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Effects of the Inhibition of Cyclooxygenase-2 on Human Esophageal Cancer Cells: Inhibition of Cell Proliferation and Induction of Apoptosis

Li Zhang · Jun Tu · Zhong-lin Yu · Yong-dong Wu · Cai-min Xu · Shu-tian Zhang

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Abstract Cyclooxygenase-2 (COX-2) has been shown to be upregulated in a variety of tumors so that COX-2 may be a potential target in the treatment of cancer. In order to further explore the mechanism, we used RNA interference to study effects of the inhibition of COX-2 on esophageal squamous cell carcinoma (ESCC) lines. Western blot analysis demonstrated that COX-2 expression was significantly reduced in ESCC cells treated with the COX-2-specific siRNA. Furthermore, the COX-2 siRNA treatment inhibited cell proliferation and induced apoptosis in ESCC cells. In addition, the combination treatment of COX-2 siRNA and acidum acetil salicylicum (aspirin) has a synergistic effect. Therefore, this combination has potential as an anticancer therapy for the treatment of ESCC.

Keywords Esophageal squamous cell carcinoma (ESCC) · Cyclooxygenase-2 (COX-2) · Acidum acetil salicylicum (aspirin) · Cell proliferation · Apoptosis · Synergistic effect

L. Zhang · Z.-l. Yu · Y.-d. Wu · S.-t. Zhang (✉)
Department of Digestive Diseases, Beijing Friendship Hospital, Capital University of Medical Sciences, Beijing 100050, China
e-mail: niuziyue1@hotmail.com

J. Tu
Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249, USA

C.-m. Xu
Department of Biochemistry and Molecular Biology, National Laboratory of Medical Molecular Biology, Institute of Basic Medicine, Chinese Academy of Medical Sciences, Beijing 100005, China

L. Zhang
Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198, USA

Abbreviations

COX-2	Cyclooxygenase-2
ESCC	esophageal squamous cell carcinoma
NSAIDs	non-steroidal anti-inflammatory drugs
PGE2	prostaglandin E2
BCA	bicinchoninic acid
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
PI	propidium iodide
ASP	acidum acetil salicylicum

Introduction

Esophageal cancer is one of the most common and devastating cancer types worldwide. It can be divided into two major groups: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. Epidemiological studies indicate that ESCC is dominant in Asian countries, especially China, Japan and Korea [1]. Given the high fatality rate and the rapidly increasing incidence of ESCC, the identification of potential therapeutic agents is highly desirable.

Recent studies have indicated that the use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of cancer of the digestive tract, including ESCC [2]. NSAIDs target the cyclooxygenase (COX) enzyme, COX-1 and COX-2. COX-2 is a key enzyme in the synthesis of prostaglandin E2 (PGE2), which is important in promoting tumorigenesis. COX-2 expression is elevated in tumors and is inducible by oncogenes, growth factors, inflammatory cytokines, chemotherapeutics and tumor promoters [3]. The upregulation of COX-2 has been associated with various premalignant and malignant lesions of epithelial origin in the colon, lung, breast,

bladder, pancreas, ovary and esophagus [4]. Previous studies using a genetic model of colorectal tumorigenesis have suggested that the upregulation of COX-2 prolongs the survival of abnormal cells, thereby favoring the accumulation of sequential genetic changes that may be responsible for colorectal carcinogenesis [5]. Additionally, recent studies have highlighted the importance of COX-2 in esophageal carcinogenesis: increased levels of COX-2 mRNA and protein are found in both premalignant and malignant tissues from esophageal tumors compared with the adjacent normal tissue [6, 7]. Furthermore the elevated protein expression of COX-2 has been correlated with a significantly reduced survival rate of patients undergoing surgery for ESCC [7, 8]. This is consistent with the hypothesis that the upregulation of COX-2 in ESCC is associated with the progression of ESCC. However, the mechanism by which the upregulation of COX-2 affects carcinogenesis in esophageal epithelial cells requires further investigation. In spite of this, the present study and prior evidence suggest that COX-2 may be involved in the early stage of carcinogenesis and that COX-2 may be an effective target in the treatment of esophageal cancer for a very significant proportion of the patient population.

In the past few years, RNAi has become an important research tool to study and manipulate a particular gene and its function. Furthermore, the use of siRNA as a potent and specific inhibitor of any target gene provides a new therapeutic approach for many unincurable diseases, particularly cancer [9–13]. In addition, NSAIDs (e.g., acidum acetil salicylicum) have been shown to inhibit both COX-1 and COX-2 [14] and NSAIDs may boost the effectiveness of chemotherapy in the treatment of cancers. Therefore, we treated ESCC cells with a COX-2-specific siRNA in combination with acidum acetil salicylicum (aspirin) to inhibit COX-2 expression and function. Previous studies in cell culture and animal models have shown that the inhibition of the COX pathway changes the characteristics of cancer cells by reducing cell proliferation, increasing apoptosis, inducing angiogenesis, subverting the immune system and promoting tumor invasion on a molecular level [15]. Therefore, in this study, we focused on the effects of siRNA and/or acidum acetil salicylicum treatment on cell proliferation and apoptosis in EC109 and EC9706 cells.

Materials and Methods

Cell Lines

The well-differentiated (EC109) and poorly differentiated (EC9706) human ESCC cell lines were obtained from the Cancer Institute, Chinese Academy of Medical Sciences. EC109 and EC9706 cells were grown in RPMI 1640 culture medium (Invitrogen, USA) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 2% L-glutamine in a humidified 37°C incubator with 5% CO2, fed every 3 days with complete medium, and subcultured when confluent.

COX-2 siRNA Synthesis and Transfection

The COX-2-specific siRNA was designed to target position 293–311 (5′-CUGCUCAACACCGGAAUUUtt-3′) of the COX-2 transcript (GenBank Accession No: **NM_000963**) and was synthesized by GeneChem (Shanghai, China). The siRNA sequence was tested for specificity in the BLAST database and did not show any homology to any other sequence. We also used a scrambled siRNA as negative control. This negative control had no significant homology to any known human, mouse or rat gene (Non-silencing-FITC: 5′-UUCUCCGAACGUGUCACGUtt-3′). The original stock of the siRNA was resuspended in the resuspension buffer provided by the manufacturer. EC109 and EC9706 cells were trypsinized and cultured in 6-well plates (2×10^5 cells/well; Nunc, Denmark). After 24 h, the cells were cultured in RPMI 1640 medium without antibiotics until the cells reached 40–50% confluence; they were then transfected with the COX-2 siRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Briefly, 15 µl of the COX-2 siRNA (20 µM) and 5 µl of Lipofectamine 2000 were diluted, individually, in 250 µl of Opti-MEM. After 5 min incubation at room temperature, the diluted COX-2 siRNA and the Lipofectamine were gently combined and incubated for 20 min at room temperature to allow complex formation. The siRNA-Lipofectamine complexes were then added to the plated cells and mixed gently. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 84 h. For the combination treatment of COX-2 siRNA and acidum acetil salicylicum, the COX-2 siRNA transfected cells were treated with 8 mmol/L acidum acetil salicylicum for 36 h after the COX-2 siRNA was transfected into EC109 and EC9706 for 48 h.

Fluorescent Detection of siRNA Transfection

Using a fluorescence microscope, the transfection efficiency of the EC109 and EC9706 cells was determined at 4 h, 5 h, 24 h, 48 h, 72 h and 84 h post-transfection with siRNA.

Western Blot

Cells were harvested and lysed in mammalian lysis buffer, and western blots were performed using conventional protocols. Briefly, the protein concentrations of the extracts were determined using a bicinchoninic acid (BCA) kit

(Pierce, USA) with bovine serum albumin as a standard. Total protein samples (30 μg) were loaded and separated in 10% SDS-PAGE gels and then transferred to PROTRAN nitrocellulose membranes (Whatman, UK). The membranes were incubated with an anti-COX-2 antibody (1:200; Santa Cruz, USA) and, after being extensively washed, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000; Santa Cruz) for 1 h at room temperature. The signal was detected by chemiluminescence using an ECL Detection Kit (Amersham, USA). The membranes that were probed for COX-2 were reprobed for β-actin to normalize for loading and/or quantification errors and to allow for the direct comparison of protein expression. The bands were quantified using a Gel EDAS analysis system (Cold Spring USA Corporation) and Gel-Pro Analyzer 3.1 software (Media Cybernetics, USA).

MTT Assay

To evaluate the effects of the siRNA and acidum acetil salicylicum on the proliferation of EC109 and EC9706 cells, the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. Briefly, EC109 and EC9706 cells were seeded in 96-well plates at a density of 2×10^5 cells/well and cultured for 24 h. The cells were then transfected with the COX-2 siRNA or the FITC-labeled negative control siRNA for 84 h. At 48 h post-transfection, the co-treated cells were treated with acidum acetil salicylicum (8 mmol/L) for an additional 36 h. The MTT solution (20 μl; 5 mg/ml) was then added to the 200 μl of media in each well. After an additional incubation for 4 h, the media was discarded, and 150 μl of DMSO was added to each well to dissolve the formazan crystals. The optical density was read with an automated microplate reader at 492 nm. The experiments were carried out in triplicate, and the results are shown as the mean ±SD of three independent experiments.

Detection of Apoptosis Rate by Flow Cytometry

To determine the effects of the siRNA and aspirin on apoptosis, EC109 and EC9706 cells were treated with the COX-2 siRNA alone or with acidum acetil salicylicum, collected by centrifugation at 200 g for 5 min, and stained with propidium iodide (PI). PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques and is suitable for flow cytometry.

The pellets were washed twice with ice-cold PBS, fixed overnight at 4°C in 70% ethanol, and stored at -20°C. The pellets were washed twice with PBS, and the cells were incubated with 5 μg/ml PI and 50 μg/ml RNase A in PBS for 1 h at room temperature in the dark. PI also binds to RNA, necessitating treatment with RNase A to distinguish between RNA and DNA staining. Flow cytometry was carried out using a FACSCalibur flow cytometer (Becton-Dickson, Mountain View, CA), 10,000 events were collected per sample, and the cells were analyzed for apoptosis rate using CELLQUEST software. The experiment was performed in triplicate.

Statistical Analysis

The statistical analysis was performed using the SPSS statistical software (SPSS, USA). The differences between the groups were assessed using the analysis of variance test. The results were considered statistically significant if the P value was <0.05 or <0.01.

Results

siRNA Transfection Efficiency

We designed a COX-2-specific siRNA using an algorithm available at the Ambion website (www.ambion.com) and following several general rules for effective siRNA design [16]. The optimal transfection time in EC109 and EC9706 cells was determined using a FITC-labeled siRNA-Lipofectamine complex. Green fluorescence was initially detected in EC109 and EC9706 cells 4–5 h post-transfection with the siRNA-Lipofectamine complex (Fig. 1). The fluorescence peaked at 48 h post-transfection and gradually decreased until 72 h post-transfection. Therefore, the optimal time for siRNA transfection in EC109 and EC9706 cells is 48–72 h. This is consistent that siRNA usually inhibits mRNA expression 2–4 days following Lipofectamine-based transfection [16].

Inhibition COX-2 Protein Expression by siRNA and/or Aspirin Treatment

Given the importance of COX-2 in ESCC carcinogenesis, we used an in vitro experimental model based on ESCC cell lines to determine if a COX-2-specific siRNA may be useful for the downregulation of COX-2 expression. From a panel of ESCC cell lines, we choose a well-differentiated cell line (EC109) and a poorly differentiated cell line (EC9706). The EC9706 cells had a high endogenous expression of COX-2 at the protein level, whereas the EC109 cells had moderate level of expression (data not shown). The effect of the COX-2 siRNA treatment was assessed by western blot and densitometry analysis. As shown in Fig. 2, COX-2 expression was reduced by approximately 40% in EC109 cells and 50% in EC9706 cells at 84 h after transfection with the COX-2 siRNA

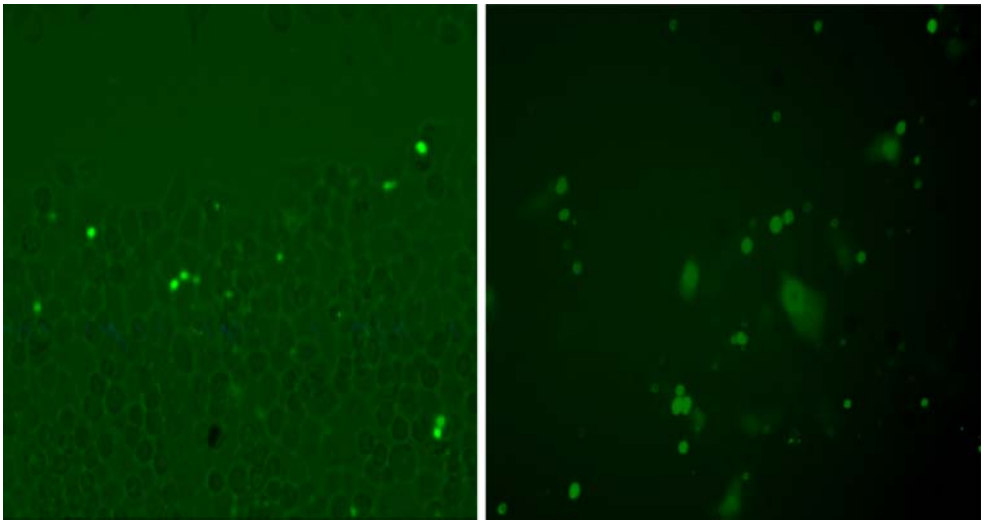

Fig. 1 siRNA transfection efficiency. EC109 and EC9706 cells were transiently transfected with siRNA (final concentration 100 nM). An immunofluorescence microscope was used to detect the FITC-labeled siRNA in EC109 cells (*left*) and EC9706 cells (*right*) at 5 h post-transfection

(100 nM), while the negative control siRNA had no effect on the expression of COX-2. COX-2 expression was normalized to β-actin expression based on the band intensity. Furthermore, this demonstrated that the transient siRNA-mediated knockdown of COX-2 in ESCC cells was highly specific since β-actin expression was unaffected. In addition, COX-2 expression was significantly decreased (by more than 70%) in EC109 and EC9706 cells transfected with the COX-2 siRNA and treated with acidum acetil salicylicum (Fig. 2). These results suggest that the co-administration of acidum acetil salicylicum and siRNA have a synergistic effect on the inhibition of COX-2 gene expression.

siRNA-Mediated COX-2 Knockdown and Aspirin Treatment Inhibits Cell Proliferation

To elucidate the effects of the siRNA and acidum acetil salicylicum on EC109 and EC9706 cell proliferation, the MTT assay was used, and cell proliferation was measured by counting the number of viable cells. There was no significant reduction in the number of viable EC109 and EC9706 cells at 24 h post-treatment. However, at 48 h, we detected a significant decrease in the number of viable cells. Furthermore, the transfection of the COX-2 siRNA resulted in a significant inhibition of cell proliferation in both the EC109 cells ($p<0.01$) and EC9706 cells ($p<0.05$) 84 h post-transfection (Fig. 3). The proliferation was not obviously influenced by treatment with the control siRNA. Additionally, treatment of siRNA-transfected cells with acidum acetil salicylicum further increased the inhibition of the cell growth ($p<0.01$; Fig. 3A and B). These data demonstrate that COX-2 expression is important for the proliferation of ESCC cells and that the inhibition of COX-2 by COX-2-specific siRNA alone or with acidum acetil salicylicum treatment may inhibit the proliferation of ESCC cells.

RNAi-mediated COX-2 Knockdown and Acidum Acetil Salicylicum Induce Apoptosis

To determine the effect of siRNA and/or acidum acetil salicylicum treatment on the rate of apoptosis in EC109 and EC9706 cells, PI staining was performed. The inhibition of COX-2 by siRNA alone or with acidum acetil salicylicum treatment resulted in a significant decrease in the growth of the EC109 and EC9706 cells due to the induction of

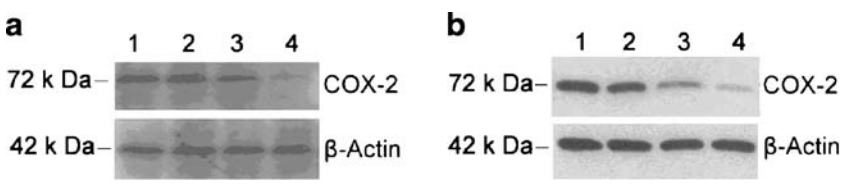

Fig. 2 COX-2 protein expression in EC109 cells (**A**) and EC9706 cells (**B**). Total cellular proteins were extracted, and the expression levels of COX-2 and β-actin were determined by western blot. The expression of β-actin was used as an internal control. All procedures are described in the Materials and Methods. All samples were treated with the siRNA and/or acidum acetil salicylicum (aspirin) for 84 h except the untreated control in lane 1. The co-treated samples were treated with COX-2 siRNA for 36 h and then treated with aspirin for 48 h. Lane 1: untreated control; lane 2: negative control siRNA; lane 3: COX-2 siRNA; lane 4: COX-2 siRNA and acidum acetil salicylicum (aspirin)

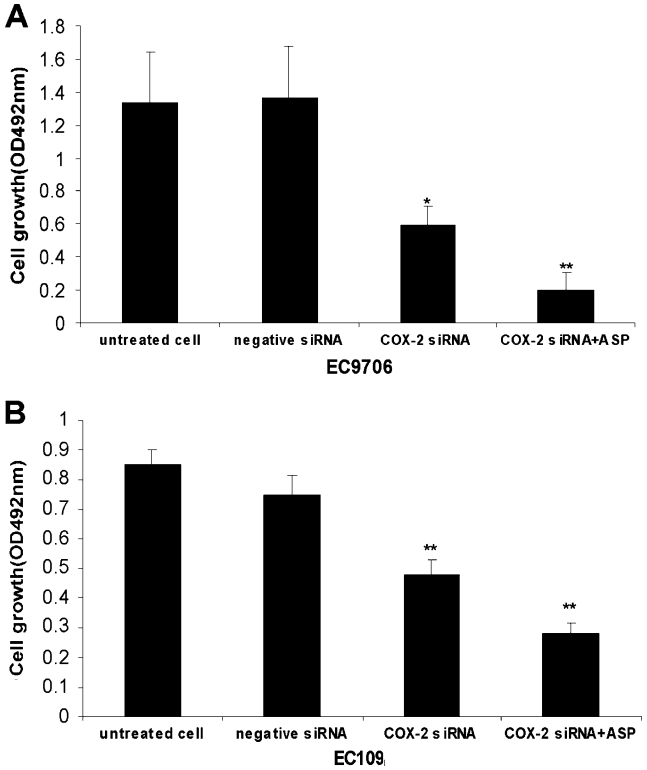

Fig. 3 The inhibition of cell proliferation in EC109 cells (**A**) and EC9706 cells (**B**) by siRNA and/or acidum acetil salicylicum (aspirin). Cell proliferation was measured by the MTT method at OD492 nm as described in the Materials and Methods. The values are expressed as the mean±SD of three independent experiments (n=3). ** (P<0.01); * (P<0.05)

apoptosis. The apoptosis rate was increased in siRNA-treated EC109 cells (4.33%) and siRNA-treated EC9706 cells (4.00%; Table 1 and Fig. 4). Apoptosis was not significantly influenced by treatment with the negative control siRNA. The rate of apoptosis was further increased when EC109 cells (11.66%) and EC9706 cells (8.38%) were treated with both the COX-2-specific siRNA and acidum acetil salicylicum (Table 1 and Fig. 4). These data indicate that the COX-2-specific siRNA alone or with acidum acetil salicylicum treatment induced apoptosis in EC109 and EC9706 cells.

Discussion

To explore the role of COX-2 in human ESCC carcinogenesis and the effect of NSAIDs, we treated two ESCC cell lines, EC109 and EC9706, with a COX-2-specific siRNA and acidum acetil salicylicum. Previous work from our laboratory has demonstrated that the COX-2 mRNA was expressed in 22 of 42 frozen specimens of human ESCC but was undetectable in all of the specimens of adjacent normal tissue by RT-PCR [17]. Additional studies using immunohistochemistry and western blots have demonstrated that the COX-2 protein level was increased in a significant number of ESCC cells [6, 18]. The experimental setup for our work involved the transient transfection of EC109 and EC9706 cells with a COX-2-specific siRNA, which avoids the selection of cells that were able to overcome the downregulation of COX-2. In this study, we demonstrated a significant reduction in the endogenous COX-2 levels in ESCC cells using an RNAi strategy. Moreover, we also demonstrated a more significant reduction in the endogenous COX-2 level following the co-administration of acidum acetil salicylicum and the siRNA compared with siRNA treatment alone. While the expression of COX-2 was regulated at the post-transcriptional level by the COX-2 siRNA, COX-2 was regulated at both the post-transcriptional and post-translational level by the combination treatment. It is well known that acidum acetil salicylicum inhibits COX-2 enzymatic activity through the irreversible inhibition of the COX active site [19]. Acidum acetil salicylicum covalently modifies the COX protein by acetylating a single serine residue in the substrate-binding channel, and this modification blocks the approach of arachidonic acid [3]. Our data suggest that the COX-2 siRNA and acidum acetil salicylicum had a synergistic effect on the inhibition of COX-2.

Tsujii and DuBois demonstrated that COX-2 overexpression in intestinal epithelial cells alters the cell adhesion properties and inhibits apoptosis [20], and additional studies have shown that the inhibition of the COX pathway could alter the characteristics of cancer cells by reducing cell proliferation, increasing apoptosis and

Table 1 siRNA-mediated COX-2 knockdown and/or aspirin induce apoptosis in ESCC cells

Conditions	Apoptosis rate (%)			
	Untreated control	Negative siRNA	COX-2 siRNA	COX-2 siRNA with aspirin
EC109	1.35±0.12	1.72 ±0.21	4.33 ±0.86	11.66 ±1.54
EC9706	0.61 ±0.11	0.46 ±0.10	4.00 ±0.99	8.38 ±1.38

Cells were labeled with PI and analyzed by flow cytometry. All samples were treated for 84 h with the negative siRNA or the COX-2 siRNA except untreated control (sample 1). The co-treated sample was treated with the COX-2 siRNA for 48 h, then aspirin was added, and the treatment continued for an additional 36 h

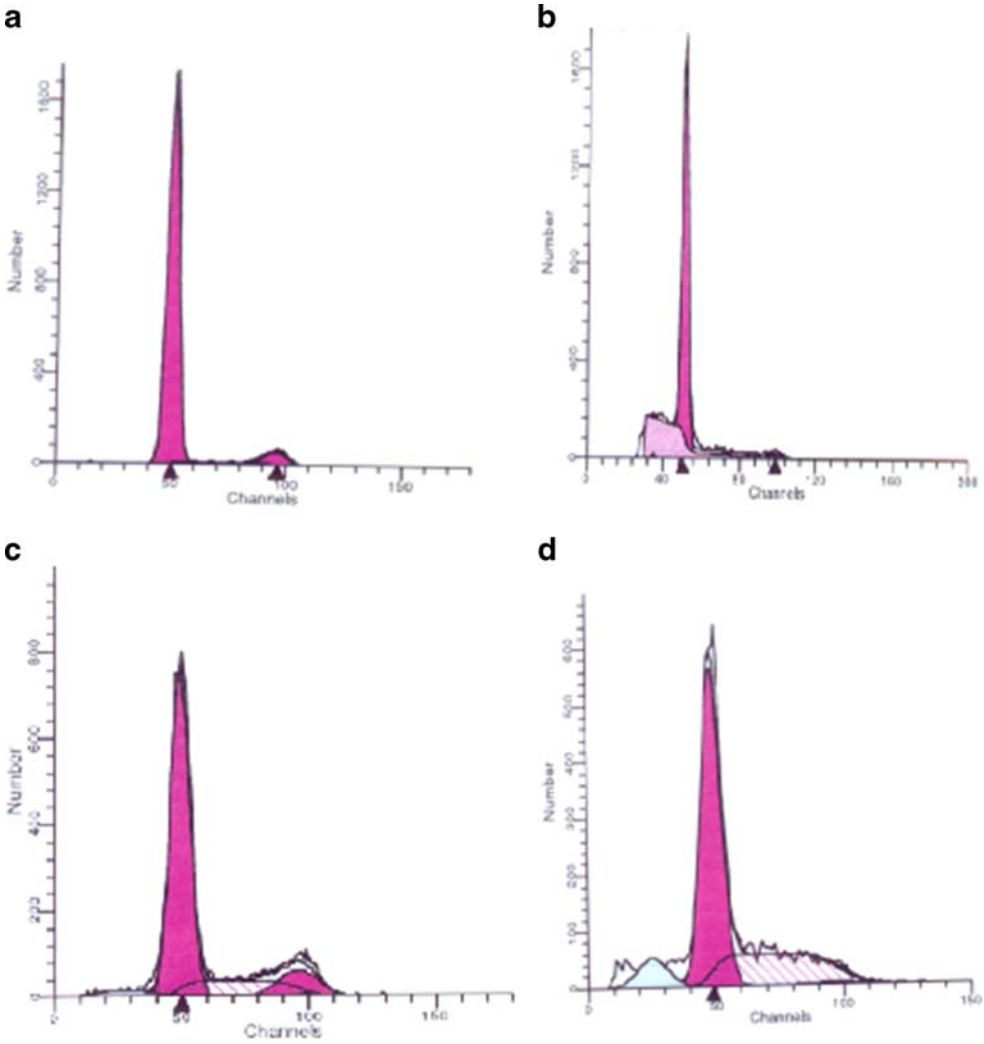

Fig. 4 siRNA-mediated COX-2 knockdown and acidum acetil salicylicum (aspirin) induces apoptosis in EC9706 (A-D). Cells were labeled with PI and analyzed by flow cytometry. All samples were treated for 84 h with the COX-2 siRNA and/or acidum acetil salicylicum (aspirin) except the untreated control in Fig. 4A. Figure 4B: samples treated with the negative control siRNA; Fig. 4C: samples treated with the COX-2 siRNA; Fig. 4D: samples treated with the COX-2 siRNA for 48 h and then treated with acidum acetil salicylicum (aspirin) for 36 h

reducing angiogenesis [4]. Moreover, it has been demonstrated that expression of COX-2, detected using immunohistochemistry, was greater in the peripheral region of the esophageal tumor nest, suggesting that COX-2 plays an important role in the proliferation of the esophageal epithelial cells [21]. Therefore, we focused our research on the effects of the inhibition of COX-2 on the proliferation and apoptosis of ESCC cells. Our data demonstrated that the treatment of EC109 and EC9706 cells with a COX-2-specific siRNA and acidum acetil salicylicum reduced the percentage of proliferating tumor cells and increased the rate of apoptosis.

Previous studies have suggested that cell proliferation and apoptosis are very critical steps in tumor metastasis. The growth of tumor cells results from an imbalance between cell proliferation and apoptosis [22]. COX-2 may prevent apoptosis not only by generating the anti-apoptotic compounds PGE_2 and PGI_2, but also by removing a pro-apoptotic substrate, arachidonic acid. Furthermore, COX-2 expression may affect the Ras signal transduction pathway because PGE_2 can activate MAPK activity [22, 23]. The decreased apoptosis of these cells favors the accumulation of sequential genetic changes, which may be responsible for carcinogenesis [22]. On the other hand, the effect of NSAIDs on cell proliferation and apoptosis was originally found in colon cancer cells, and NSAIDs have been shown to have antitumor action against colon cancer in vivo [24]. Acidum acetil salicylicum has been shown to induce apoptosis in esophageal cancer cell lines in a time- and dose-dependent manner [19]. Growth inhibition of 10 esophageal cancer cells by acidum acetil salicylicum was dose- and time-dependent and associated with the induction of apoptosis [19]. Results from our lab further confirmed the similar effect of aspirin in EC109 and EC9706 cell lines by time- and dose-dependent manner [17]. Therefore, acidum acetil salicylicum can affect esophageal carcinogenesis although the mechanisms underlying these effects remain to be elucidated. The concentration of acidum acetil salicylicum (8 mmol/L) applied is relevant in an *in vivo* treatment including animal model (Manuscript in prepara-

tion.). This study sought to avoid the pitfalls of NSAID treatment that the doses of NSAIDs, found to exert these effects *in vitro*, have generally been well in excess of pharmacological concentrations [11]. We employed RNAi to specifically knock down endogenous COX-2 in combination with pharmacological concentration of acidum acetil salicylicum in cell and animal models. Except that, a recent study from our lab demonstrated that the acidum acetil salicylicum -mediated inhibition of proliferation is associated with apoptosis in EC109 cells but not in EC9706 cells, even though apoptosis is a common mechanism that contributes to the reduced cell proliferation [25]. We also had investigated the effect of COX-2 specific inhibitors (i.e. nimesulide) in this study. Nimesulide could inhibit ESCC cell proliferation and induce apoptosis, which is probably through COX-2 inhibition [25]. This result suggested that while COX-2 plays a critical role in esophagus tumor carcinogenesis, the mechanisms by which COX-2 may acts in different tumor cells are different.

In summary, our studies demonstrated that the inhibition of COX-2 by RNAi has effects to inhibit cell proliferation and induction apoptosis in ESCC cells. Additionally, the COX-2 siRNA and acidum acetil salicylicum had a synergistic effect on the inhibition of COX-2. Furthermore, the combination treatment has an antiproliferative and pro-apoptotic effect on esophageal cancer cells, although other mechanisms are also involved. These studies suggest that a combination therapy including a traditional NSAID (e.g., acidum acetil salicylicum) co-administrated with COX-2-specific siRNA has the potential to be a new and highly effective treatment for ESCC. The response of esophageal tumors to exposure of COX-2 siRNA and/or COX-2 inhibiting compounds in vivo, however, awaits further evaluation.

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