RESEARCH ARTICLE

Targeting of COX-2 Expression by Recombinant Adenovirus shRNA Attenuates the Malignant Biological Behavior of Breast Cancer Cells

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Abstract

Background: Cyclooxygenase-2 (COX-2), considered to have tumor-promoting potential, is highly expressed in a variety of tumors, including breast cancer. Since the functions and action mechanisms of COX-2 in breast cancer have not been fully elucidated, in the present study, the effects of target inhibiting COX-2 with recombinant adenovirus Ad-COX-2-shRNA on malignant biological behavior were investigated in representative cell lines. Materials and Methods: Breast cancer MDA-MB-231 and MCF-7 cells were transfected with Ad-COX-2-shRNA and COX-2 expression was tested by RT-PCR and Western blotting. Changes in proliferation, apoptosis and invasion of breast cancer cells were detected with various assays including MTT, colony forming, flowcytometry and Transwell invasion tests. The expression of related proteins involved in the cell cycle, apoptosis, invasion and signaling pathways was assessed by Western blotting. Results: COX-2 expression was significantly reduced in both breast cancer cell lines infected with Ad-COX-2-shRNA, with obvious inhibition of proliferation, colony forming rate, G2/M phase passage and invasion, as well as induction of apoptosis, in MDA-MB-231 and MCF-7 cells, respectively. At the same time, proteins related to the cell cycle, anti-apoptosis and invasion were significantly downregulated. In addition, c-myc expression and phosphorylation activation of Wnt/β-catenin and p38MAPK pathways were reduced by the Ad-COX-2-shRNA. Conclusions: COX-2 expression is associated with proliferation, apoptosis and invasion of breast cancer cells, and its mechanisms of action involve regulating expression of c-myc through the p38MAPK and Wnt/β-catenin pathways.

Keywords: COX2-shRNA - proliferation - apoptosis - invasion - breast cancer cells - signaling pathways

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Introduction

Breast cancer is one of the most common woman malignancies in the worldwide (Parkin et al., 2005). Because many breast cancer patients are diagnosed at an advanced stage of cancer, in which invasion and metastasis have already occurred, the 5 years of survival rate for breast cancer has shown limited improvement in the past twenty years. However, mechanisms of invasion and metastasis in breast cancer remain poorly understood, and it is necessary to gain a better understanding of the underlying causes in order to develop better targeted treatments.

Cyclooxygenase 2 (COX2), an inducible form of cyclooxygenases which are the rate limiting enzyme in the production of prostaglandins (PEGs), is considered to be involved in inflammation and progression of various carcinomas including breast cancer (Holmes et al, 2011; Siegfried et al., 2007; Singh et al., 2011). The up-regulation of COX2 has found to participate in the growth, invasion and suppress the apoptosis as well as increased motility and invasion of breast cancer (Singh et al., 2006; Siegfried et al., 2007; Su et al., 2009 Singh et al., 2011). The polymorphisms of COX-2 gene are related with the cancer susceptibility (Shalaby et al., 2014). Furthermore, nonsteroidal anti-inflammatory drugs (NSAIDs) are beneficial in breast cancer therapy. The selective and non-selective inhibitors of COX2 such as aspirin, nimesulide and celecoxib were experimented as prevention and therapeutic agents against breast cancer and other tumors (Bresalier et al., 2005; Harris et al., 2005; Siegfried et al., 2007; Cronin-Fenton et al., 2010). Other research showed that nimesulide also could restrain the activity and expression of aromatase in some breast cancer cell lines (Su et al., 2009). However, the clinical use of classical nonselective COX-2 inhibitors is often limited by side-effects such as gastrointestinal ulcers thought to result from COX-1-inhibition (Harris et al.,

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2005), and selective COX-2 inhibitors also caused lethal cardiovascular untoward reaction (Bresalier et al., 2005).

Recently, molecular targeted therapy, which acts on specific deregulated signal transduction pathways, has shown some advantages on the treatment of cancer (Llovet et al., 2008). The RNA interference (RNAi) is a useful way of inhibiting target gene expression after gene transcription (Schutze et al., 2004). Furthermore, recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications in target gene therapy way as well as investigate the molecular mechanism (He et al., 1998).

C-myc is one of the most commonly upregulated oncogenes in cancer (Ferguson et al., 2012). It plays an important role in the malignant biological behaviors (Janghorban et al., 2014). Studies have focused on c-myc's effect on regulatory proteins of the G1-S phase transition of the cell cycle. This transition is promoted when cyclin dependent kinases are activated. C-myc directly promotes cyclin D1 expression (Asara et al., 2013; Daksis et al., 1994), and cyclin D1 activation kinases regulate the phosphorylation of retinoblastoma protein (pRb) (Hochegger et al., 2008). Recent advances strongly support that C-myc plays multiple functions on cell proliferation, apoptosis and invasion (Han et al., 2012). Matrix metalloproteinases (MMPs), the zinc containing enzymes to degrade extracellular matrix components, play a crucial role in the invasion and metastasis of cancer cells. MMP-9 from the family is constantly upregulated in a variety of cancers (Jovanovic et al., 2010), and this has aroused our interest to investigate the correlation of COX-2 and MMP-9 in breast cancer.

C-myc promoter activation may be controlled by several kinase-mediated pathways such as extracellular signal-regulated kinase 1/2 (ERK1/2) (Bermudez et al., 2008) and intracellular signal p38MAPK (Chen et al., 2006). Wnt/β-catenin is one of the intracellular signaling pathways that may control transcription factor binding to the c-myc promoter. Interestingly, the Wnt/β-catenin pathway has been reported to be a target of COX-2 inhibitors (Xia et al., 2010).

In current study, we investigated the effects of target inhibiting COX-2 with recombinant adenovirus Ad-COX-2-short hairpin RNA (Ad-COX-2-shRNA) on the breast cancer cell malignant biological behaviors including proliferation, cell cycle, apoptosis and invasion, and explored whether the mechanism involves regulating c-myc expression.

Materials and Methods

Cell culture, medium and reagents

The breast cancer cell MDA-MB-231 and MCF-7 was obtained from the Key Laboratory of Laboratory Diagnostics of Education Ministry (Chongqing Medical University, Chongqing City, China). The cells were cultured in DMEM (Gibco, Grand Island, NY USA) supplemented with 10% FBS and 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA USA) at 37°C with 5% CO_2.

Infection of MDA-MB-231 and MCF-7 cells by Ad-COX-2-shRNA

The recombinant adenoviruses Ad-COX-2-shRNA and empty control Ad-HK were kindly provided by Dr. Dong in our university (Dong et al., 2013). When approximately 80% to 90% fused, the MDA-MB-231 and MCF-7 cells were infected by prepared Ad-COX-2-shRNA or Ad-HK respectively. Cells were examined for the green fluorescent protein (GFP), which was a marker carried by Ad-COX-2-shRNA and Ad-HK, with fluorescence microscopy (Nikon Eclipse TE200; Nikon Corp., Tokyo, Japan; excitation and emission filters were 490 and 550nm) 48 hours after transfection to identify whether the cells were successfully transfected.

Cell viability assay

The MTT assay was performed in hexaploid to assess the viability of breast cancer cells infected by Ad-COX-2-shRNA and Ad-HK. About 3,000 of each group of cells in 300μl of the medium were seeded in 96-well plates. About 20μl of the MTT reagent (5mg/ml; Progema, Madison, WI, USA) was added, and the mixture was incubated for 1 h. Absorbance was measured at 590nm using a microplate reader daily for the following 5 days, then the growth curve was drawn.

Colony-forming assay

The two breast cancer cells from each group were collected and seeded in triplicate in a soft agar medium at 200 cells/culture dish for 10-14 days. When the clone was observed, the cells were washed twice by PBS and stained by the Wright's method. The colony forming rate was obtained as: (colony number/seeded cell number) ×100%. Each experiment was repeated thrice.

Cell cycle and apoptosis analysis

The two breast cancer cells from each group were harvested by centrifugation. After being washed twice with ice-cold phosphate buffered saline (PBS; pH 7.4) and resuspended, cell cycle distribution was analyzed by flowcytometry with PI, apoptosis was analyzed by flowcytometry with AnnexinV-PE/7-AAD and the CellQuest software package. Each experiment was triplicate.

Transwell invasion assay

Invasion assays were performed with 8 μm-pore Transwell chambers (Corning, NY, USA) as described with some modification (Marra et al., 2009; Wei et al., 2010). The breast cancer cells from each group were cultured at 3×10^5/well in the upper chamber of type I-collagen coated 24-well culture inserts in triplicate. After 24 h, the cells were dried for 5 min, fixed with dehydrated alcohol, and stained with hematoxylineosin. The cells that invaded the collagen coated-inserts were counted. Mean values for five randomly selected fields were obtained for each well. The experiments were repeated thrice.

PCR analysis

Total RNA was extracted from MDA-MB-231 and

MCF-7 cells using Trizol (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized from 1μg of total RNA with a Prime Script Kit (TAKARA, Dalian, China). COX-2 gene expression was quantified by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), β-actin was used as an endogenous control. All samples were run in triplicate for each experiment. Gene expression analysis was performed with the Quantity One Software (BIO-RAD, USA).

The PCR conditions were: 95° for 30 s, followed by 30 cycles of 95° for 10 s, 55° for 30 s and 72° for 20 s. The mRNA expression levels of the target gene were normalized to those of β-actin. The specific primers for COX-2 were 5'-AGTCCCTGAGCATCTACGGTTTG-3' (forward) and 5'-CCTATCAGTATTAGCCTGCTTGTCT-3' (reverse). Those for β-actin were 5'-CCTTCTACAATGAGCTGCGT-3' (forward) and 5'-CCTGGATAGCAACGTACATG -3' (reverse).

Western blot analysis

The MDA-MB-231 and MCF-7 cells were infected by Ad-COX-2-shRNA or Ad-HK. After 12 hr at 37° C, the cells were lysed with RIPA buffer consisting of 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate and Complete Proteinase Inhibitor Mixture Tablets (Roche Applied Science, German). Protein concentration was determined with the BCA Assay (Beyotime, China). Equal amount of total protein was separated with 10% SDS polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane. Membranes were incubated overnight in TBS containing 0.1% Tween-20 and 5% dried skim milk at 4°C to block the nonspecific binding site. Then membranes were incubated overnight with primary antibodies at 4°C and finally with the appropriate secondary antibodies for 2 h at room temperature. Signal pathways were detected using an enhance chemiluminescence (ECL) kit (Millipore, USA). Quantification of protein signals was performed by the Gel-Pro Analyzer software (Quantity One, USA). Primary antibodies used in the western blot analysis were human COX-2, β-actin, cyclinD1, PCNA, bcl-2, survivin, MMP-9, c-myc, P-P38MAPK, P38MAPK, β-catenin, P-GSK-3β and GSK-3β (Santa Cruz, CA USA). The specific pathway inhibitors used in the Western blot analysis were SB203580 and LY294002 (Beyotime, China).

Statistical analyses

Results were expressed as means±SD. All statistical analyses were performed by SPSS 16.0 using the independent sample t test for comparing the two sample groups. For all tests, $P<0.05$ was considered statistically significant.

Results

Changes of COX-2 mRNA and protein expression in breast cancer cells after recombinant adenovirus infection

The breast cancer cells were infected by Ad-COX-2-shRNA and Ad-HK, respectively. After 48 hr, there were about 95% of the MDA-MB-231 cells and 99% of the MCF-7 cells were transfected, which were indicated by GFP expression cells (data not shown). However, the infection efficiency of the recombinant adenovirus of Ad-COX-2-shRNA and Ad-HK in MDA-MB-231 and MCF-7 cells was basically consentaneous. The expression of COX-2 mRNA was analyzed by RT-PCR. Figure 1 A-C displayed that the expressions of COX-2 mRNA were significantly decreased in both breast cancer cell lines infected by Ad-COX-2-shRNA compared to the Ad-HK and blank groups. Consistently, the expressions of COX-2 protein were also significantly reduced in both breast cancer cell lines infected by Ad-COX-2-shRNA compared to the Ad-HK and blank groups analyzed by Western blot (Figure 1D).

Recombinant adenovirus inhibited the proliferation and induced the apoptosis of breast cancer cells

The effect of Ad-COX-2-shRNA on the viability of breast cancer cells was detected by the MTT assay. It was shown that Ad-COX-2-shRNA effectively reduced both of the proliferation and colony-forming in breast cancer cells. The cell proliferation was significantly inhibited at

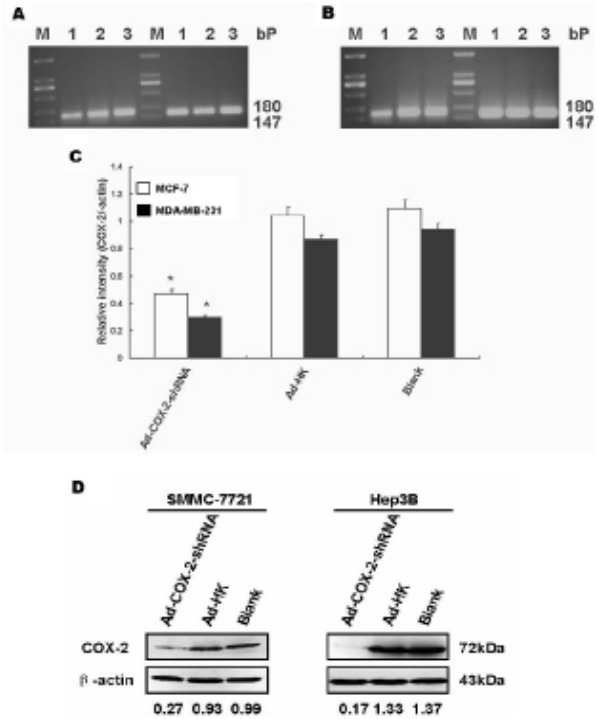

Figure 1. Ad-COX-2-shRNA Reduced the Expressions of COX-2 mRNA and Protein in Breast Cancer Cells. A and B: RT-PCR identification of downregulated COX-2 mRNA expression by Ad-COX-2-shRNA. The mRNA levels of COX-2 were decreased in MCF-7 (A) and MDA-MB-231 (B) cells infected by Ad-COX-2-shRNA. The lane M was the marker, lane 1 was Ad-COX-2-shRNA group, lane 2 was Ad-HK group, and lane 3 was blank group. The 180 bp and 147 bp bands were the COX-2 and β-actin. C: The relative intensity was indicated by the ratio of COX-2 mRNA/β-actin mRNA. D: Western blot showed the downregulated COX-2 protein expression by Ad-COX-2-shRNA. The 72 kDa and 43 kDa bands were the COX-2 and internal control β-actin protein band respectively, and the relative intensity of COX-2/β-actin was calculated. A, B and E showed one representative result of three independent experiments with similar results, respectively

Table 1. The Effects of Ad-COX-2-shRNA on Colony Forming, Cell Cycle and Apoptosis of Breast Cancer Cells (X±SD, n=3)

Cell line	Treatment	Colony forming rate (%)	Cell cycle phase (%)		Apoptosis rate (%)
			G0/G1	G2/M	
MDA-MB-231	Blank	94.0±4.6	51.8±3.7	19.1±1.7	1.5±0.2
	Ad-HK	91.0±5.1	50.2±3.4	20.5±2.3	1.3±0.4
	Ad-COX-2-shRNA	48.6±3.1*	68.5±2.3*	6.5±0.4*	7.9±1.8*
MCF-7	Blank	123.6±7.4	26.4±1.3	29.3±2.1	2.9±0.8
	Ad-HK	118.3±8.2	27.1±2.5	27.8±2.4	3.1±0.8
	Ad-COX-2-shRNA	17.7±2.6*	52.2±4.1	4.5±1.2*	25.8±2.6*

*P<0.05 vs blank and Ad-HK groups, respectively.

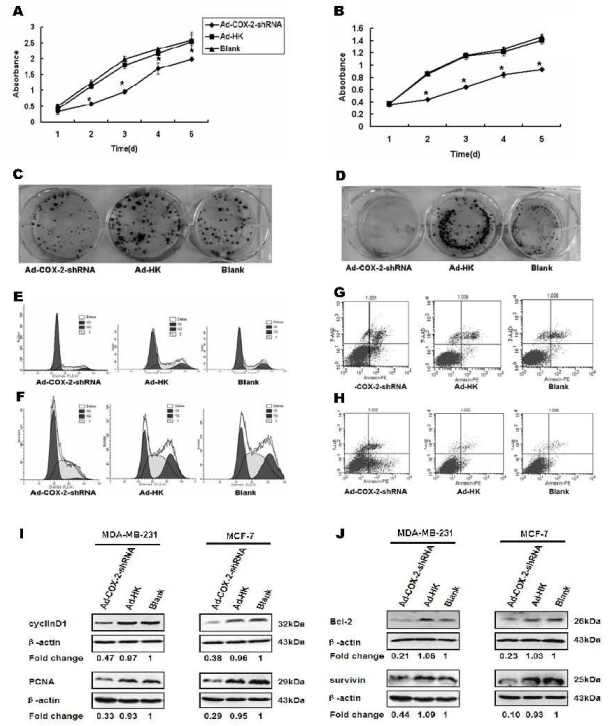

Figure 2. Effects of Ad-COX-2-shRNA on the Growth, Cell Cycle and Apoptosis in Breast Cancer Cells. A: The Ad-COX-2-shRNA inhibited the proliferation rate of MDA-MB-231 cells (*P < 0.05). B: The proliferation rate of MCF-7 cells was attenuated by Ad-COX-2-shRNA (*P < 0.05). C and D: The colony-forming assay results of MDA-MB-231 cells (C) and MCF-7 cells (D). E and F: The cell cycle distribution by flowcytometry analysis in MDA-MB-231 cells (E) and MCF-7 cells (F). Ad-COX-2-shRNA blocked the cells at the G0/G1 phase and resulted in significant reduction of G2/M phase cells. G and H: The apoptosis percentage was obviously increased in MDA-MB-231 cells (G) and MCF-7 cells (H) infected by Ad-COX-2-shRNA. I: The levels of cell cycle regulators (cyclin D1 and PCNA) determined with Western blot were decreased in breast cancer cells by Ad-COX-2-shRNA. J: The antiapoptosis-related proteins (bcl-2, survivin) were reduced by Ad-COX-2-shRNA in breast cancer cells. C-J showed one representative result of three independent experiments with similar results, respectively

Table 2. The Effects of Ad-COX-2-shRNA on Invasion of Breast Cancer Cells with Transwell Chamber Test (X±SD, n =3)

Cell line	Treatment	Invasive cell number
MDA-MB-231	Blank	352.4±20.8
	Ad-HK	341.6±17.3
	Ad-COX-2-shRNA	121.5±10.7*
MCF-7	Blank	162.5±15.6
	Ad-HK	151.2±14.3
	Ad-COX-2-shRNA	70.0±6.6*

*P<0.05 vs blank and Ad-HK groups, respectively.

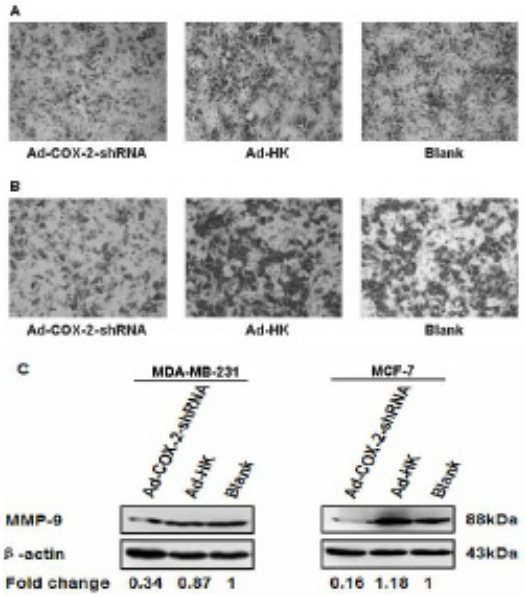

Figure 3. Decreased Invasiveness and MMP-9 Expression of Breast Cancer Cells Caused by Ad-COX-2-shRNA. A: The amount of invasive cells was notably decreased in the MDA-MB-231 cells infected by Ad-COX-2-shRNA with Transwell invasion assay (×100). B: Ad-COX-2-shRNA reduced the amount of invasive cells in MCF-7 cells (×100). C: Ad-COX-2-shRNA downregulated the invasiveness-related protein MMP-9 expression of breast cancer cells, and the relative intensity of MMP-9/β-actin was calculated. The data were representative of three independent experiments showing similar results

2-5 days after recombinant adenovirus Ad-COX-2-shRNA infection (P<0.05) (Figure 2 A and B). The colony-forming rate of the MDA-MB-231 and MCF-7 cells infected by the Ad-COX-2-shRNA was also significantly decreased (P<0.05) (Figure 2 C and D, Table 1).

The effect of Ad-COX-2-shRNA on cell cycle distribution and apoptosis in the two breast cancer cell lines was analyzed by flow cytometry. Apparently, Ad-COX-2-shRNA led to an accumulation of G0/G1 phase and a profound reduction of G2/M phase in both breast cancer cell lines after Ad-COX-2-shRNA treatment for 48h (P<0.05) (Figure 2 E and F, Table 1). Furthermore, the apoptosis rate was significantly higher than those of control groups in MDA-MB-231 and MCF-7 cells treated by Ad-COX-2-shRNA (P<0.05) (Figure 2G and H, Table 1).

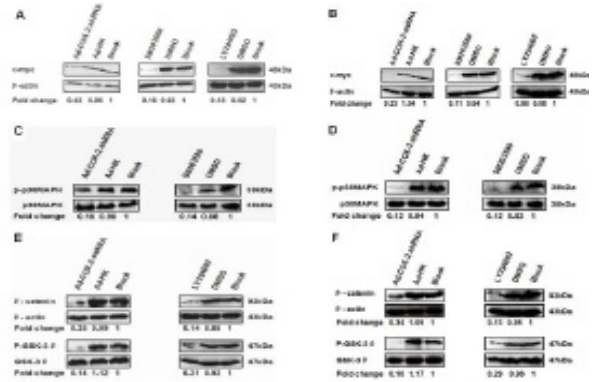

Figure 4. Ad-COX-2-shRNA Inhibited the Expression of C-myc Protein through p38MAPK and Wnt/β-catenin Signaling Pathways. A and B: The c-myc protein expression was inhibited by Ad-COX-2-shRNA, p38MAPK inhibitor and Wnt/β-catenin inhibitor in MDA-MB-231 cells (A) and MCF-7 cells (B). C: The p38MAPK pathway was inhibited by Ad-COX-2-shRNA and p38MAPK inhibitor in MDA-MB-231 cells. D: The p38MAPK pathway was inhibited by Ad-COX-2-shRNA and p38MAPK inhibitor in MCF-7 cells. E: The Wnt/β-catenin pathway was attenuated by Ad-COX-2-shRNA and the Wnt/β-catenin inhibitor in MDA-MB-231 cells. F: The Wnt/β-catenin pathway was inhibited by Ad-COX-2-shRNA and the Wnt/β-catenin inhibitor in MCF-7 cells. The data were representative of three independent experiments showing similar results

Ad-COX-2-shRNA affected the expressions of cell cycle and apoptosis regulators in MDA-MB-231 and MCF-7 cells

The protein levels of a panel of factors that specifically act on the cell cycle phase transition and apoptosis were analyzed. It was showed that cell cycle regulating factors were changed by Ad-COX-2-shRNA in breast cancer cells (Figure 2I). In Ad-COX-2-shRNA treated MDA-MB-231 cells (left), the changes of cell cycle were accompanied by a downregulation of cyclin D1 and PCNA. The same modulation of these factors was also found in MCF-7 cells (right). These results have indicated that cyclin D1 and PCNA are as part of the modulatory mechanism triggered by COX-2 on cell cycle of breast cancer cells.

With regard to apoptosis, several studies have shown that treatment with multi-kinase inhibitor sorafenib is frequently associated with the downregulation of antiapoptotic Bcl-2 family member bcl-2 and survivin (Tai et al., 2011). In our study, the changes of apoptosis rate increase were accompanied with the downregulation of both major antiapoptotic proteins in breast cancer cells by Ad-COX-2-shRNA compared to the control groups (Figure 2J). It has been suggested that Bcl-2 and survivin are involved in the induction of apoptosis of breast cancer cells treated by Ad-COX-2-shRNA.

Ad-COX-2-shRNA decreased the invasion and MMP-9 expression of breast cancer cells

The effect of recombinant adenovirus Ad-COX-2-shRNA on the invasion of breast cancer cells was determined by Transwell chamber experiment. As showed in Figure 3 and Table 2, the Ad-COX-2-shRNA significantly decreased the invasive cells of breast cancer cells compared with control groups ($P<0.05$).

The invasiveness-related protein expression of MMP-9 was detected by Western blot, and it was noted that the expression of MMP-9 was downregulated in both breast cancer cell lines infected by recombinant adenovirus (Figure 3C). These results have indicated that Ad-COX-2-shRNA could effectively decrease the invasiveness of breast cancer cells accompanied with inhibiting the MMP-9 protein expression.

Ad-COX-2-shRNA impaired c-myc expression of breast cancer cells through p38MAPK and Wnt/β-catenin signaling pathways

Recent work has established a role for c-myc in cell cycle progression, metabolism, apoptosis and genomic instability (Hoffman et al., 2008). In present study, analysis of c-myc protein showed that Ad-COX-2-shRNA strongly downregulated the c-myc protein expression in breast cancer cells. Similar effects were found by p38MAPK inhibitor SB203580 and Wnt/β-catenin inhibitor LY294002, respectively (Figure 4A and B). Above results indicated that Ad-COX-2-shRNA as well as p38MAPK and Wnt/β-catenin inhibitors impaired c-myc expression of breast cancer cells, therefore, we further investigated whether COX-2 regulated the c-myc expression through p38MAPK and Wnt/β-catenin signaling pathways.

The Ad-COX-2-shRNA obviously inhibited p38MAPK phosphorylation (Figure 4C) with a similar effect of the specific p38MAPK inhibitor SB203580 (60μM, 1h of treatment), and Wnt/β-catenin phosphorylation was also inhibited (Figure 4E) by Ad-COX-2-shRNA and the specific inhibitor LY294002 (70μM, 1 h of treatment) in MDA-MB-231 cells. Consistently, in MCF-7 cells, we also observed that Ad-COX-2-shRNA profoundly inhibited p38MAPK phosphorylation with a similar effect of p38MAPK inhibitor SB203580 (40μM; 1 h of treatment) (Figure 4D), and Wnt/β-catenin phosphorylation was inhibited by Ad-COX-2-shRNA and the specific inhibitor LY294002 (50μM; 1 h of treatment) (Figure 4F). These findings have proven that target inhibiting COX-2 could downregulate the c-myc protein expression through the p38MAPK and Wnt/β-catenin pathways in breast cancer cells.

Discussion

Experimental studies and clinical trials have demonstrated that COXIBs show antitumor activities in breast cancer and other tumors (Cervello et al., 2004; Fodera et al., 2004; Cusimano et al., 2007; Cronin-Fenton et al., 2010). However, the chemotherapy approach to cancer treatment is often hindered by the lack of specificity and side effects (Bombardier et al., 2000; Niederberger et al., 2004).

The advancement in the field of RNA interference (RNAi) has opened up a new strategy to target silence genes involved in tumour progression which may avoid these side effects (Charames et al., 2006; Strillaeeit et al., 2006). Besides the selection of target gene, the choice of carrier is equally important for cancer gene therapy, and efficient gene delivery system is the basis of gene therapy. The adenoviruses, lentivirus, retrovirus,

lipidosome and so on can be used as RNAi vectors. Recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications. Adenoviral vectors have a high transduction efficiency, are capable of containing DNA inserts up to 8 kB, have extremely high viral titers (on the order of 10^{10}-10^{13}), and infect both replicating and differentiated cells. Also since they lack integration, they can not bring about mutagenic effects caused by random integration into the host genome like lentivirus and retrovirus. In most recombinant vectors, transgenes are introduced in place of E1 or E3, the former supplied exogenously. The E1 deletion renders the viruses defective for replication and incapable of producing infectious viral particles in target cells; the E3 region encodes proteins involved in evading host immunity and is dispensable for viral production perse (He et al., 1998).

Therefore, recombinant adenovirus Ad-COX-2-shRNA was used in current study, and the expression of COX-2 protein was significantly silenced by it. The infection efficiency and target gene inhibition efficiency of Ad-COX-2-shRNA on both breast cancer cell lines were good. It provided a foundation for our further study of the function and action mechanism of COX-2. The COX-2 overexpression has been linked to promote cell proliferation and increase the motility and invasion of breast cancer (Sobolewski et al., 2010). To study the anti-carcinogenesis effects of Ad-COX-2-shRNA on breast cancer, the malignant biological behaviors were investigated by a series of *in vitro* experiments including proliferation curve, colony-forming rate, apoptosis percentage, cell cycle distribution and invasiveness. The proliferation curves showed that the growth of MDA-MB-231 and MCF-7 cells was effectively inhibited after Ad-COX-2-shRNA infected. Consistently, the colony-forming rate, which indicated the growth ability of individual cancer cell, was also significantly reduced in both breast cancer cell lines treated with Ad-COX-2-shRNA. On the other hand, the breast cancer cells infected by Ad-COX-2-shRNA were arrested in the G0/G1 phase accompanied with the significant decrease of G2/M phase cells. The percentage of apoptotic cells significantly increased. These results suggested that the growth inhibition of breast cancer cells by Ad-COX-2-shRNA resulted from the interference transition of G1 to S phase, which caused the reduction of G2/M cells, and the induction of apoptosis. Moreover, one of the most important properties of cancer infiltration and metastasis is the ability to degrade and move through extracellular ground substances. In our study, the cellular invasive abilities of breast cancer cells infected by Ad-COX-2-shRNA were significantly decreased. All above results have proven that COX-2 plays an important role in the carcinogenesis of breast cancer, and targeting silence COX-2 gene could effectively inhibit the growth and invasiveness, and facilitate the apoptosis. Our results were consistent with the previous studies with COX-2 selective inhibitor (Fodera et al., 2004).

In the literature, an accumulation of cancer cells in G0/G1 has been reported to be a consequence of treatment with COX-2 inhibitors. The selective COX-2 inhibitor 48 NS-398 has been demonstrated to exert effects, including a reduction of cyclin D1 and PCNA, thus perturbing the G1/S transition (Sobolewski et al., 2011). In current study, Ad-COX-2-shRNA was downregulated the expressions of PCNA and cyclin D1 proteins following the growth inhibition in breast cancer cells. Our data supported above study. Moreover, the COX-2 has been found to promote apoptosis resistance e.g. by altering the relative levels of survivin and of pro- and anti-apoptotic proteins of the Bcl-2 family in human breast cancer cells, lung adenocarcinoma cells and rat intestinal epithelial cells, respectively (Lin et al., 2001; Komatsu et al., 2005; Song et al., 2014). It has been reported that selective COX-2 inhibitor celecoxib could interfere with the prostaglandin (PG)-mediated upregulation of anti-apoptotic proteins (Lin et al., 2001; Patel et al., 2002). However, some studies have found that the pro-apoptotic effects of celecoxib do not critically rely on COX-2 inhibition in hematopoietic, epithelial and prostate cancer cells (Waskewich et al., 2002).

To further understand the effect of COX-2 on the expression of apoptosis related proteins in breast cancer cells, the Ad-COX-2-shRNA was transfected into breast cancer cells, the percentage of apoptotic cells was increased about 5~8 times, and antiapoptotic protein levels of survivin and Bcl-2 were significantly decreased in both of MDA-MB-231 and MCF-7 cells. Our results have supported that COX-2 plays some important role in the apoptosis through upregulating the expression of survivin and Bcl-2 in breast cancer cells.

In addition to cell proliferation and apoptosis, COX-2/PGE2 pathway is also involved in tumor invasiveness and metastasis (Murata et al., 1999; Shirahama et al., 2001; Half et al., 2002). The process of tumor cell invasion requires the ability of the cells to release MMPs, and a good correlation has been found between the expression of MMP and the invasive ability of various malignant tumors (Jovanovic et al., 2010). MMP-9, one of the two IV collagenase types of MMPs, has been extensively studied in human cancers, and a large body of evidence indicates its expression correlates well with tumor invasion and metastasis. Our results demonstrated that when COX-2 was specifically silenced by Ad-COX-2-shRNA, the invasion ability was decreased, and the expression of MMP-9 was also significantly downregulated in MDA-MB-231 and MCF-7 cells. These findings suggest that the expression of MMP-9 regulated by COX-2 correlates with the invasiveness of breast cancer cells.

However, the mechanisms of increased related proteins of cell proliferation, invasiveness, metastasis and apoptosis caused by COX-2 have largely been unknown, which led us to investigate the possible impacts of the Ad-COX-2-shRNA on upstream transcription factors regulating the expression of the related proteins. One of these factors is the oncogene c-myc, which is known to be a positive regulator of cyclin D1 and PCNA transcription (Daksis et al., 1994). C-myc also regulates the expressions of bcl-2 and survivin in hepatic cancer cells (Yamanaka et al., 2006). However, the relation of c-myc and bcl-2, survivin was not still fully clear. In our research, the c-myc, PCNA, cyclin D1, bcl-2 and survivin were all decreased in breast cancer cells infected by Ad-COX-2-

shRNA. Furthermore, it was reported that the MMP-2 and MMP-9 were indirectly upregulated by c-myc (Han et al., 2012). Our experiments showed that following the c-myc decrease, the expression of MMP-9 was downregulated in breast cancer cells infected by the Ad-COX-2-shRNA. Hence, it has been speculated that the expression of c-myc mediated by COX-2 is involved in the expression regulation of proliferation, invasiveness and apoptosis resistance related proteins in breast cancer cells. However, the c-myc was only one of the transcription factors. There are other transcription factors that regulate related proteins of cell proliferation, invasiveness and apoptosis resistance. Certainly, this requires further experimental verification.

According to previous reports, the COX-2 was able to activate the c-myc promoter through the p38MAPK and Wnt/β-catenin pathways in normal and other cancer cells (Chen et al., 2006; Xia et al., 2010). In present study, when c-myc expression was downregulated by target silencing COX-2 with Ad-COX-2-shRNA, the phosphorylated activations of p38MAPK and GSK3-β which stabilized β-catenin in Wnt/β-catenin signal pathway were obviously inhibited in breast cancer cells. Consistently, similar effects were observed with the specific phosphorylation inhibitors of p38MAPK and Wnt/β-catenin pathways, respectively. These results have supported that COX-2 is able to activate the c-myc promoter through the p38MAPK and Wnt/β-catenin pathways in breast cancer cells. However, besides p38MAPK and Wnt/β-catenin pathways, other signal pathways are also involved in the regulation of c-myc, such as ERK1/2 signal pathway (Bermudez et al., 2008). Further studies are needed to identify all the mechanisms responsible for the c-myc alterations.

In summary, our investigations have demonstrated that COX-2 expression is associated with the proliferation, apoptosis and invasion of breast cancer cells. The mechanism involves regulating the expression of c-myc through the activation of p38MAPK and Wnt/β-catenin pathways, and then results in the expression regulation of related downstream proteins. The COX-2 has profound potentials in the tumorgenesis and target therapy of breast cancer, and further explorations may lead to novel prevention and therapeutic approaches for breast cancer.

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