

Anti-cancer effect of low dose of celecoxib may be associated with *lnc-SCD-1:13* and *lnc-PTMS-1:3* but not *COX-2* in NCI-N87 cells

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Abstract. In order to investigate the mechanism of celecoxib and whether long non-coding RNAs (lncRNAs) were involved in the effects of celecoxib treatment in NCI-N87 cells, NCI-N87 cells were treated with 15, 30 and 60 μ M celecoxib and an MTT assay was performed to assess cell viability. Following treatment with 15 μ M celecoxib, the cell cycle and apoptosis were analyzed by flow cytometry, and the mRNA levels of *lnc-SCD-1:13*, *lnc-PTMS-1:3*, cyclooxygenase-2 (*COX-2*), integrin $\alpha 3$ (*ITGA3*) and DSH homolog 1 (*DVL1*) were detected by reverse transcription quantitative PCR (RT-qPCR) in NCI-N87 cells. MTT analysis demonstrated that celecoxib significantly inhibited cell viability in treated cells compared with untreated cells. Flow cytometry analysis revealed that, compared with untreated cells, the percentage of cells in the G₀/G₁ phase was significantly increased, and the percentage of cells in the S and G₂ phase was decreased. In addition, the percentage of early and late apoptotic cells was increased in cells treated with 15 μ M celecoxib compared with the control. RT-qPCR analysis also demonstrated that the mRNA levels of *lnc-SCD-1:13*, *lnc-PTMS-1:3*, *ITGA3* and *DVL1* were increased following treatment with celecoxib (15 μ M; $P < 0.05$). However, there were no significant differences in the expression of *COX-2* mRNA between cells treated with celecoxib (15 μ M) and untreated cells. The present study demonstrated that a low dose of celecoxib may be involved in regulating cell growth independent of *COX-2* in NCI-N87 cells. Furthermore, *ITGA3* and/or *DVL1* co-expressed with *lnc-SCD-1:13* and *lnc-PTMS-1:3* may be associated with the effects of treatment with a low dose of celecoxib in NCI-N87 cells.

Introduction

Gastric cancer (GC) is a common malignancy worldwide, characterized by high invasiveness and aggressiveness (1).

It is estimated that there are ~990,000 new cases of GC and that ~738,000 succumb from this type of cancer per year (2). Although a number of conventional therapeutic methods, including surgical excision and chemotherapy achieve satisfactory therapeutic effects for patients with early GC, a greater number of patients with GC at an advanced stage have poor prognosis (3). Therefore, despite increasing knowledge of the genetic and biochemical basis of GC, it is also essential to search for novel therapeutic targets and to develop more effective diagnostic and treatment methods.

A series of studies have reported that nonsteroidal anti-inflammatory drugs (NSAIDs) have an anti-cancer effect in various types of cancer, including breast cancer (4), esophageal cancer (5), colorectal cancer (6), prostate cancer (7) and GC (8). In addition to aspirin, celecoxib, a cyclooxygenase-2 (*COX-2*) inhibitor, has been demonstrated to be involved in uncontrolled cell proliferation, apoptosis, angiogenesis and metastasis (9,10). Previous studies have suggested that celecoxib is able to induce cell apoptosis through the phosphoinositide 3 kinase/Akt signaling pathway (11) and to inhibit invasion through the adenine nucleotide translocator-dependent signaling pathway in GC cells (12). In addition, celecoxib has been reported to be able to prevent the development of GC in rats (13). Increasing evidence suggests that long non-coding RNAs (lncRNAs) are able to regulate tumor-suppressing or oncogenic effects, and lncRNAs may be considered as novel biomarkers and therapeutic targets for cancer (14,15). However, an understanding of the function of lncRNAs in the treatment of GC with celecoxib remains limited.

A previous bioinformatics study has demonstrated that *DVL1* and/or *ITGA3*, as well as their co-expressed lncRNAs, including *lnc-SCD-1:13* and *lnc-PTMS-1:3*, were abnormally expressed in patients with GC following treatment with celecoxib. In the present study, the effects of a low dose of celecoxib on the viability, cell cycle and apoptosis of human GC NCI-N87 cells was investigated. In addition, the mRNA levels of *lnc-SCD-1:13*, *lnc-PTMS-1:3*, *COX-2*, *ITGA3* and *DVL1* were analyzed in order to investigate further whether lncRNAs are involved in the treatment of NCI-N87 cells with celecoxib.

Materials and methods

Cell culture. NCI-N87 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences

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(Shanghai, China). The cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a 37°C incubator with a humidified atmosphere of 5% CO₂ (Thermo Fisher Scientific, Inc.).

MTT assay. The cells (5x10³) were seeded into each well of a 96-well plate and cultured in DMEM supplemented with 10% FBS. Following 24 h of culture at 37°C and 5% CO₂ with saturated humidity, the cells were treated with 0 (control group), 15, 30 and 60 µM celecoxib (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and subsequently incubated for 72 h (37°C, 5% CO₂ with saturated humidity) (11). MTT (10 µl, 5 mg/ml; Shanghai Sangon Biotech Co., Ltd, Shanghai, China) was added to each well at the same time, and the cells were subsequently incubated for 4 h at 37°C. Following the removal of the medium, dimethyl sulfoxide (DMSO; 100 µl; Shanghai Sangon Biotech Co., Ltd) was added into each well for 10 min to solubilize the formazan crystals at room temperature. The zero control (medium, MTT, DMSO) and blank control (no reagent) were set up. The absorbance was read at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All experiments were performed in triplicate.

Flow cytometry. The NCI-N87 cells were treated with a low dose of celecoxib (15 µM) for 72 h at 37°C. For cell cycle detection, the treated cells were collected by centrifugation (250 x g for 6 min) at 25°C and subsequently fixed with ice-cold 70% ethanol overnight. Next, the cells were centrifuged (111 x g for 5 min) at 25°C and re-suspended in 500 µl phosphate buffered saline (PBS). The cells were subsequently treated with 50 µg/ml RNase A (Shanghai Sangon Biotech Co., Ltd.) for 30 min at 37°C. Finally, the cells were stained with propidium iodide (PI; BD Pharmingen, San Diego, CA, USA) in the dark for 15 min at 4°C and detected using a FACSCalibur flow cytometer (BD Pharmingen). An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Pharmingen) was used for the detection of apoptotic cells. The treated cells were digested with 0.25% trypsin-EDTA and collected by centrifugation at 250 x g for 6 min at 25°C. The cells were then washed once with PBS. Subsequently, the cells were resuspended with 1X binding buffer (from the annexin V-FITC apoptosis detection kit) and stained with FITC-annexin V and PI in the dark for 15 min at 25°C. Following this, 1X binding buffer (400 µl) was added to the cells, and apoptosis was detected using a FACS-Calibur flow cytometer (BD Pharmingen).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The cells were treated with a low dose of celecoxib (15 µM) for 72 h at 37°C. Total RNA was extracted using 1 ml TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China), and cDNA was obtained once using a reverse transcription kit (Takara Biotechnology Co., Ltd), according to the manufacturer's protocol. Primer sequences for *ITGA3*, *DVL1*, *COX-2*, *lnc-SCD-1:13*, *lnc-PTMS-1:3* and glyceraldehyde-3-phosphate dehydrogenase are listed in Table I. SYBR Premix Ex Taq (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform qPCR. The thermocycler conditions

Table I. Primer sequences for real-time quantitative polymerase chain reaction.

Gene	Primer sequences (5'-3')
GAPDH-hf	GAAGGTGAAGGTCGGAGTC
GAPDH-hr	GAAGATGGTGATGGGATTTC
ITGA3-hf	GGACCTTACAACGCCGAGTG
ITGA3-hr	GGAGGCTCTTTGGCTTGTTTT
DVL1-hf	AGCACCTCATCCAGACTCATCC
DVL1-hr	GATGCTGATGCCCAGAAAGTGAT
COX-2-hf	CCCTGAGCATCTACGGTTTG
COX-2-hr	CAGTATTAGCCTGCTTGCTCT
lnc-SCD-1:13-hf	AAGTCTTGAAGTTGGGTGTT
lnc-SCD-1:13-hr	GAAGATGGCAGAGCAGAAAG
lnc-LRR1-1:2-hf	GTGTCCGCACTAAGTTCGGCATCA
lnc-LRR1-1:2-hr	GTGTCCGCACTAAGTTCGGCATCA
lnc-PTMS-1:3-hf	ATCCCAAACGGCAGAAGACA
lnc-PTMS-1:3-hr	CAGAGCAGGGAGCCAGGTGA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITGA3, integrin subunit α3; DVL1, dishevelled segment polarity protein 1; COX-2, cyclooxygenase-2; lnc, long non-coding RNA; hf, forward; hr, reverse.

used were as follows: 50°C for 3 min, 95°C for 3 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Relative quantification and calculations were performed using the comparative threshold (Cq) cycle method (2^{-ΔΔCq}) (16).

Statistical analysis. SPSS statistical analysis software (version 12.0; SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. Data are expressed as the mean ± standard error and were analyzed by one-way analysis of variance followed by least significance difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of celecoxib on the viability of NCI-N87 cells. MTT analysis was performed to observe the effect of celecoxib treatment on NCI-N87 cell viability. The results demonstrated that cell viability was significantly inhibited in the celecoxib treatment group compared with the untreated cells (P<0.05; Fig. 1A). To further understand the nature of the decrease in cell viability associated with the addition of celecoxib, cell cycle analysis was performed. The results suggested that compared with the control, there was an increased percentage of cells in the G₀/G₁ phase (P<0.05) and a decreased percentage of cells in the S and G₂ phase in the celecoxib (15 µM) treatment group (P<0.05; Fig. 1B, Table II).

Effect of celecoxib on apoptosis in NCI-N87 cells. The apoptotic rate was analyzed by flow cytometry, and the results indicated that the percentage of early and late apoptotic cells was significantly increased in cells treated with celecoxib (15 µM) compared with untreated cells (P<0.05; Fig. 2).

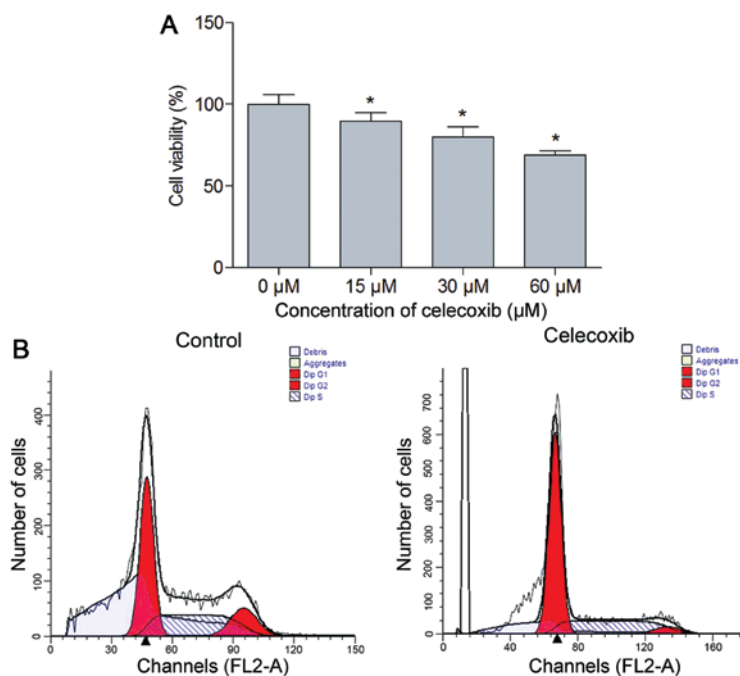


Figure 1. Celecoxib inhibits NCI-N87 cell viability. (A) MTT analysis demonstrated that cell viability was significantly inhibited in the celecoxib treatment group compared with untreated cells. (B) Flow cytometry analysis indicated an increase in the percentage of cells in the G_0/G_1 phase and a decrease in the percentage of cells in the S and G_2 phases in the celecoxib treatment group compared with the control. * $P < 0.05$ vs. untreated cells. Dip, diploid.

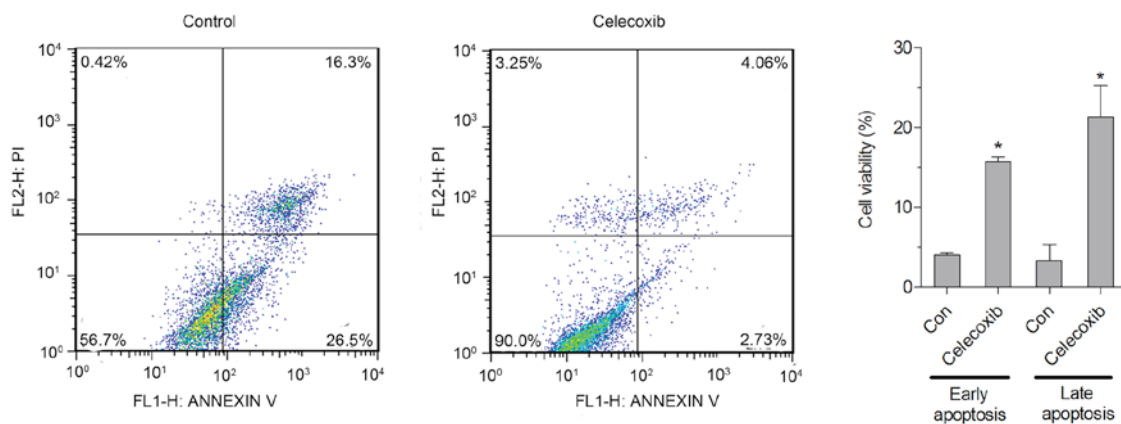


Figure 2. Celecoxib (15 μ M) promotes early and late apoptosis in NCI-N87 cells. * $P < 0.05$ vs. control. Con, control. PI, propidium iodide.

Table II. Cell cycle analysis of celecoxib treatment and control groups.

Group	G_0/G_1 phase (%)	S phase (%)	G_2 phase (%)
Control	47.07 \pm 0.70	39.05 \pm 4.34	13.88 \pm 3.65
Celecoxib	65.56 \pm 0.16 ^a	29.19 \pm 0.98 ^a	5.25 \pm 1.16 ^a

^a $P < 0.05$ vs. control. Data are expressed as the mean \pm standard error.

(15 μ M) and untreated cells (Fig. 3). The mRNA levels of *Inc-SCD-1:13*, *Inc-PTMS-1:3*, *ITGA3* and *DVL1* were also detected. The expression of these genes was indicated to be aberrant in GC by our previous bioinformatics analysis (17). RT-qPCR analysis demonstrated that there was an increase in mRNA expression of *Inc-SCD-1:13*, *Inc-PTMS-1:3*, *ITGA3* and *DVL1* in cells treated with celecoxib (15 μ M) compared with untreated cells ($P < 0.05$, Fig. 3). Expression of *COX2* was not significantly altered in cells treated with celecoxib (15 μ M) compared with untreated cells (Fig. 3).

Discussion

Effect of a low dose of celecoxib on the mRNA levels of *Inc-SCD-1:13*, *Inc-PTMS-1:3*, *COX2*, *ITGA3* and *DVL1* in NCI-N87 cells. There was no significant difference in the expression of *COX2* in cells treated with celecoxib

Celecoxib, a novel NSAID that is able to inhibit COX-2 activity, is considered to be an agent for the chemoprevention of GC (18). A previous study demonstrated that increased COX-2 expression is an independent prognostic factor for

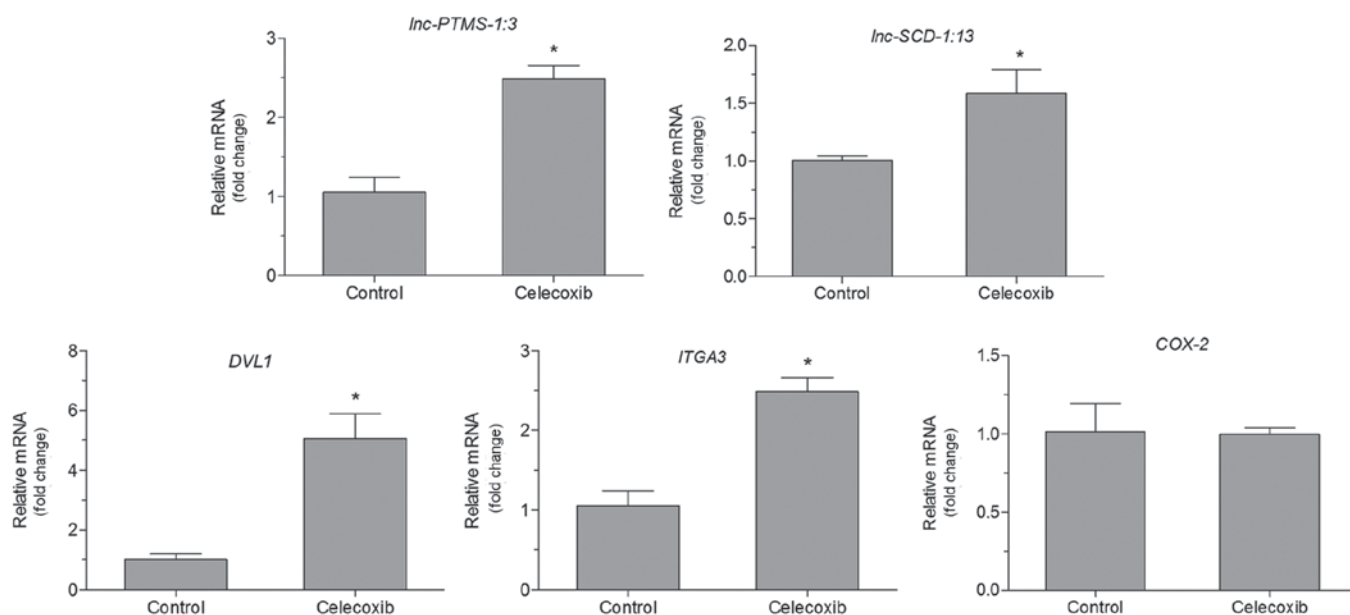


Figure 3. Celecoxib (15 μ M) increases mRNA expression of *lnc-SCD-1:13*, *lnc-PTMS-1:3*, *ITGA3* and *DVL1*, but not *COX-2* in NCI-N87 cells. * $P < 0.05$ vs. control. lnc, long non-coding RNA; ITGA3, integrin subunit $\alpha 3$; DVL1, dishevelled segment polarity protein 1; COX-2, cyclooxygenase-2.

poor prognosis and is associated with reduced survival in patients with GC (19). Therefore, increasing attention had been directed to the mechanisms of celecoxib action on GC. In the present study, it was demonstrated that a low dose of celecoxib (15 μ M) was able to significantly inhibit viability of NCI-N87 cells by arresting the cell cycle at the G_0/G_1 phase and promoting apoptosis. Similarly, Cho *et al* (20) demonstrated the anti-cancer effect of celecoxib on GC cells (AGS and MKN-45) by regulating cell-cycle arrest and apoptosis. In addition, Liu *et al* (11) suggested that celecoxib induced apoptosis through the mitochondrial and death receptor pathways in GC cells. These results indicated that a low dose of celecoxib may have a chemopreventive function in the development of GC by regulating cell cycle and apoptosis.

The association of COX2 with tumor development by promoting cell proliferation and invasion or metastasis has been well established (21,22). Previous studies have demonstrated that COX-2 is overexpressed in human GC (23,24), and *in vitro* downregulation of COX-2 is able to induce growth inhibition (25). However, the present study indicated that 15 μ M celecoxib was not able to inhibit COX2 mRNA expression, but was able to suppress cell viability. Consistent with the findings of the present study, Kim *et al* (26) also demonstrated that COX2 expression is not inhibited by 10 μ M celecoxib, but expression is suppressed by 25 μ M celecoxib. Taken together, these results indicated that the anti-cancer effect of celecoxib may not be fully dependent on COX-2 suppression. Previous studies have also demonstrated that chemopreventive effect of celecoxib on cancer may be associated with a COX-2-independent mechanism (13,27-29). Combined with the results of the present study, it is possible to hypothesize that a low dose of celecoxib may inhibit cell growth independent of COX-2. However, the exact mechanism requires further elucidation.

To further investigate the molecular mechanisms underlying the inhibition of cell viability induced by a low dose of celecoxib, the mRNA levels of differentially expressed genes (*ITGA3* and

DVL1) and lncRNAs (*lnc-SCD-1:13* and *lnc-PTMS-1:3*) were detected in NCI-N87 cells. ITGA3 has been previously been demonstrated to be involved in the development of GC (30). Integrins, a family of adhesion receptors, are associated with cell adhesion and migration as well as signal transduction (31,32). Several previous studies have also demonstrated that decreased expression of ITGA3 is associated with cancer growth and development (33,34). In addition, overexpression of DVL1 was observed in the metastasis of colorectal cancer (35). Dishevelled homologs (DVL1, DVL2, DVL2) are core signaling molecules of the WNT/planar cell polarity (PCP) signaling pathways (36). Accumulating evidence has demonstrated that PCP signaling is associated with tumorigenesis (31). Tang *et al* (37) reported that microRNA (miR)-200b and miR-22 were able to synergistically inhibit the growth of GC through the Wnt-1 signaling pathway. Notably, a previous study by our group demonstrated that ITGA3 and/or DVL1 were co-expressed with *lnc-SCD-1:13* and *lnc-PTMS-1:3* in GC cells. Further studies have reported that lncRNAs are involved in the pathogenesis of GC (38-40). These results suggested that genes co-expressed with *lnc-SCD-1:13* and *lnc-PTMS-1:3* may be involved in the effects of a low dose of celecoxib on GC. However, the associations of ITGA3 and DVL1 with *lnc-SCD-1:13* and *lnc-PTMS-1:3* remain unclear, and should be investigated in further studies.

In summary, the present study demonstrated that a low dose of celecoxib may exert an anti-cancer effect by regulating the cell cycle and apoptosis independent of COX-2 in GC cells. Furthermore, *ITGA3* and/or *DVL1* co-expressed with *lnc-SCD-1:13* and *lnc-PTMS-1:3* may be involved in the effects of a low dose of celecoxib on GC.

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