

Effects of silencing cyclooxygenase-2 expression via RNA interference on the tumorigenicity of the SMMC-7721 human hepatocarcinoma cell line

LI-LI LIU^{1,2*}, LI-RONG LIN^{1*}, MAN-LI TONG^{1,2*}, ZUO-GEN FU^{1,2}, GUI-LI LIU^{1,2}, WEI-HONG ZHENG³, FAN LIU², DAI-WEI ZHANG³, ZHONG-YING ZHANG¹ and TIAN-CI YANG^{1,2}

¹Center of Clinical Laboratory, Zhongshan Hospital, Medical College of Xiamen University;

²Medical College of Xiamen University; ³Department of Neurology, Zhongshan Hospital, Medical College of Xiamen University, Xiamen 361004, P.R. China

Received December 5, 2011; Accepted February 2, 2012

DOI: 10.3892/or.2012.1702

Abstract. We constructed a vector carrying a shRNA sequence against cyclooxygenase-2 (COX-2) that was subsequently transfected into the human hepatocarcinoma cell line SMMC-7721. Furthermore, we established a COX-2-deficient stable cell line and a model of tumor-shRNA transplantation in nude mice. Negative shRNA was used as the control. The tumor volume in the experimental group was smaller compared to that in the control group. Hematoxylin and eosin staining indicated that the cells in the experimental group differentiated better than those in the control group. The COX-2 mRNA level in the tumor tissues injected with SMMC-7721/COX-2i was markedly downregulated compared to that in the tumor tissues injected with SMMC-7721/Negative shRNA. The inhibition rate reached 68.6%. Immunohistological study showed a significantly strong COX-2 expression in the control group tumor cells, whereas the experimental group exhibited moderate expression, indicating the inhibition of COX-2 expression after transfection of cells with shRNA against COX-2. Western blot analysis further proved the inhibition of COX-2 expression. In conclusion, RNAi-mediated regulation of COX-2 expression could efficiently inhibit liver-transplanted tumor growth in BALB/c nude mice.

Introduction

Cyclooxygenase (COX), also known as prostaglandin (PG) peroxidase synthase, is an important rate-limiting enzyme

in synthesizing PG. Aside from its production of various PGs during arachidonic acid metabolism, this enzyme also affects various physiological and pathological processes (1,2). COX-2 is overexpressed in most tumor cells (3), promoting cell proliferation and suppressing apoptosis. This gene also facilitates tumor angiogenesis and metastasis (4). COX-2 inhibitors can block tumor proliferation and induce tumor cell apoptosis, indicating the antitumor role of these inhibitors (5-7). COX-2 is of great clinical value as a potential target protein for the prevention and cure of tumor and inflammation (8-10). RNA interference (RNAi) involves post-transcriptional gene silencing triggered by double-stranded RNA, which can specifically inhibit the expression of a target gene (11-13). In our previous study, we used a gastric cancer xenograft model in BALB/c nude mice and transfected small interfering RNA (siRNA) against the COX-2 gene with lipid-cationic polymer (LCP), a gene vector for siRNA transfection, to silence the expression of this gene. Results indicate that LCP-mediated siRNA transfection could efficiently inhibit COX-2 expression and suppress gastric tumor tissue growth (14). Meanwhile, we have synthesized a cationic polymer with branch structures, and have constructed a Sofast gene vector which is a gene vector with a high transfection efficiency, low cytotoxicity and good stability, that has the potential to be widely used in gene transfection both *in vitro* and *in vivo* (15-17). In the present study, we constructed a novel pCOX-2 shRNA vector that specifically inhibits COX-2 expression, as well as the negative control vector pNegative-shRNA. We used our patent-protected Sofast reagent to transfect the human hepatocarcinoma (HCC) cell line SMMC-7721 to establish a COX-2-deficient stable cell line. We then established a model of nude-transplanted tumor-shRNA. We probed the functions of COX-2 in tumor development and provide fundamental evidence for the targeted cancer therapy.

Materials and methods

Biomaterials. Plasmid pGenesil-1 was purchased from Clontech (USA). The SMMC-7721 cell line was obtained from the American Type Culture Collection (Manassas, VA). The fetal

Correspondence to: Dr Tian-Ci Yang or Dr Zhong-Ying Zhang, Center of Clinical Laboratory, Zhongshan Hospital, Medical College of Xiamen University, Xiamen 361004, P.R. China
E-mail: yangtianci@xmu.edu.cn
E-mail: zhangzy1121@xmu.edu.cn

*Contributed equally

Keywords: cyclooxygenase-2, RNA interference, SMMC-7721, nude-transplanted tumor

calf serum, Dulbecco's modified Eagle's medium (DMEM), and Opti-MEM were all from Gibco (USA). BALB/c nude mice were from the Experimental Animal Center, Medical College of Xiamen University in China. TRIzol reagent was obtained from Invitrogen (USA). Reagents for the reverse transcription-polymerase chain reaction (RT-PCR) kits were from Qiagen (USA). Antibiotic G418, penicillin, and streptomycin were purchased from Xiamen Sunma Biotechnology Co., Ltd. (China). BALB/c nude mice were approved by the Review Board, Medical College of Xiamen University. The Sofast transfection reagent was constructed in our laboratory, which has been used for transferring macromolecular nucleic acids both *in vivo* and *in vitro* (15-17).

Cell culture. SMMC-7721 cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C under a humidified atmosphere of 5% CO₂.

Vector construction. shRNA was synthesized by the Chinese Military Academy of Medical Sciences. The detailed sequences are as follows: COX-2 shRNA: COX-2 Ri-F 5'-GATCCC TGCTCAACACCGGAATTTTCAAGACGAAATTCCG GTGTTGAGCAGTTTTTTA-3', COX-2 Ri-R 5'-AGCTTAA AAAACTGCTCAACACCGGAATTCGTCTTGAAAA TTCCGGTGTGAGCAGG-3'; whereas for negative short hairpin RNA (shRNA): Negative Ri-F 5'-GATCCGACTTC ATAAGGCGCATGCTTCAAGACGGCATGCGCCTTAT GAAGTCTTTTTTA-3'; Negative Ri-R 5'-AGCTTAAAA AGACTTCA TAAGGCGCATGCCGTCTTGAAGCATG CGCCTTATG AAGTCG-3'. The shRNA sequences were cloned into the pGenesil-1 vector. The recombinant vector was then transformed into DH5 α cells for propagation. After sequence verification, the pCOX-2 shRNA and pNegative shRNA were ready for maxi preparation.

Gene transfection and stable cell line selection. SMMC-7721 cells were seeded in 6-well culture plates at a concentration of 5.0x10⁵ cells/ml for 16-24 h. Plasmid DNA [2 μ g/100 μ l (pCOX-2 shRNA and pNegative shRNA)] was mixed with the Sofast gene vector at room temperature for 15 min to form the DNA/vehicle complex, and then subsequently dropped into the wells. Cells were cultured continuously at 37°C for 48 h. Antibiotic G418 (800 μ g/ml) was initially used to screen the positive clones. After 10 to 12 days, cells were maintained continuously in the medium with G418 (400 μ g/ml), until the cell colony was macroscopic. The cells that expressed green fluorescent protein were marked under a fluorescence microscope and selected for propagation under the pressure of G418 (400 μ g/ml). Cells that stably expressed pCOX-2 shRNA or pNegative-shRNA were named SMMC-7721/COX-2i or SMMC-7721/Negi, respectively.

Establishment of nude transplant tumor model. Human HCC cell lines SMMC-7721/COX-2i and SMMC-7721/Negi were cultured under the pressure of G418 and harvested by trypsin digestion. After washing with PBS twice and centrifugation at 2000 rpm for 5 min, the cell pellet was re-suspended in PBS at a final cell density of 2x10⁷ cell/ml. A total of 20 BALB/c nude mice, 5-6 weeks old, were equally divided into two groups. In

the experimental group, 2x10⁶ SMMC-7721/COX-2i cells were injected subcutaneously into the dorsal flank region of the mice, whereas in the control group, the mice were injected with the same number of SMMC-7721/Negi cells. All mice were raised under specific pathogen-free conditions. They were housed at room temperature on a 12 h light and 12 h dark cycle and were fed with water and food regularly. The mice diet, physiological conditions, and tumorigenicity were observed daily. After 4 weeks, all animals were sacrificed by intraperitoneal injection of pentobarbital. The tumor tissues were separated, and the tumor size was determined by measuring the volume of water drained from the tumor tissues. All the animal experiments were approved by the Animal Care and Use Committee of Xiamen University. Their care was in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of Xiamen University.

Pathology changes and tumor volume. The sizes of the tumor tissues were measured, and hematoxylin and eosin (H&E) staining was used to observe the tumor sections.

Detection of COX-2 mRNA levels. A total of 100 mg fresh tumor tissues were frozen in liquid nitrogen and ground with a mortar and pestle to yield tissue powder. Each 100 mg of tissue or 10⁷ cells of SMMC-7721 were lysed with 1 ml TRIzol reagent. Total RNA was isolated from the tissues according to the manufacturer's instruction. The RNA precipitate was then dissolved in 10-15 μ l of RNase-free water. A spectrophotometer was used to determine the quantity and quality of the RNA precipitate. A one-step RT-PCR for the COX-2 gene was performed using a kit from Qiagen. The resultant complementary DNA (cDNA) was then quantified by Bio-Rad C1000 (Bio-Rad Laboratories). β -actin was amplified as an internal control. The PCR primers used were: COX-2-F (F for forward) (5'-TCAAGTCCCTGAGCATCTAC-3'), COX-2-R (R for reverse) (5'-CATTCCTACCACCAGCAACC-3'), β -actin-F (5'-GAAACTACCTCAACTCCATC-3'), and β -actin-R (5'-CG AGGCCAGGATGGAGCCGCC-3'). The amplified products of the COX-2 gene and β -actin were 488 and 219 bp, respectively. PCR was performed as follows: i) 30 min at 50°C, 5 min at 94°C, and ii) 35 cycles, with 1 cycle consisting of 55 sec at 94°C, 55 sec at 54°C, and 55 sec at 72°C. The amplified products were separated with 1% agarose gel for 30 min followed by ethidium bromide (Xiamen Sunma Biotechnology Co., Ltd., China) staining. The gel was visualized and analyzed by UVipro (UVP).

Detection of COX-2 expression by immunohistochemistry (IHC). Cancer tissue microarrays for the immunohistochemical analysis were constructed through the following method: sections (5 μ m in size) were taken from a tissue array block, affixed to 3-aminopropyl triethoxysilane-coated slides, and air-dried overnight at 37°C. After dewaxing and antigen retrieval, endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. Immunohistochemistry (IHC) was performed on the two-step plus 1 polyhorseradish peroxidase (HRP) anti-mouse/rabbit IgG detection system (ZSGB-Bio Co., Ltd., China). The monoclonal anti-COX-2 (1:1,000 dilution) was produced by our laboratory, and its specificity has been previously confirmed (18). After overnight incubation,

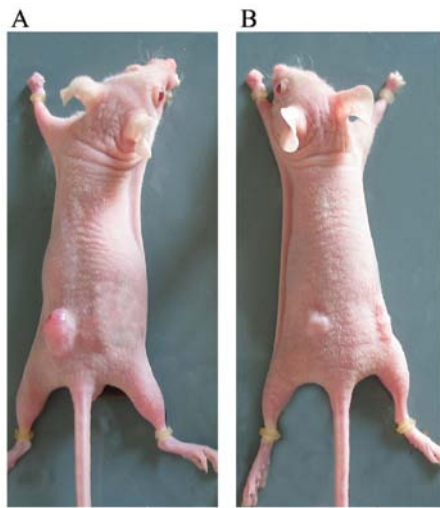


Figure 1. Establishment of nude-transplanted tumor model after 4-week post-inoculation. (A) The control group was injected with SMMC-7721/Negi; (B) the experimental group was injected with SMMC-7721/COX-2i.

the sections were washed thoroughly with PBS with 0.05% Tween-20. Subsequent steps were performed according to the manual of the aforementioned detection system. The antigen-antibody complex was visualized with the diaminobenzidine substrate.

COX-2 protein levels by western blot analysis. Fresh tissues from mice with transplanted tumors were ground and solubilized in lysis buffer (7 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 1.5% Triton X-100, 65 mmol/l dithiothreitol, 40 mmol/l Tris, 1 mmol/l phenylmethylsulfonyl fluoride, and 3 mmol/l EDTA), and then sonicated for 20 min at 0°C, and centrifuged at 12,000 x g for 15 min at 4°C. Protein concentrations were determined using the Bradford assay. For western blot analysis, 20 µg cell lysates were loaded and separated on 12% polyacrylamide gels, and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) according to a standard protocol. The blots were blocked for 1 h at room temperature in 5% skim milk. The target proteins were probed with primary antibodies and HRP-labeled secondary antibodies. β-actin was used as an indicator of equal protein loading. Antibody-positive bands were visualized using ECL Western blot detection reagents (Pierce).

Ethical approval. This study was approved by the Ethics Committees at the Medical College of Xiamen University.

Statistical analysis. All statistical analyses were performed using SPSS 13.0. ANOVA was used to determine the univariate differences among multiple groups, and the t-test was performed to probe the differences between the two different groups. $P < 0.05$ was considered to indicate significant differences. All studies were performed in triplicates or were repeated thrice.

Results

Establishment of nude-transplanted tumor model. A total of 20 BALB/c nude mice were inoculated with human HCC cells

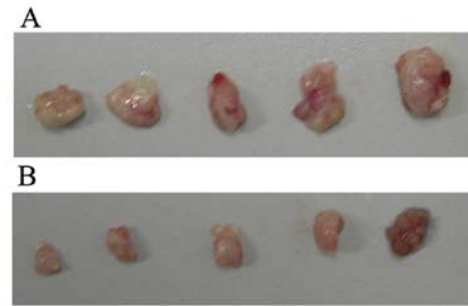


Figure 2. Nude-transplanted tumor tissues four weeks post-inoculation. (A) Control group; (B) experimental group. There was a significant difference in the tumor volumes between the two groups ($P < 0.05$).

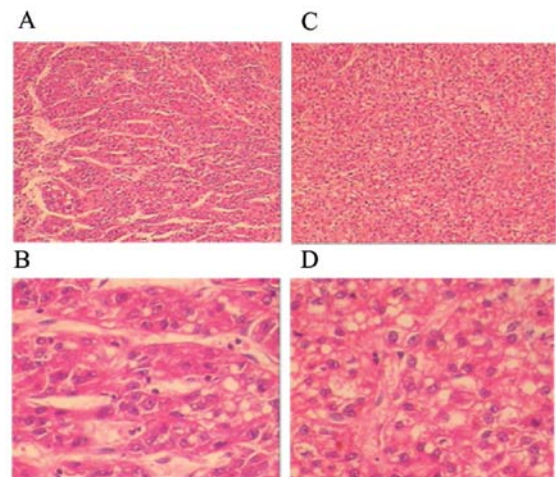


Figure 3. Sections of nude-transplanted tumors after H&E staining. (A and B) Experimental group. Cells were more highly differentiated, and tumor cells were arranged in nests; (C and D) control group. Cells were heteromorphic with multiple nucleoli. Cells were loose and irregularly arranged, (original magnification of A and C, x100; B and D, x400).

through subcutaneous injection into the dorsal flank region. The experimental group was injected with SMMC-7721/COX-2i, whereas the control group was injected with SMMC-7721/Negi. The SMMC-7721/Negi-inoculated mice developed tumors 14 days post-inoculation. After 4 weeks, the tumor tissues were separated. The rate of tumor formation was 100% in both groups. The control group formed a tumor body with an average diameter of 13 ± 3 mm, compared with the 11 ± 3 mm tumor body in the experimental group. The tumor volumes were determined by measuring the volume of water drained from the tumor tissues. The average tumor volumes of the control and experimental groups were 1087.13 ± 288.25 and 529.86 ± 154.33 mm³, respectively. There was a significant difference between the control and experimental groups ($P < 0.05$). The inhibition rate reached 51.2% (Figs. 1 and 2).

H&E staining show that the experimental group cells differentiated better than the cells in the control group. Cells in the experimental group were relatively small with lower nucleus-to-cytoplasm ratio compared with those in the control group. The blood capillary and sinus in the experimental group were more abundant as well, and the tumor cells were arranged in nests. In the control group, the tumor cell nuclei were not uniform in size and were deeply stained with less

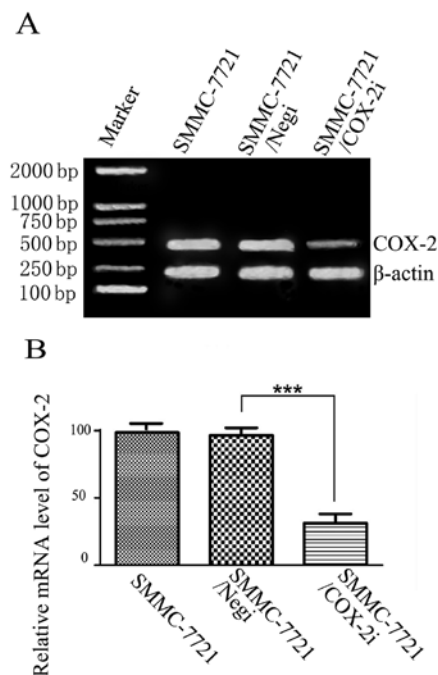


Figure 4. COX-2 mRNA levels by RT-PCR in tumor tissues. (A) Agarose electrophoresis (1%) was used to separate the RT-PCR products. The gel was visualized and quantified by UVipro (UVP); (B) the COX-2 mRNA levels in tumor tissues injected with SMMC-7721/COX-2i were markedly decreased compared with tumor tissues injected with SMMC-7721/Negi ($P<0.05$). The COX-2 mRNA levels in the control SMMC-7721 cells and in the tumor tissues injected with SMMC-7721/Negi were identical ($P>0.05$).

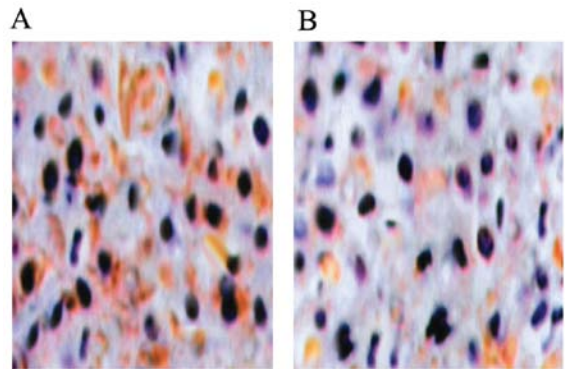


Figure 5. Immunohistochemistry was performed to detect the COX-2 expression in tumor tissues (original magnification, $\times 400$). (A) Control group. The cell cytoplasm in the control group was tawny-stained, and the nucleus was counterstained blue, indicating a strong COX-2 expression in the tumor cells; (B) experimental group. The cytoplasm was slightly stained.

intercellular substances. Cells were heteromorphic with multiple nucleoli, evident mitotic cells, and a loose irregular arrangement (Fig. 3).

COX-2 mRNA level by RT-PCR. The amplified products from RT-PCR were separated by agarose gel electrophoresis. The gel was visualized and analyzed by UVipro (UVP). The COX-2 mRNA levels in the tumor tissues injected with SMMC-7721/COX-2i were markedly lower than that in the tumor tissues injected with SMMC-7721/Negi ($P<0.05$). The inhibition rate reached 68.6%. The COX-2 mRNA levels in the control SMMC-7721 cells were identical to that in the tumor tissues

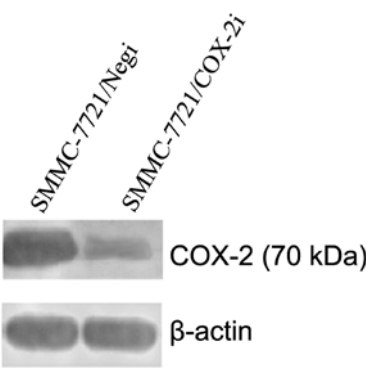


Figure 6. Detection of COX-2 expressions in the tumor tissues by western blot analysis. The COX-2 protein expression was significantly inhibited in the tumor cells in the experimental group.

injected with SMMC-7721/Negi. No significant differences were noted between these two groups ($P>0.05$). These findings indicate that the pCOX-2 shRNA vector could efficiently downregulate the COX-2 mRNA levels (Fig. 4).

Detection of COX-2 expression by IHC. The IHC results show that the cell cytoplasm in the control group was tawny-stained, and the nucleus was counterstained blue, indicating a strong COX-2 expression in the tumor cells. In the experimental group, the cytoplasm was slightly stained, and the nucleus was counterstained blue, indicating that COX-2 expression was efficiently inhibited by expressing shRNA against COX-2 (Fig. 5). To calculate the positive rate of COX-2 expression, 200 cells in each group were selected, among which the COX-2 positive cells were marked. Results show that 29.5% cells in the experimental group were COX-2 positive, whereas 78.5% were positive in the control group. Moreover, the COX-2 protein level in the experimental group was marked lower than that in the control group ($P<0.05$) (Table I).

Alteration of COX-2 protein expression determined by western blot analysis. Western blot analysis detected a deep-colored protein band with a molecular weight of 70 kDa in the control group, identical to the molecular mass of COX-2. In the experimental group, a lighter protein band was detected at the same position of 70 kDa. These findings indicate that COX-2 protein expression was significantly inhibited in the experimental group tumor of cells (Fig. 6).

Discussion

The COX-2 gene in humans localizes on the human chromosome at 1q25.2-25.3 with an overall length of 8.3 kb, consisting of 10 exons and 9 introns. The COX-2 gene is transcribed and forms a 4.5 kb mRNA, which encodes an open reading frame containing 604 amino acids with 17 amino acid residues. COX can produce various PGs through arachidonic acid metabolism, and function during physiological and pathological processes. Recent research demonstrates that there are at least two COX genes encoded in the cell, COX-1 and COX-2. COX-1 is important to maintain the normal physiological functions, such as protecting gastrointestinal cells and adjusting renal blood flow(18).

Table I. Positive rate of COX-2 protein expression in the tumor cells by immunohistochemistry.

	Total cells, n	Positive cells, n	%	Statistics	
Control group	200	157	78.5	$\chi^2=96.657$	P<0.001
Experimental group	200	59	29.5		

COX-2 is overexpressed in multiple precancerous lesions and malignant tumors, such as adenoma tumor, rectal, gastric, and breast cancer, to name a few, indicating that this gene is probably involved in tumor development. Under normal physiological conditions, the *COX-2* gene, whose expression can be induced by several intracellular or extracellular stimulations, is not expressed in most tissues and cells (19). *COX-2* upregulation is probably related to the activation of mitogen-activated protein kinase and protein kinase C (20). Existing reports indicate that the *COX-2* protein levels could dramatically increase by ten times 24 h after stimulation. The activated *COX-2* participates in multiple physiological or pathological processes by catalyzing arachidonic acid to generate various PGs. This protein is closely related to the development of inflammation and tumorigenesis. *COX-2* is of great clinical value because it could be used as a target protein for curing and preventing inflammation and tumor development. Therefore, the detection and use of a *COX-2* inhibitor to prevent tumor development have been the focus of cancer research (1,21,22). Silencing *COX-2* expression through RNAi to cure cancer is expected to be an efficient, safe, and low-cost strategy. In our previous study, we had successfully established a nude-transplanted tumor model with human gastric cancer BGC-823 cells. Tumor tissues were transfected with siRNA against *COX-2* mediated by LCP. The effects of *COX-2* on the gastric transplanted tumors in nude mice were determined. Tumor volume and the nucleus-to-cytoplasm ratio in the experimental group are smaller than those in the control group, signifying the efficient inhibition of *COX-2* expression and suppression of tumor growth. The results indicate that *COX-2* can be used in tumor gene therapy.

Traditional non-steroidal anti-inflammatory drugs, such as aspirin, indomethacin, and sulindac, exhibit anticancer effects by suppressing *COX-2* expression (23,24). However, these drugs have side effects on *COX-1* expression, inevitably generating toxicity to the gastrointestinal tract and the kidneys. *COX-2* inhibitors, such as meloxicam and SC-58635, can block tumor cell proliferation, induce tumor cells to undergo apoptosis, and improve the susceptibility of tumor cells to gamma-rays (25). Combination of a *COX-2* inhibitor and chemotherapeutics will make tumor cells more susceptible to chemotherapeutics. The development of better chemoprophylaxis with the combination of a *COX-2* inhibitor and an antioxidant reagent or iNOS inhibitor is anticipated. *COX-2* inhibitors with better characteristics, such as good curative effect, safety, and lower cost are continuously being developed. Regulation of *COX-2* expression in cancer prevention and treatment may have wide applications.

In the present study, we constructed a novel pCOX-2 shRNA vector that specifically inhibits *COX-2* expression, as well as the negative control vector pNegative-shRNA. We used our patent-protected Sofast reagent to transfect the human HCC cell line

SMMC-7721. We obtained the SMMC-7721/*COX-2i* cell line that could stably express *COX-2* shRNA and the SMMC-7721/Negi that could stably express pNegative-shRNA. We inoculated BALB/c mice with these two stable cell lines. After 4 weeks, all mice in the two groups successfully generated tumors. The tumor tissues were then separated. The tumor volume in the experimental group was smaller than that in the control group. H&E staining indicates that the cells in the experimental group differentiated better than the cells in the control group. The nuclei in the cells of the control group were significantly larger than those in the experimental group, indicating that expressing shRNA against *COX-2* could efficiently inhibit tumor growth. The *COX-2* mRNA levels in tumor tissues injected with SMMC-7721/*COX-2i* were markedly lower than that in tumor tissues injected with SMMC-7721/Negi, and the inhibition rate reached 68.6%. The immunohistological study showed a significantly stronger *COX-2* expression in the tumor tissues from the control group, whereas *COX-2* expression in the experimental group was inhibited. Western blot analysis further confirmed the alteration of the *COX-2* protein level between the two groups. Results indicate that expressing shRNA against *COX-2* could efficiently silence the target gene expression efficiently.

The relationship between *COX-2* expression and liver cancer development has recently been well elucidated (26,27). Existing literature indicates that a *COX-2*-specific inhibitor could suppress tumor growth both *in vitro* and in an HCC animal model. However, all these inhibitors have not yet been clinically applied (28,29). Researchers have found that *COX-2* inhibitors could adversely affect cardiovascular and renal functions (30). Use of RNAi instead of *COX-2* inhibitor to regulate *COX-2* expression, is expected to decrease toxicity and presents a new method for preventing and curing tumors through regulation of *COX-2* expression, which merits further investigation.

Acknowledgements

This study was supported by the National Natural Science Foundation's Major Research Planning (grant no: 91029729), the National Natural Science Foundation (grant no. 81171625, 81101324), and the Technology Foundation's Major Project of Social Development in Fujian Province (grant no. 2011Y4009).

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