX-2 protein. Chinese-German J Clin Oncol, 2010, 9: 125–132.