Esophageal squamous cell carcinoma cell proliferation induced by exposure to low concentration of cigarette smoke extract is mediated via targeting miR-101-3p/COX-2 pathway

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Abstract. Cigarette smoke has been implicated as a major risk factor for esophageal squamous cell carcinoma (ESCC). Several lines of evidence have suggested that the promoting effect of cigarette smoking extract (CSE) on ESCC is mediated by upregulation of cyclooxygenase-2 (COX-2) expression. Yet, the underlying molecular and cellular mechanisms of how CSE stimulates COX-2 expression and facilitates ESCC development are largely unknown. In the present study, we revealed microRNA (miR)-101-3p expression was downregulated upon exposure to low concentration of CSE in Eca109 cancer cells, and suppression of miR-101-3p was required for low CSE-induced cell proliferation, presenting as overexpression of miR-101-3p reversing CSE stimulated cancer cell growth. Luciferase assay revealed that COX-2 was a direct target for miR-101-3p and overexpression of miR-101-3p decreased cellular COX-2 protein expression. Furthermore, we found that COX-2 inhibitor and knockdown of COX-2 by siRNA interference could abolish CSE-induced cell proliferation, indicating that promotion of cancer cell proliferation by low concentration of CSE was dependent on COX-2 activity. Finally, downregulation of miR-101-3p expression and upregulation of COX-2 was found in ESCC specimens from patients with smoking history. Taken together, our findings revealed a new post-transcriptional mechanism by which CSE regulated COX-2 expression to favor cancer cell proliferation, suggesting miR-101-3p as a potential biomarker and therapeutic target for smoke-related ESCC.

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Introduction

Esophageal cancer (EC) is the eighth most common cancer worldwide and ranks sixth among all cancers in mortality due to its high fatality rate (1). EC is mainly comprised of two histologic subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, which shows striking variation by both geography and etiologic factors (2). In China, often referred to as the 'esophageal cancer belt', 90% of all cases are ESCC (3). According to the epidemiologic statistics (4), cigarette smoking is one of the major risk factors for ESCC development. Abundant evidence has shown that the exposure of cancer cells to cigarette smoke extracts (CSE) could affect cell proliferation, invasion, metastasis, cell death and immune response by modulating several critical signaling pathways (5).

Cyclooxygenase-2 (COX-2), an immediate-early response gene that is induced by a variety of stimuli such as mitogens, cytokines and growth factors. Although evidence strongly supports that smoking extracts upregulate COX-2 expression, thereby facilitating cell malignant transformation and cancer development, the detailed mechanisms of how CSE elevates COX-2 expression are largely unknown. To date, researchers have found that CSE function along with β-adrenoceptors and α7-nAChR to stimulate COX-2 and its derived prostanoids by activation of COX-2 gene regulators NF-κB and CREB through p38MAPK, ERK and cAMP-dependent pathways (6). Another study also found that methylation of COX-2 promoter regulates COX-2 expression in ESCC in response to the stimulation of CSE (7). Those mechanisms mainly relate to transcriptional regulation of COX-2 by CSE, yet, post-transcriptional mechanisms by which CSE upregulates COX-2 expression have not been explored.

MicroRNAs (miRNAs) are endogenous, small (~22 nucleotide long) non-coding RNAs, which serve as key regulators of gene expression at the post-transcriptional level by binding to the 3'-untranslated region (UTR) of corresponding target mRNA (8). Growing evidence has showed that exposure of cells to CSE causes extensive alterations in miRNA expression (9). Yet, the detailed miRNAs and correspondent targets contributing to CSE-induced-cancinogensis are not fully defined. In the present study, through microarray analysis of differential microRNA expression in human esophageal

epithelial cell line treated with or without CSE, we found 47 downregulated and 13 upregulated miRNAs. Furthermore, we revealed CSE-miR-101(-)-COX-2(+) axis, from which low concentration of CSE facilitated cancer cell proliferation, thereby providing insights into the mechanisms of miR-101-3p contributing to CSE-associated ESCC development.

Materials and methods

Cell line and culture. The human immortalized non-tumorigenic esophageal epithelial cell line (Het-1A) was purchased from the American Type Culture Collection (ATCC; lot no. CRL-2692) and was cultured in BEGM culture medium including BPE, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B and retinoic acid. HEK293T cell line was preserved in our laboratory. The human ESCC cell line (Eca109) was obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Eca109 cells were grown in RPMI-1640 containing 10% fetal bovine serum (both from Gibco, New York, NY, USA) 100 units of penicillin/ml and 100 mg of streptomycin/ml (Invitrogen, Carlsbad, CA, USA), and all cells were incubated at 37˚C in a humidified chamber supplemented with 5% CO_2.

Cigarette smoke exact preparation. Cigarettes were purchased from Hunan Zhongyan Industrial Co., Ltd. [Hongmei Brand; tar content, 12 mg; and nicotine content, 1.3 mg/cigarette; smoke gas (CO): 13 mg]. CSE was prepared by a modification based on the method of Su *et al* (10). In brief, three cigarettes with filters were combusted with a modified syringe-driven apparatus. The smoke from cigarettes was bubbled through 30 ml of sterile phosphate-buffered solution (PBS) which was pre-warmed to 37˚C by application of a vacuum to the vessel containing the PBS. Each cigarette was smoked for 5 min, and three cigarettes were used per 30 ml of PBS to generate a CSE-PBS solution. Control solutions were prepared with the same protocols used to generate CSE, except that the cigarettes were unlit. CSE stock was adjusted to pH 7.4, then filtered through a 0.20-μm pore filter. The concentration of nicotine in the CSE stock solution was measured by high-performance liquid chromatography (HPLC) at the Department of Pharmacy, The Second Xiangya Hospital of Central South University, and the concentration of nicotine was ~10,000 ng/l in CSE stock solution. The CSE solutions were diluted with RPMI-1640 medium and used immediately as subsequently described. Final concentrations of these solutions are expressed as percent values, which were calculated with the following equation: (ml CSE solution ÷ total ml) x 100. Total milliliters in this equation are the sum of milliliters of CSE solution and milliliters of RPMI-1640 (10). Solutions with concentration ranging from 0.01 to 20% were used in the present study.

Establishment of the stable miR-101-3p overexpressing cell lines. Commercial pLVX-IRES-ZsGreen1 for miR-101-3p overexpression was purchased from Clontech (Palo Alto, CA, USA). Lentiviral vectors of pLVX-pre-miRNA-101-3p which expressed miR-101-3p precursor were constructed by Yinrunbio (Changsha, China). A pre-mixed Lentiviral Packaging System (Biosettia, San Diego, CA, USA) used for viral packaging was transfected into HEK293T cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Lentivirus was added to Het-1A or Eca109 cells at 50% confluency in 100 mm dishes along with Polybrene at a final concentration of 8 μg/ml. After 72 h transduction, Het-1A or Eca109 cells with Lenti-miR-101-3p or Lenti-NC (negative control) expression were sorted by fluorescence-activated cell sorting (FACS) using green fluorescence protein ZsGreen1 as selecting marker, and then used for subsequent experiments including proliferation, quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting.

Quantitative real-time polymerase chain reaction (qRT-PCR) and miRNA microarray analysis. Total RNA from the frozen tissue specimens and cultured cells was extracted using the TRIzol kit (Invitrogen) according to the manufacturer's instructions. cDNA synthesis and qPCR was performed with Invitrogen NCode™ miRNA SYBR®-Green qRT-PCR analysis (Invitrogen). The forward primer for miR-101-3p was 5'-CCGGTACAGTACTGTGATAACTGAA-3'. Fold-change ($2^{-\Delta\Delta Ct}$) normalized to control U6 small nuclear RNA (snRNA) levels was used to compare differential miRNA expression. For miRNA microassay analysis, total RNA was isolated with TRIzol reagent from Het-1A cells with or without CSE treatments. Samples were labeled and hybridized with miRCURY LNA™ Array v.16.0 (Exiqon, Denmark). GenePix 4000B scanner and GenePix Pro 6.0 software (Axon Instruments, Union City, CA, USA) were used to scan images for the analysis. Each chip was normalized to the U6 signal intensity. miRNAs with a significant value of 0.05 or lower and a fold-change value of 2 or higher were considered to be differentially expressed.

Luciferase reporter gene assays. The putative miR-101 binding sites at the 3'-UTRs of COX-2 mRNAs (NM_000963.1 nt 3481-3954 with seed sequence at nt 3689-3696) was cloned downstream of luciferase gene into the pGL3-control Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). Primer used for cloning were: 5'-GGACT AGTGCTATCTGTAACCAAGATGG-3' (forward) and 5'-CCCAAGCTTCACATAGGCCTATCCTAAGG-3' (reverse) (11). The pGL3-COX-2-3-UTR-MU plasmid, which carried the mutated sequence in the complementary sites for the seed region of miR-101-3p, was generated based on pGL3-COX-2-3-UTR-WT plasmid by site-specific mutagenesis (QuikChange™ II; Stratagene, La Jolla, CA, USA).

Eca109 cells with Lenti-miR-101-3p and Lenti-NC expression were plated into 24-well plates ($3x10^4$ cells/well). After 24 h, the cells were co-transfected with the 50 ng control pRL-TK plasmid containing the *Renilla* luciferase gene (Promega) and 300 ng pGL3-COX-2-3-UTR (WT/MUT) plasmid DNA or 300 ng pGL3 control-luciferase plasmid using Lipofectamine 2000. At 48 h post-transfection, luciferase activity was detected using the Dual-Luciferase Reporter Assay system (Promega). All transfection experiments were performed in triplicate and repeated at least three times.

Cell viability. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

After incubation with CSE at different concentrations for 0, 24, 48 and 72 h, Het-1A or Eca109 cells with Lenti-miR-101-3p or Lenti-NC overexpression were washed with PBS three times to remove CSE and then incubated with 2.5% MTT solution (5 mg/ml) for another 4 h at 37˚C. Thereafter, DMSO was added to solubilize the crystals for 20 min at room temperature. The optical density was determined with a spectrophotometer (Thermo Scientific Varioskan Flash, USA) at a wavelength of 490 nm. All experiments were performed three times in triplicate.

siRNA interference. To suppress expression of COX-2, the following previously described pairs of oligonucleotides were used: siMock, 5'-GUAAGACACGACUUAUCGCdTdT-3' and 5'-GCGAUAAGUCGUGUCUUACdTdT-3'; siCOX-2, 5'-UGAAAGGACUUAUGGGUAAdTdT-3' and 5'-UUACCCAUAAGUCCUUUCAdTdT-3' (12). Each pair of oligonucleotides (5 μM each) was dissolved in an annealing buffer (5 mM Tris-HCl, pH 7.5, 1 mM EDTA), heated at 65˚C for 5 min and then slowly cooled to room temperature and stored at -80˚C. Eca109 cells were transfected with 100 pmol of each duplex/well of a 24-well using oligofectamine reagent (Invitrogen) according to the manufacturer's recommendation.

Protein extraction and western blotting. All cells were rinsed with PBS (pH 7.4) and were lysed on ice for 30 min in RIPA lysis buffer (Beyotime, China) supplemented with a protease inhibitor cocktail (Roche, Switzerland). The tissue samples were frozen solid with liquid nitrogen, ground into powder, and lysed on ice for 30 min in RIPA lysis buffer containing the protease and phosphatase inhibitor. When necessary, sonication was used to facilitate lysis. Cell lysates or tissue homogenates were centrifuged for 30 min (14,100 x g, 4˚C). The supernatant was collected, and the protein concentration was calculated using the BCA protein assay kit (Conway Century, China). The protein levels were analyzed via western blotting using the COX-2 antibody (CST, Danvers, MA, USA). The protein levels were normalized by probing the same blots with a β-actin antibody (Sigma, St. Louis, MO, USA). Protein bands were analyzed using the ImageJ (National Institutes of Health, Bethesda, MD, USA).

Flow cytometric analysis. Approximately 1-2x10^6 single cells were harvested and washed in cold PBS twice, then fixed in 70% ethanol overnight. Cells were washed the next day in cold PBS once and then incubated in propidium iodide (PI) buffer (PBS containing 40 μg/ml PI and 100 μg/ml RNAase) at 37˚C for 30 min prior to analysis by flow cytometry (BD FACSCanto II analyzer; BD Biosciences). The percentage of sub-G1 population indicative of cell death was analyzed with WinMDI 2.9. The mean value was calculated from three independent experiments.

Patients and samples. From October 2011, 8 pairs of fresh human samples of ESCC and corresponding adjacent non-tumor tissue (3 cm from the cancer tissue) were obtained from patients at The Second Xiangya Hospital of Central South University. All the 8 patients in the study had a long history of smoking but almost no drinking. None of them received any chemotherapy or radiation therapy before surgery. All the patients were informed of the purpose and procedure of the present study and agreed to donate excess tissue. The study was approved by the Ethics Committee of the Second Xiangya Hospital [file no. 184 (2010)] and written informed consent was obtained from all surgical patients to use resected samples for research.

Statistical analysis. All statistical analyses were carried out using SPSS version 18.0 statistical software (Aspire Software International, Leesburg, VA, USA). Student's t-test was used to determine statistical significance. All data represent mean ± SD. All statistical tests were two-sided and P-values <0.05 were considered to indicate a statistically significant result.

Results

CSE induces a decrease in miR-101-3p expression. Microarray analysis revealed 60 miRNAs that were differentially regulated in 20% CSE treated immortalized non-tumorigenic esophageal epithelial cell line Het-1A compared to sham-exposed cells, among which 47 miRNAs are downregulated and 13 miRNAs are upregulated (Table I). A representative heat map of differentially expressed miRNAs between CSE-exposed and sham-exposed Het-1A cells is shown in Fig. 1A. Microarray results were validated by qRT-PCR analysis of the following miRNAs: hsa-miR-3687, hsa-miR-200c-3p (upregulated) and hsa-miR-101-3p, hsa-miR-140-3p and hsa-miR-320a (downregulated) (Fig. 1B). Among those miRNAs, we focused on miR-101-3p, functions of which have not been defined upon cells exposure to CSE, yet the expression has been found downregulated in many types of cancers, including ESCC (13). We then studied the effects of different concentration of CSE on the expression of miR-101-3p in Het-1A and cancer cell line Eca109, and qRT-PCR results showed that higher concentrations of CSE could induce a decrease in miR-101-3p expression in both cell lines (Fig. 1C), notably, low concentration of CSE (0.25%), not medium concentration of CSE, also induced a significant decrease in miR-101-3p expression in Eca109, but not Het1A cells (Fig. 1C). The differences of miR-101-3p expression to CSE treatments facilitated us to study phenotype changes of cells to different concentrations of CSE solution.

Cancer cell proliferation induced by exposure to low concentration of CSE was mediated via suppression of miR-101-3p. As contradictory results on cell growth and cell death have been reported when cells were exposed to different models and concentrations of CS-extract, we first applied MTT assay to study cell proliferation effects in both Het-1A and Eca109 cells under different concentrations of our CSE solution. Results showed that CSE inhibited proliferation of the normal esophageal epithelial Het-1A cells, and massive cell death was found at 5% and the higher concentration of CSE treatment, which presented as a plateau or decrease in cell proliferation curve (Fig. 2A). Eca109 cells are comparatively resistant to CSE treatment with a plateau or decrease in cell proliferation at 10% and higher concentrations. Notably, low concentration of CSE (0.25%) treatment of Eca109 did not inhibit cell growth but stimulated cell proliferation (Fig. 2B).

Table I. The differential expression profiles of miRNAs in CSE-induced Het-1A cells compared to sham-control cells using miRNA array analysis.

ID	Name	Fold-changes	Normalized Co.	Normalized Exp.
Upregulated				
14808	hsa-miR-3687	2.43	0.45	1.86
42551	hsa-miR-122-3p	2.26	0.58	1.28
145789	hsa-miR-550a-3-5p/ hsa-miR-550a-5p	2.68	0.14	0.31
147604	hsa-miR-4285	2.64	0.16	0.43
147947	hsa-miR-4308	2.16	0.49	1.30
42454	hsa-miR-138-2-3p	2.85	0.26	0.56
42514	hsa-miR-937	3.0	0.41	1.18
17427	hsa-miR-200c-3p	2.15	0.12	0.35
148000	hsa-miR-3195	2.31	0.63	1.35
146043	hsa-miR-24-1-5p	2.01	0.63	1.45
147739	hsa-miR-3161	2.39	0.12	0.24
45764	hsa-miR-302e	2.77	0.89	2.12
145914	hsa-miR-135b-5p	2.43	0.09	0.24
Downregulated				
145638	hsa-miR-29a-5p	0.48	4.62	2.23
31026	hsa-miR-101-3p	0.47	0.92	0.43
10943	hsa-miR-136-5p	0.45	0.96	0.43
17463	hsa-miR-151a-3p	0.44	1.62	0.71
10964	hsa-miR-155-5p	0.45	0.69	0.31
17810	hsa-miR-29b-1-5p	0.38	1.37	0.51
10975	hsa-miR-182-5p	0.45	0.63	0.29
29190	hsa-miR-708-5p	0.39	0.93	0.36
42663	hsa-miR-20a-3p	0.23	0.32	0.08
145852	hsa-miR-210	0.28	1.40	0.40
145742	hsa-miR-935	0.40	0.94	0.37
27536	hsa-miR-190a	0.47	0.44	0.21
10936	hsa-miR-130b-3p	0.47	1.26	0.59
10986	hsa-miR-193a-3p	0.47	4.59	2.14
42682	hsa-miR-25-3p	0.47	2.60	1.23
147735	hsa-miR-4289	0.47	0.38	0.18
17885	hsa-miRPlus-A1086	0.49	27.97	13.21
28950	hsa-miR-455-3p	0.48	0.31	0.15
42630	hsa-miR-140-3p	0.28	1.05	0.30
27533	hsa-miR-320a	0.12	0.98	0.12
9938	hsa-let-7i-5p	0.32	6.90	2.20
11020	hsa-miR-22-3p	0.34	46.80	15.70
27720	hsa-miR-15a-5p	0.40	7.95	3.15
4610	hsa-miR-126-3p	0.10	0.32	0.03
17961	hsa-miR-629-5p	0.28	0.50	0.14
19582	hsa-miR-106b-5p	0.44	4.63	2.04
13147	hsa-miR-96-5p	0.48	1.24	0.60
29490	hsa-miR-7-5p	0.48	1.69	0.81
42887	hsa-miR-331-3p	0.46	1.41	0.65
145670	hsa-miR-18b-5p	0.48	0.49	0.23
42532	hsa-miR-22-5p	0.41	2.45	1.01
148620	hsa-miR-454-3p	0.35	0.58	0.20
148418	hsa-miR-3607-5p	0.48	1.07	0.51
10972	hsa-miR-181b-5p	0.45	0.81	0.36
17918	hsa-miR-222-5p	0.24	0.31	0.07
30687	hsa-miR-93-5p	0.40	2.79	1.11
145943	hsa-miR-100-5p	0.40	7.97	3.16
145636	hsa-miR-181d	0.39	1.73	0.67
145845	hsa-miR-20a-5p	0.31	4.24	1.33
13143	hsa-miR-301a-3p	0.32	0.94	0.30
17888	hsa-let-7a-3p	0.50	0.30	0.15
46870	hsa-miR-320d	0.38	0.67	0.26
146112	hsa-miR-30b-5p	0.47	5.84	2.74
148098	hsa-miR-374b-5p	0.44	2.26	0.99
46439	hsa-miR-1243	0.24	0.77	0.19
10987	hsa-miR-193b-3p	0.49	0.73	0.35
145841	hsa-miR-23b-3p	0.43	27.31	11.86

miRNA, microRNA; CSE, cigarette smoke extract; hsa, *homo sapiens*; Con., control; Exp., experiment.

Considering the miR-101-3p regulation upon CSE exposure, which showed significant downregulation at low and high concentration of CSE treatment in Eca109 cells, we wondered whether promotion of Eca109 cell proliferation under low concentration of CSE as well as induced cell death under high concentration of CSE were depended on miR-101-3p downregulation. First, we applied MTT assay to study cell proliferation under low concentration of CSE exposure in Eca109 cells, and results showed overexpression of miR-101-3p could reverse the increased cell proliferation induced by low concentration (0.25%) of CSE treatment ($p<0.05$) (Fig. 2C), indicating an important role of miR-101-3p in promotion of cell proliferation under low concentration of CSE treatment. Next, we studied cell apoptosis under high concentration of CSE exposure by flow cytometric analysis of sub-G1 population, however, overexpression of miR-101-3p could not rescue cell death induced by high concentration (20%) of CSE treatment (Fig. 2D). Collectively, our data suggested that suppression of miR-101-3p plays a crucial role in low-CSE-induced cell proliferation, yet the role of miR-101-3p in response to high-CSE is not known.

miR-101-3p inhibits COX-2 expression by targeting 3'-UTR of COX-2 mRNA. As miR-101 was reported to inhibit COX-2 post-transcriptional expression by binding to the 3'-UTR of COX-2 mRNA in prostate and colon cancer cells (14,15), to verify whether COX-2 is a direct target of miR-101-3p in esophageal cancer cell lines, COX-2 3'-UTR containing the miR-101 binding sites (WT), and binding site mutant (Fig. 3A) were cloned downstream of the luciferase open reading frame to obtain constructs for luciferase activity assay. The assay results showed that increased expression of miR-101-3p

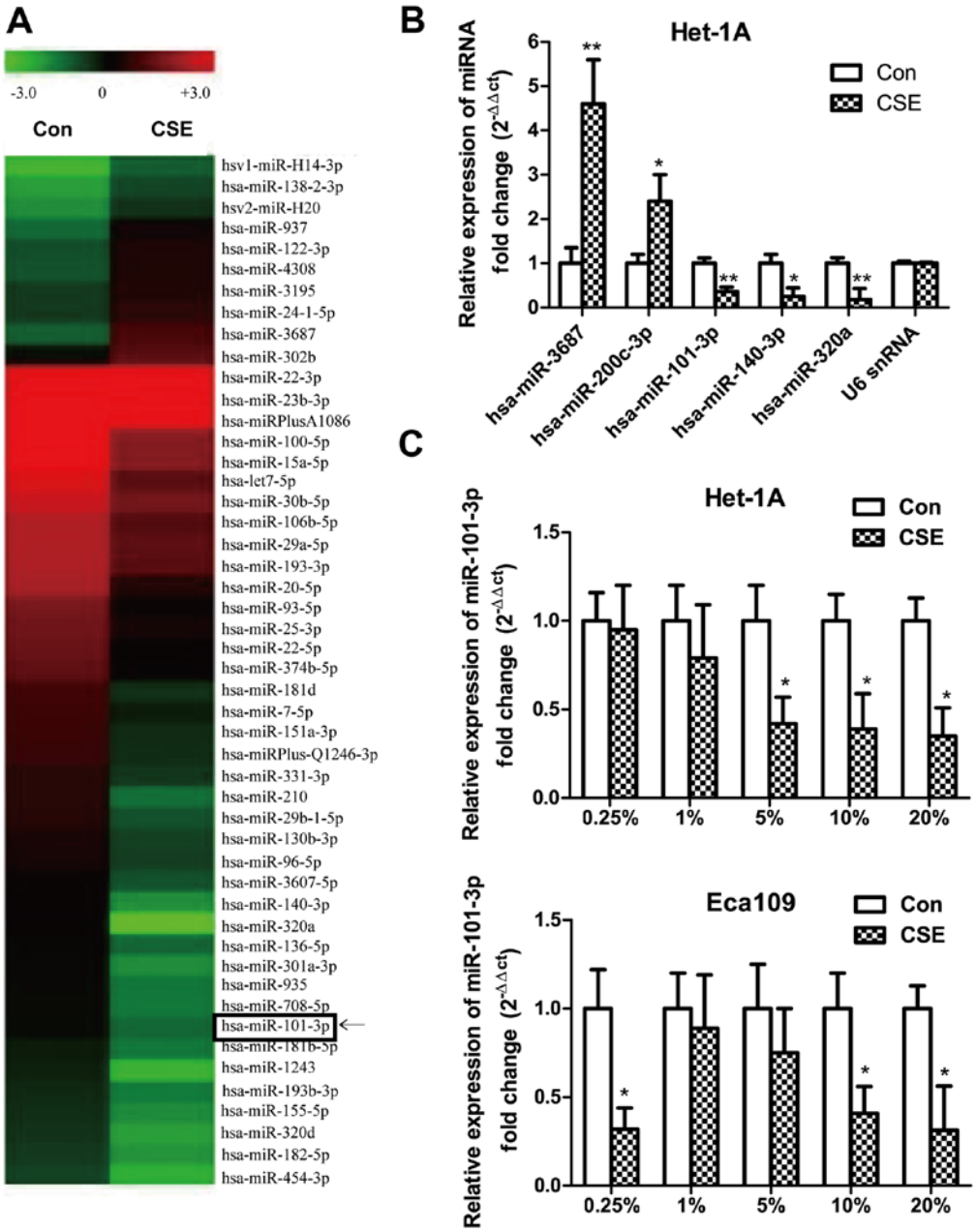

Figure 1. CSE downregulated miR-101-3p expression. (A) miRNA microarray analysis of Het-1A cells with or without 20% CSE treatment for 48 h, representative heat map (presented as normalized intensity) with 49 miRNAs is shown. Colors represent relative miRNA expression as indicated in the color key. Red and green represent low and high miRNA expression, respectively. Arrow points to the miR-101-3p. (B and C) Quantitative RT-PCR validation of differential indicated miRNAs expression in Het-1A cells (B) or analysis of miR-101-3p expression in Het-1A and Eca109 cells with different concentration of CSE solution treatment for 48 h (C). Data are representative of three biological replicates. $^{**}p<0.01$, $^{*}p<0.05$.

significantly downregulated luciferase activity in Eca109 cells that co-transfected with COX-2 3'-UTR-WT construct, while the luciferase activities of binding site mutants were unaffected by lentiviral transduction of miR-101-3p (Fig. 3B). Without exogenous expression of miR-101-3p, the luciferase activity in cells with luciferase construct of COX-2 3'-UTR-WT was lower than that in cells with construct of binding site mutant, suggesting a functional effect of endogenous miR-101-3p (Fig. 3B). The protein expression of COX-2 was also showed downregulated upon lentiviral transduction of miR-101-3p in both esophageal epithelial cell line Het-1A and esophageal squamous cancer cell line Eca109 (Fig. 3C), confirming miR-101-3p negative regulation of COX-2 protein expression in esophageal cells. MTT analysis showed proliferation of Het-1A and Eca109 cells were significantly inhibited concomitant with miR-101-3p overexpression (Fig. 3D and E). Furthermore, the level of COX-2 protein in Eca109 cells significantly increased under the stimulus of 0.25% CSE for 48 h, and attenuated by miR-101-3p overexpression (Fig. 3F), indicating COX-2 is a direct target of miR-101-3p under low concentration of CSE.

Promotion of cancer cell proliferation induced by low concentration of CSE is dependent on COX-2 expression. As COX-2 is a direct target of miR-101-3p under low concentration of CSE, we wondered whether promotion of Eca109 cell proliferation

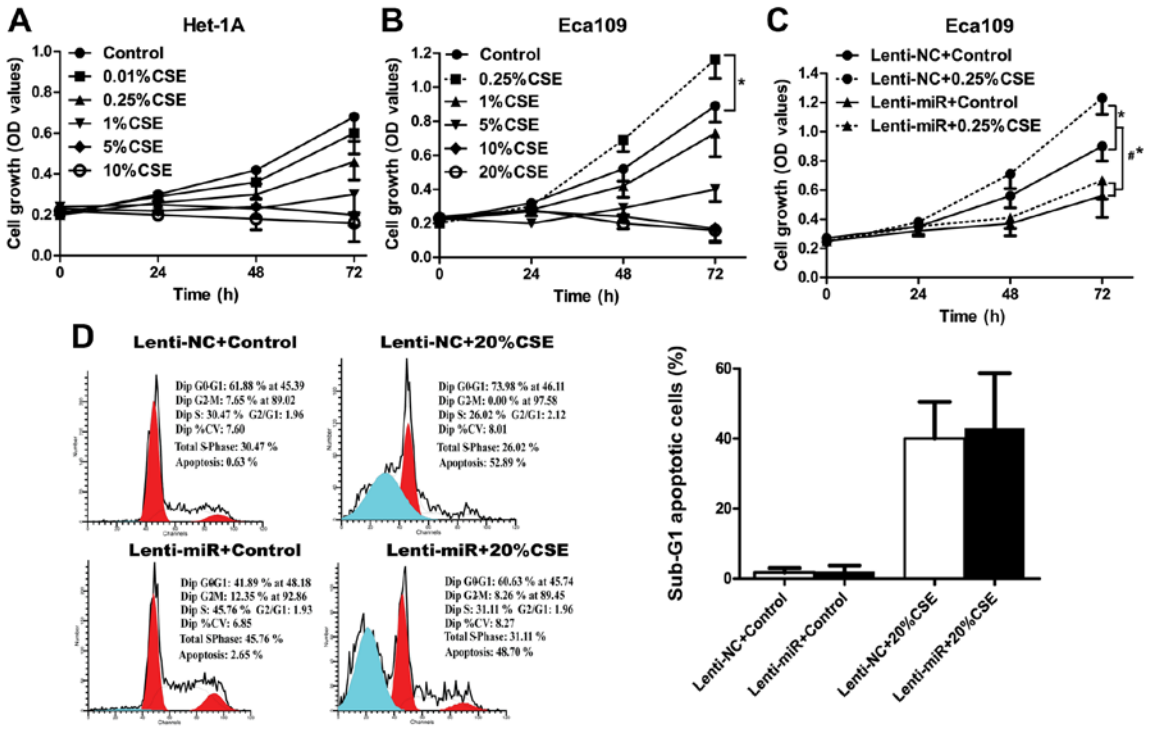

Figure 2. Cancer cell proliferation induced by exposure to low concentration of CSE is mediated via suppression of miR-101-3p. (A and B) MTT analysis of cell growth in (A) Het-1A and (B) Eca109 cells incubation with indicated concentration of CSE for 0, 24, 48 and 72 h. Error bars show the mean ± SD from three biological replicates, *p<0.05 shows 0.25% CSE treatment compared to control solution. (C) Eca109 cell proliferation induced by low concentration of CSE was via suppression of miR-101-3p. Eca109 cells with Lenti-miR-101-3p and Lenti-NC expression were treated with 0.25% CSE for 0, 24, 48 and 72 h and cell growth was measured by MTT. Error bars show the mean ± SD from three biological replicates. #, Compared promoting rate at 72 h between Lenti-miR-101-3p and Lenti-NC group by two-sided t-test. *p<0.05. (D) miR-101-3p had no significant effects on regulation of cell apoptosis under high concentration of CSE. Eca109 cells with Lenti-miR-101-3p and Lenti-NC expression were treated with 20% CSE for 48 h then cell were subjected to flow cytometry for sub-G1 analysis.

under low concentration of CSE were depended on COX-2 activity. Indeed, COX-2 inhibitor (NS398) reversed 0.25% CSE-induced cancer cell proliferation ($p<0.05$) (Fig. 4A). Moreover, knockdown of COX-2 also inhibited 0.25% CSE-induced cancer cell proliferation (Fig. 4B). Collectively, promotion of cancer cell proliferation induced by low concentration of CSE was dependent on COX-2 upregulation.

Downregulation of miR-101-3p and upregulation of COX-2 in ESCC tissue samples. We studied 8 patients who had long history of smoking but almost no drinking. We detected the level of miR-101-3p in the 8 paired ESCC patients (tumor and adjacent non-tumor tissue) by qRT-PCR analysis. Generally, in ESCC patients, miR-101-3p expression was significantly lower in 7/8 of cancer tissues compared to adjacent non-tumor tissue (Fig. 5A). The t-test showed that miR-101-3p was significantly reduced in ESCC tissues than their adjacent non-cancerous tissues. Then we used western blotting to examine COX-2 expression in tissue samples collected from those ESCC patients (Fig. 5B). The 6 of 8 samples showed higher expression of COX-2 protein in ESCC tissues than in the non-cancerous esophageal tissues.

Discussion

Exposure of cells to CSE causes extensive alterations in miRNA expression, and those changes in microRNA (miRNA) expression are an early event following exposure to cigarette smoke (9). To unravel miRNA and its function may provide better understanding of how environmental factors contribute to cancer initiation and development. miR-101-3p has been reported downregulated in many types of cancers, including ESCC (13), prostate (14) and colorectal cancer (15). Through post-transcriptional inhibition of targets, such as cyclooxygenase-2 (COX-2/PTGS2) (16), EZH2/ENX-1 (13), Mcl-1(17), mTOR (18), miR-101-3p could suppress cancer cell proliferation, migration and invasion. In the present study, we reported miR-101-3p was downregulated upon CSE exposure in both normal epithelia cell Het-1A and cancer cells, expanding the view of the miRNA changes that contribute to CSE-induced ESCC development.

A large body of evidence has suggested that the promoting effect of smoking on ESCC is mediated by the induction of COX-2 activity. COX-2 has been found transcriptional upregulated in Eca109 cells upon cell treatment of chloroform or ethanol extract of CS, and upregulation of COX-2 stimulates Eca109 cells proliferation (19). In this present study, the model of generation of CSE is the extraction in buffered media, but not organic extract, we also confirmed a correlation of CSE on COX-2 expression when cells were exposed to a low concentration (0.25%) of CSE. Yet, the mechanisms of how CSE regulated COX-2 expression are different from published literature, revealing a new post-transcriptional mechanism by identifying COX-2 targeting microRMAs. Our finding adds

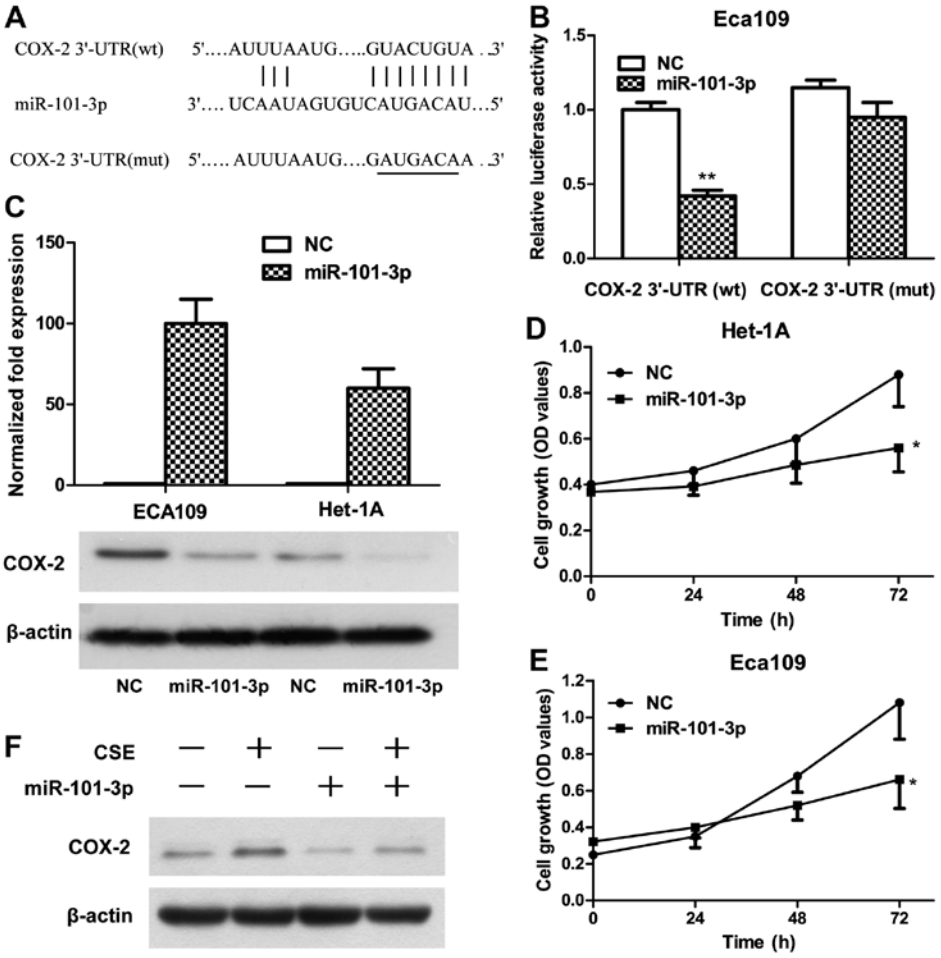

Figure 3. miR-101-3p targeted COX-2 expression by binding to 3'-UTR of COX-2 mRNA. (A) Schematic representation of the putative binding sites in COX-2 mRNAs 3'UTR for miR-101-3p. The designed COX-2-mutant (MUT) sequence (AUGACA) without miR-101-3p binding sites is also shown. (B) Eca109 cells with Lenti-miR-101-3p and Lenti-NC expression were cotransfected with 300 ng dual luciferase reporter plasmids carrying either WT or MUT 3'-UTR of COX-2. At 48 h post-transfection, luciferase activity was detected using the Dual-Luciferase Reporter Assay system (Promega). The relative firefly luciferase activity was normalized with *Renilla* luciferase activity. Data are shown as mean ± SD (n=3). $^{**}p<0.01$. (C) q-RT-PCR (upper panel) and western blotting (down panel) analysis of miR-101-3p and COX-2 expession in Het-1A and Eca109 cells lines with Lenti-NC and Lenti-miR-101-3p overexpression. (D and E) Het-1A (D) and Eca109 (E) cell lines with Lenti-NC and Lenti-miR-101-3p overexpression were cultured in 96-well plates for indicated times and were analyzed by MTT assay. Error bars show the mean ± SD from three biological replicates, $^{*}p<0.05$. (F) Western blot analysis of Eca109 cells with or without stable miR-101-3p overexpression treated with 0.25% CSE for 48 h. β-actin protein was detected as loading control. Representative images of three independent biological experiments are shown.

an important piece to the puzzle of how environmental factors regulate COX-2 expression, thereby contributing to ESCC development.

In our finding, low concentration of CSE (0.25%) induced a significant decrease in miR-101-3p expression in Eca109 cells but not in the non-tumorigenic esophageal epithelial cell line Het1A, indicating dysregulation of miR-101-3p may be exclusively related to ESCC cancer cell. Considering the anatomy of esophagus, esophageal cancer cells, unlike oral/airway epithelial cells, may not directly expose to high concentration of smoking extract, therefore, the effect of low concentration of smoking extract on ESCC cells may better imitate the real situation of how environment factor facilitate ESCC development. CSE solutions of 2.5% used in the present study approximately correspond to direct exposure to cigarette smoking from those who smoke 0.5 pack/day (10). Yet, whether 0.25% CSE or which concentration of CSE could mimic the *in vivo* concentration of esophageal epithelial cell exposure when passive or active inhalation of cigarette smoke are unknown but warrant further investigation. In the present study, we confirmed that the proliferation of Eca109 cells when exposed to low concentration of CSE was dependent on suppression of miR-101-3p and upregulation of its target COX-2 (Figs. 2C and 4), yet this effect was not found in Het-1A cells, indicating miR-101-3p may function as a tumor suppressor in the ESCC development but not in the initiation stage.

In the present study, we noticed that both normal esophageal epithelial Het-1A and Eca109 cancer cells induced downregulation of miR-101-3p when exposed to high concentration of CSE, yet the functions of downregulation of miR-101-3p in this circumstance are not known. We speculated that identification of new targets of miR-101-3p may provide clues for unveiling novel functions for miR-101-3p, and warrant better understanding of cellular responses to environmental factors, such as high CSE exposure.

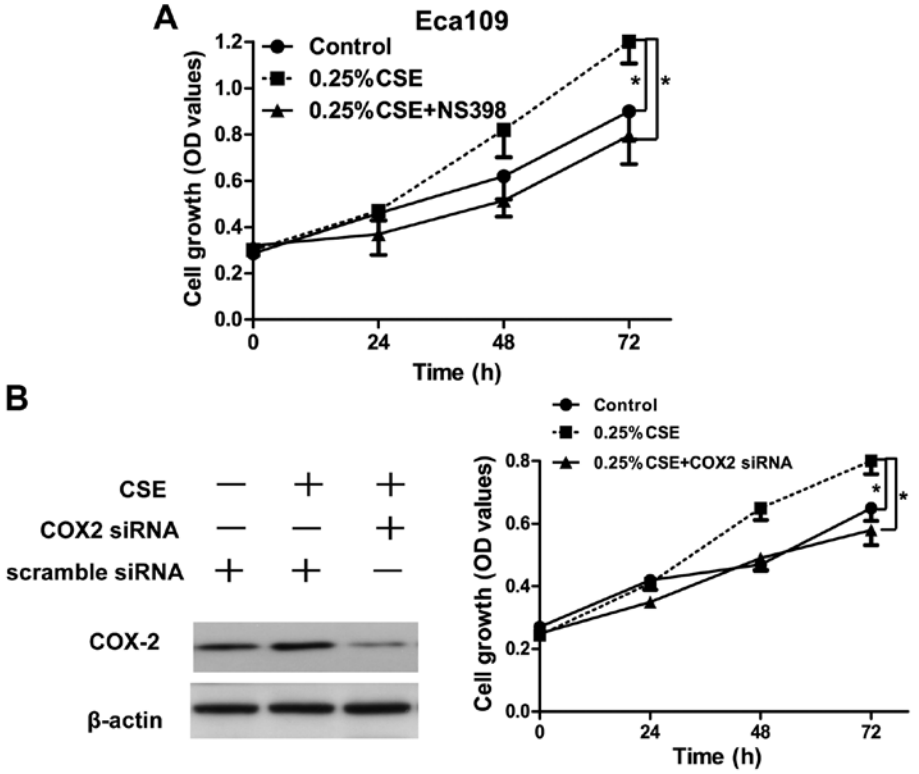

Figure 4. The low concentration of CSE-induced cell proliferation is dependent on upregulation of COX-2. (A) Eca109 cells were cultured in the absence (DMSO) or presence of NS398 (10 μM) for 18 h, then treated with 0.25% of CSE for 0, 24, 48 and 72 h, and cell growth was measured by MTT. Data are shown as mean ± SD of three independent biological replicates. *p<0.05. (B) Eca109 transfected with scrambled-siRNA or COX-2 siRNA for 24 h, then subjected to 0.25% CSE treatment for 48 h and cell lysates were collected for western blot analysis of COX-2 and β-actin expression (left panel), or subject to 0.25% CSE for further incubation of indicated times then analyzed by MTT assay (right panel). Error bars show the mean ± SD from three biological replicates, *p<0.05.

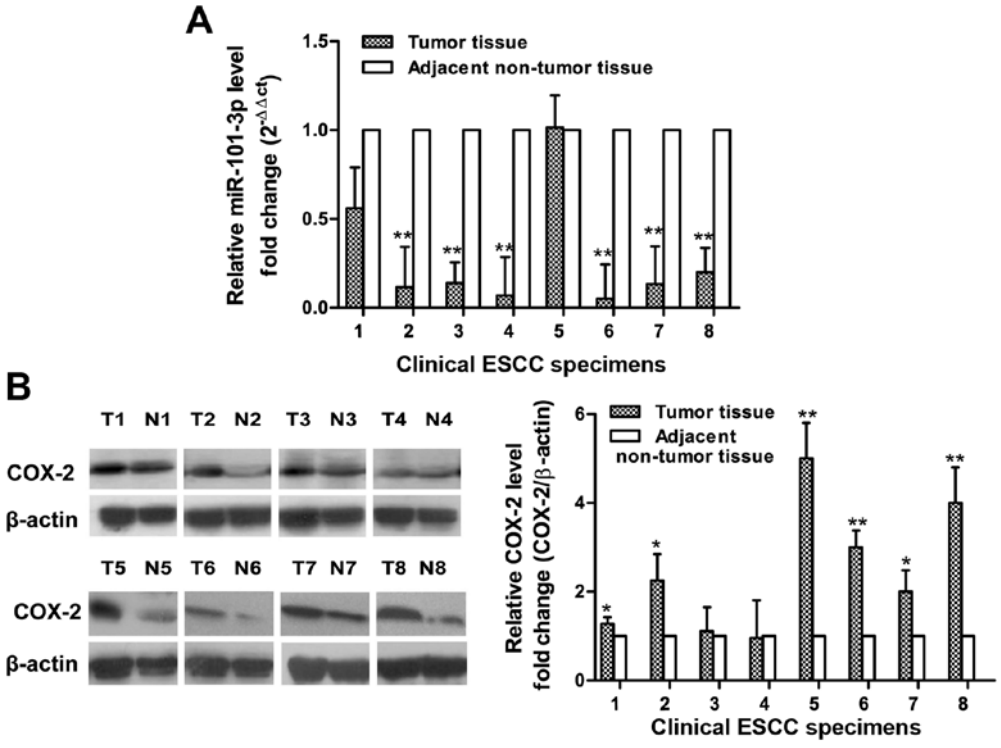

Figure 5. Downregulation of miR-101-3p and upregulation of COX-2 in ESCC tissue samples. (A) Quantitative real-time PCR analysis of miR-101-3p expression in paired ESCC and adjacent non-tumor tissues obtained from 8 patients who had a long history of smoking but almost no drinking. **p<0.01 tumor vs. adjacent non-tumor. (B) Western blot analysis of COX-2 protein expression in 8 paired ESCC tissues and adjacent normal esophageal tissues (N, adjacent non-tumor tissues; T, tumor tissues). The protein expression of COX-2 in ESCC specimens were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) and normalized to the expression levels of β-actin. *p<0.05, **p<0.01.

In conclusion, we revealed CSE-miR-101(-)-COX-2(+) axis, from which low concentration of CSE facilitated cancer cell proliferation. These findings provide new regulatory mechanism involved in the smoking-induced excessive proliferation of ESCC, providing novel clues into blocking this pathological process.

Acknowledgements

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