Identification of key genes and long non-coding RNAs in celecoxib-treated lung squamous cell carcinoma cell line by RNA-sequencing

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Abstract. Celecoxib is an inhibitor of cyclooxygenase-2, a gene that is often aberrantly expressed in the lung squamous cell carcinoma (LSQCC). The present study aims to provide novel insight into chemoprevention by celecoxib treatment. The human LSQCC cell line SK-MES-1 was treated with or without celecoxib and RNA-sequencing (RNA-seq) was performed on the Illumina HiSeq 2000 platform. Expression levels of genes or long non-coding RNAs (lncRNAs) were calculated by Cufflinks software. Subsequently, differentially expressed genes (DEGs) and differentially expressed lncRNAs (DE-LNRs) between the two groups were selected using the limma package and LNCipedia 3.0, respectively; followed by co-expression analysis based on their expression correlation coefficient (CC). Enrichment analysis for the DEGs and co-expressed DE-LNRs were performed. Protein-protein interaction (PPI) network analysis for DEGs was performed using STRING database. A set of 317 DEGs and 25 DE-LNRs were identified between celecoxib-treated and non-treated cell lines. A total of 12 pathways were enriched by the DEGs, including 'protein processing in endoplasmic reticulum' for activating transcription factor 4 (*ATF4*), 'mammalian target of rapamycin (mTOR) signaling pathway' for vascular endothelial growth factor A (*VEGFA*) and 'ECM-receptor interaction' for fibronectin 1 (*FN1*). Genes such as *VEGFA*, *ATF4* and *FN1* were highlighted in the PPI network. *VEGFA* was linked with lnc-AP000769.1-2:10 (CC= -0.99227), whereas *ATF4* and *FN1* were closely correlated with lnc-HFE2-2:1 (CC=0.996159 and -0.98714, respectively). lncRNAs were also enriched in pathways such as 'mTOR signaling pathway' for lnc-HFE2-2:1. Several important molecules were identified in celecoxib-treated LSQCC cell lines, such as *VEGFA*, *ATF4*, *FN1*, lnc-AP000769.1-2:10 and lnc-HFE2-2:1, which may enhance the anti-cancer effects of celecoxib on LSQCC.

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Abbreviations: ^{18}F-FDG, 2-(^{18}F)-fluoro-2-deoxy-d-glucose; *ATF4*, activating transcription factor 4; CC, correlation coefficient; CDDP, *cis*-diamminedichloroplatinum (II); CHOP, C/EBP homologous protein; COX, cyclooxygenase; DEG, differentially expressed gene; DR5, death receptor 5; ER, endoplasmic reticulum; FC, fold change; *FN1*, fibronectin 1; GO, gene ontology; lncRNA, long noncoding RNA; LSQCC, lung squamous cell carcinoma; mTOR, mammalian target of rapamycin; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; PPI, protein-protein interaction; RNA-seq, RNA-sequencing; *VEGFA*, vascular endothelial growth factor A

Key words: lung squamous cell carcinoma, celecoxib, long noncoding RNA, chemosensitivity, RNA sequencing, mammalian target of rapamycin signaling

Introduction

Lung cancer is one of the most frequently diagnosed cancers with high mortality worldwide, with >1,500,000 new cases diagnosed annually (1,2). Based on the cancer statistics data from 2015, lung and bronchial cancers are expected to have the highest mortality among all cancers in the USA, with the estimated mortality rate of 158,040 (3). Lung squamous cell carcinoma (LSQCC) is the second most common type of lung cancer, with an annual mortality rate of ~400,000 worldwide (4). Chemotherapy and radiotherapy are the most common treatments for LSQCC; however, patient responses to these therapies are limited (5). Therefore, there is a requirement for additional agents to be developed that may enhance the response to these treatments.

Cyclooxygenase (COX)-2 serves an important role in the tumorigenesis of various types of cancer, and COX-2 inhibitors may effectively prevent tumor progression (6). Celecoxib is a selective COX-2 inhibitor; at the early stages of non-small-cell lung cancer (NSCLC), celecoxib was reported to increase the anti-cancer properties of preoperative chemotherapies, such as paclitaxel and carboplatin (7). In addition, celecoxib treatment upregulated the expression of death receptor 5 (DR5), decreased cell survival and induced apoptosis in NSCLCs (8).

Increased expression of COX-2 has been reported in LSQCC, and its inhibitor celecoxib is predicted as the beneficial target for chemotherapy (9). Although these previous studies have implied that celecoxib enhances the response sensitivity of chemotherapy in LSQCC, the specific molecular mechanisms of this inhibitor are still unknown. Several previous studies have indicated that celecoxib treatment may result in cell cycle arrest by downregulating the expression of p21 and p27, which may account for the reduction of cyclin-dependent kinase activity (10,11). In addition, celecoxib may contribute to the inhibition of angiogenesis by suppressing the expression of angiogenic factors in tumor cells (12). However, additional studies are required to comprehensively determine the mechanism of celecoxib on chemoprevention.

Long noncoding RNAs (lncRNAs) are a recently described RNA transcript species that is different from mRNAs and microRNAs. They are not transcriptional 'noise', but are important genes that function in numerous biological processes (13,14). Currently, several lncRNAs have been identified with significant functions in lung cancer; for example, the upregulation of *PVT1* was reported to promote oncogenesis in NSCLCs (15), and prostate cancer-associated transcript 6 was predicted as an oncogenic lncRNA in the growth and invasion of lung cancer cells (16). In addition, a recent study demonstrated that the novel lncRNA *onco-lncRNA 230* was able to induce invasion and apoptosis in LSQCC, and it was suggested as a possible new diagnostic marker for the disease (17). These imply that regulation of lncRNAs serves a key role in LSQCC. However, lncRNA expression under celecoxib treatment has not been reported. A previous study demonstrated that celecoxib treatment (50 μM) induced significant overexpression of *Bcl-2*, Bcl-extra large and survivin following 24 h treatment, whereas no significant alterations in expression were identified in the activation of caspase-3, caspase-8 or caspase-9 (18). Another study revealed that the expression of multidrug resistance-associated-4, a member of the ATP-binding cassette transporters, was significantly upregulated in human LSQCC SK-MES-1 cells following treatment with 5 and 50 μmol/l of celecoxib for 24, 48 and 72 h (19). These data suggested that celecoxib treatment may induce a series of variations in the metabolism of SK-MES-1 cells; however, no lncRNAs have been identified. Therefore, the present study used RNA-sequencing (RNA-seq), which facilitates transcript analysis in various cancers (20), to identify differentially expressed genes (DEGs) and differentially expressed lncRNAs (DE-LNRs) between SK-MES-1 cells cultured with or without celecoxib treatment. In addition, potential correlations were calculated, followed by pathway exploration of the lncRNAs. This study aimed to provide novel insight into celecoxib chemoprevention and to identify potential targeting markers for COX-2 induced LSQCC.

Materials and methods

Cell culture and drug treatment. The human LSQCC cell line SK-MES-1 was purchased from the Cell Bank of Type Culture Collection Chinese Academy of Sciences (Shanghai, China). Cells were cultured in the RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), at 37˚C and 5% CO_2.

Table I. Primer sequences of genes and lncRNA determined using reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'→3')
VEGFA	F: CTGTCTAATGCCCTGGAGCC
	R: ACGCGAGTCTGTGTTTTTGC
FN1	F: TTGCTCCTGCACATGCTTTG
	R: CATGAAGCACTCAATTGGGCA
Lnc-AP000769. 1-2:10	F: GGGGAAGTAGTCTCGGGTAT
	R: GTCGTTATGAAGGCAATGTG
GAPDH	F: TGACAACTTTGGTATCGTGGAAGG
	R: AGGCAGGGATGATGTTCTGGAGAG

FN1, fibronectin 1; lnc, long noncoding; VEGFA, vascular endothelial growth factor A.

Cells at the logarithmic growth phase (at a confluency of 70-75%) were divided into two groups: i) Two identical SK-MES-1 cell samples were treated with 10 μM celecoxib in 1% DMSO medium (celecoxib-treated group); and ii) two identical SK-MES-1 cell samples were treated with equal amounts of DMSO (Control group). Both groups were cultured for 48 h at 37˚C.

RNA extraction and RNA-seq. A total of $5x10^7$ cells were utilized to isolate RNA using the RNeasy kit (catalog no. 74106; Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. RNA purity was analyzed with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.), and the RNA was reverse transcribed into cDNA for library preparation using NEBNext Ultra RNA Library Prep kit for Illumina (catalog no. E7530L; New England BioLabs, Inc., Ipswich, MA, USA), following the manufacturer's protocol. Briefly, RNA (5 μg) from each sample was sheared into small fragments (200 nucleotides) prior to cDNA synthesis using fragment buffer. Subsequently, the cDNA was blunt-ended and phosphorylated. A single 3' adenosine moiety and Illumina adapters were added on the repaired ends, followed by 15 cycles of polymerase chain reaction (PCR) according to the protocol of the kit. preamplification were performed using the NEB Phusion DNA polymerase (New England BioLabs, Inc.). RNA-seq was performed on the Illumina Hiseq 2000 Sequencing System (Illumina, Inc., San Diego, CA, USA) using the 2x50 paired-end sequencing method.

Pretreatment of RNA-seq data. Quality control (QC) of raw sequencing reads was performed using the next generation sequencing (NGS) QC Toolkit, as previously described (21). Briefly, the adaptor sequences in the reads were removed, and the low-quality reads with the base quality score <20 were filtered out. High quality sequences were defined having bases with a quality score >20 that accounted for >90% of its length. Subsequently,

Table II. Differentially expressed lncRNAs in the celecoxib-treated group and Control group.

lncRNA	Value_1	Value_2	Log_2 (FC)	q-value
A, Upregulated				
lnc-C14orf166B-3:4	183.118	400.277	112.823	$5.35x10^{-4}$
lnc-CNN3-3:1	112.248	179.237	0.675	$5.06x10^{-8}$
lnc-CTSL1-2:2	327.718	549.936	0.747	$2.63x10^{-2}$
lnc-CXCL3-1:1	128.057	34.035	141.023	$8.89x10^{-13}$
lnc-ENTPD6-2:1	101.243	188.522	0.897	$1.7x10^{-2}$
lnc-ERN1-1:1	750.668	164.504	113.187	$8.84x10^{-8}$
lnc-HES1-10:1	258.042	694.213	142.777	$5.96x10^{-4}$
lnc-HFE2-2:1	343.839	851.858	130.888	0
lnc-KIAA1257-3:1	132.568	21.211	0.678	$6.09x10^{-4}$
lnc-KSR1-1:1	108.716	165.887	0.610	$1.57x10^{-2}$
lnc-MOGAT2-5:1	930.449	140.722	0.597	$2.81x10^{-2}$
lnc-MT2A-1:2	1990.13	3034.1	0.608	$2.03x10^{-8}$
lnc-RAB44-3:1	517.827	879.917	0.765	$1.22x10^{-8}$
lnc-RBM3-1:1	726.858	139.598	0.942	$1.57x10^{-2}$
lnc-RP11-231C14.2.1-3:1	164.477	551.775	174.619	$1.89x10^{-2}$
lnc-RSPH9-4:1	658.082	105.15	0.676	$1.33x10^{-8}$
lnc-TRIB3-1:2	366.972	569.299	0.634	$7.30x10^{-3}$
B, Downregulated				
lnc-AP000769.1-2:10	895.237	589.174	-0.604	$6.98x10^{-5}$
lnc-BOLA3-2:2	136.839	0	$-1.80x10^{-8}$	$4.31x10^{-2}$
lnc-E2F2-1:1	200.807	118.647	-0.759	$1.50x10^{-6}$
lnc-FOXG1-7:1	39.749	0	$-1.80x10^{-8}$	$4.16x10^{-13}$
lnc-GNLY-4:2	90.639	306.009	-156.656	$3.67x10^{-3}$
lnc-KIAA0226-2:1	219.713	0.367	-258.257	$1.62x10^{-4}$
lnc-LTBP3-2:5	11.504	730.114	-0.656	$5.95x10^{-3}$
lnc-TOR1A-2:1	23.017	131.966	-0.803	$1.40x10^{-2}$

FC, fold change; lncRNA, long noncoding RNA.

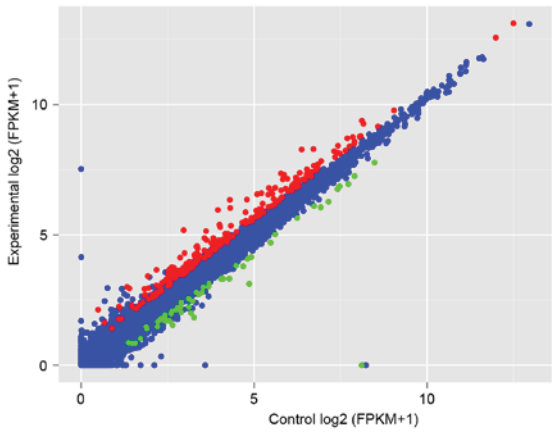

Figure 1. Scatter plot of FPKM in celecoxib-treated SK-MES-1 cells and untreated Control cells. Red dots represent upregulated genes in the celecoxib-treated group; green dots represent downregulated genes; and blue dots represent genes without differential expressions. The x-axis denotes cells in the Control group, and the y-axis denotes celecoxib-treated cells. FPKM, fragments per kilobase of exon per million fragments mapped.

the clean reads were aligned against the University of California Santa Cruz *Homo sapiens* reference genome (hg19 assembly, http://www.genome.ucsc.edu/index.html) using TopHat2 software (v2.0.9; http://ccb.jhu.edu/software/tophat) with the default parameters (22).

Identification of DEGs. Cufflinks (v2.2.1; http://cufflinks.cbcb.umd.edu/index.html) software was used to calculate the fragments per kilobase of exon per million fragments mapped (FPKM), from which the gene expression values were obtained (23). The linear models for microarray analysis (limma; v3.10.3) package in R (http://www.bioconductor.org/packages/release/bioc/html/limma.html) was used to select DEGs between the two groups (24), with the thresholds of q-value<0.05 and $|\log_2(FC)|>0.58$; where FC is fold change.

Selection of DE-LNRs. LNCipedia 3.0 (http://www.lncipedia.org), an online storage of lncRNA annotation (25), was used

Table III. Top 10 identified KEGG enrichment pathways of identified DEGs.

A, Upregulated DEGs

KEGG ID	Pathway name	Count[a]	Genes	P-value
970	Aminoacyl-tRNA biosynthesis	10	*AARS, CARS, EPRS, GARS, IARS*	4.49×10^{-7}
4141	Protein processing in endoplasmic reticulum	15	*ATF4, DDIT3, DNAJB2, ERN1, HERPUD1*	1.05×10^{-6}
260	Glycine, serine and threonine metabolism	6	*CBS, CTH, PHGDH, PSAT1, PSPH, SHMT2*	3.78×10^{-5}
3060	Protein export	4	*HSPA5, SEC11C, SEC63, SRPRB*	1.09×10^{-3}
520	Amino sugar and nucleotide sugar metabolism	5	*GFPT1, GFPT2, GMPPB, HKDC1, NAGK*	2.79×10^{-3}
250	Alanine, aspartate and glutamate metabolism	4	*ASNS, GFPT1, GFPT2, GPT2*	3.84×10^{-3}
4150	mTOR signaling pathway	5	*DDIT4, EIF4EBP1, RPS6KA2, ULK1, VEGFA*	3.97×10^{-3}
860	Porphyrin and chlorophyll metabolism	4	*EPRS, FTH1, HMOX1, UROS*	1.11×10^{-2}
5020	Prion diseases	3	*EGR1, HSPA5, IL1A*	3.39×10^{-2}
450	Selenocompound metabolism	2	*CTH, MARS*	4.62×10^{-2}

B, Downregulated DEGs

KEGG ID	Pathway name	Count[a]	Genes	P-value
4350	TGF-β signaling pathway	4	*ID1, ID3, TGFβ2, THBS1*	1.94×10^{-4}
5323	Rheumatoid arthritis	4	*CCL2, CSF1, CXCL6, TGFβ2*	2.65×10^{-4}
5144	Malaria	3	*CCL2, TGFβ2, THBS1*	7.36×10^{-4}
5200	Pathways in cancer	6	*AXIN2, E2F2, EGLN3, FN1, MITF, TGFβ2*	7.47×10^{-4}
4512	ECM-receptor interaction	3	*COL1A1, FN1, THBS1*	3.23×10^{-3}
5146	Amoebiasis	3	*COL1A1, FN1, TGFβ2*	6.01×10^{-3}
5219	Bladder cancer	2	*E2F2, THBS1*	9.63×10^{-3}
4380	Osteoclast differentiation	3	*CSF1, MITF, TGFβ2*	1.01×10^{-2}
4060	Cytokine-cytokine receptor interaction	4	*CCL2, CSF1, CXCL6, TGFβ2*	1.32×10^{-2}
4115	p53 signaling pathway	2	*RRM2, THBS1*	2.41×10^{-2}

[a]The number of genes that were enriched in a specific pathway category. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene.

to acquire information on lncRNAs. Subsequently, Cufflinks was used to screen the DE-LNRs. Similar to the selection of DEGs, the cut-off values were $q<0.05$ and $|\log_2(FC)|> 0.58$.

Enrichment analysis of the DEGs. To explore potential functions and pathways that the DEGs may participate in, function enrichment and pathway enrichment were implemented based on the Gene Ontology (GO; http://www.geneontology.org) (26) database and the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/pathway.html) database, respectively, and the Database for Annotation, Visualization and Integration Discovery (DAVID; v6.8; http://david.abcc.Ncifcrf.gov) (27). Selection criteria for a significant GO or KEGG pathway category were $P<0.05$ with ≥2 genes enriched in a category.

Protein-protein interaction (PPI) network construction. The Search Tool for the Retrieval of Interacting Genes (STRING; v10.0, http://string-db.org) database was searched to discover potential interactions of proteins encoded by the identified DEGs (28). With the selection criterion of a combined score >0.7, a PPI network was established, which was drawn using Cytoscape (v3.2.0; http://cytoscape.org) software (29).

Co-expression analysis of lncRNAs and mRNAs. For the identified DE-LNRs and DEGs, their correlation coefficient (CC) was calculated by Pearson correlation. The co-expressed DE-LNRs and DEGs were selected under the condition of $|CC|>0.98$. Subsequently, enrichment analysis of the co-expressed DEGs was performed to predict biological functions of the DE-LNRs.

Validation of identified DEGs and lncRNA. To further confirm the identification of DEGs and DE-LNRs, expression levels of fibronectin 1 (*FN1*), vascular endothelial growth factor A (*VEGFA*) and lncAP000769.1-2:10 were determined using reverse transcription-quantitative PCR (RT-qPCR) in SK-MES-1 cells treated with 10 μM celecoxib or DMSO for 48 h. Total RNA was isolated from cells at a confluency of 70-75% using TRIzol agent (Takara Biotechnology Co., Ltd., Dalian, China), and reverse transcribed to cDNA using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. qPCR was

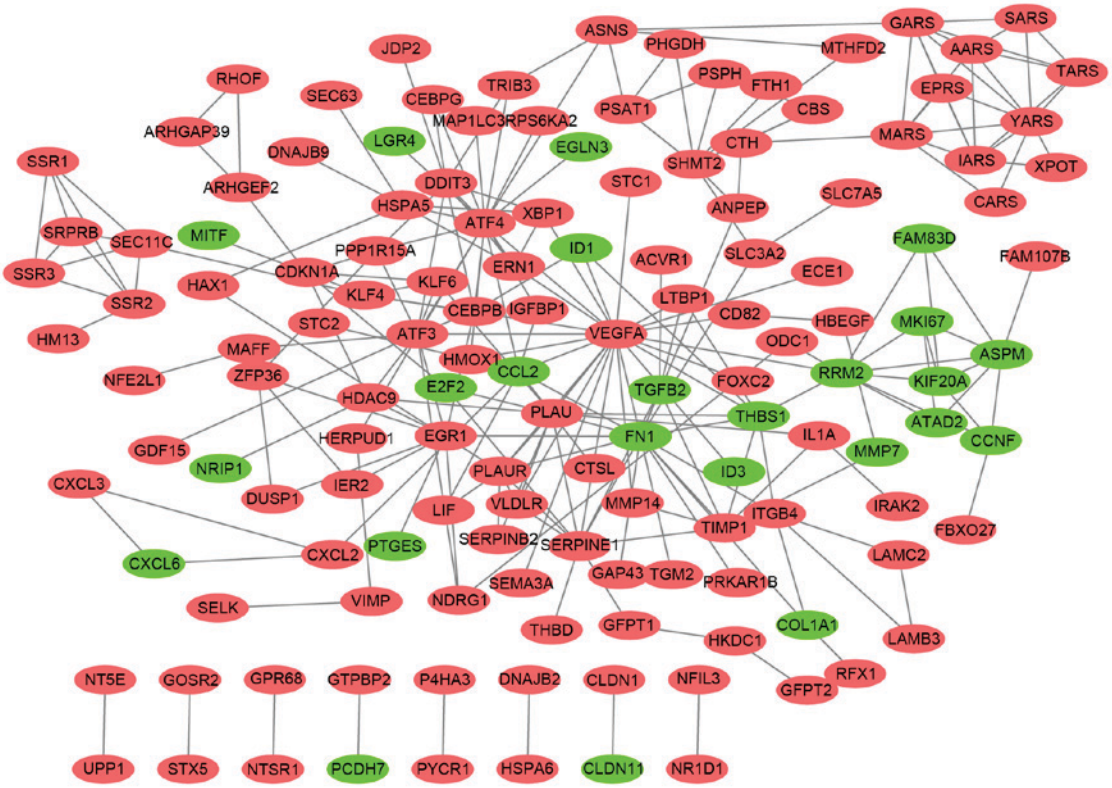

Figure 2. Protein-protein interaction network of the differentially expressed genes. Red circles represent upregulated genes, and green circles represent downregulated genes.

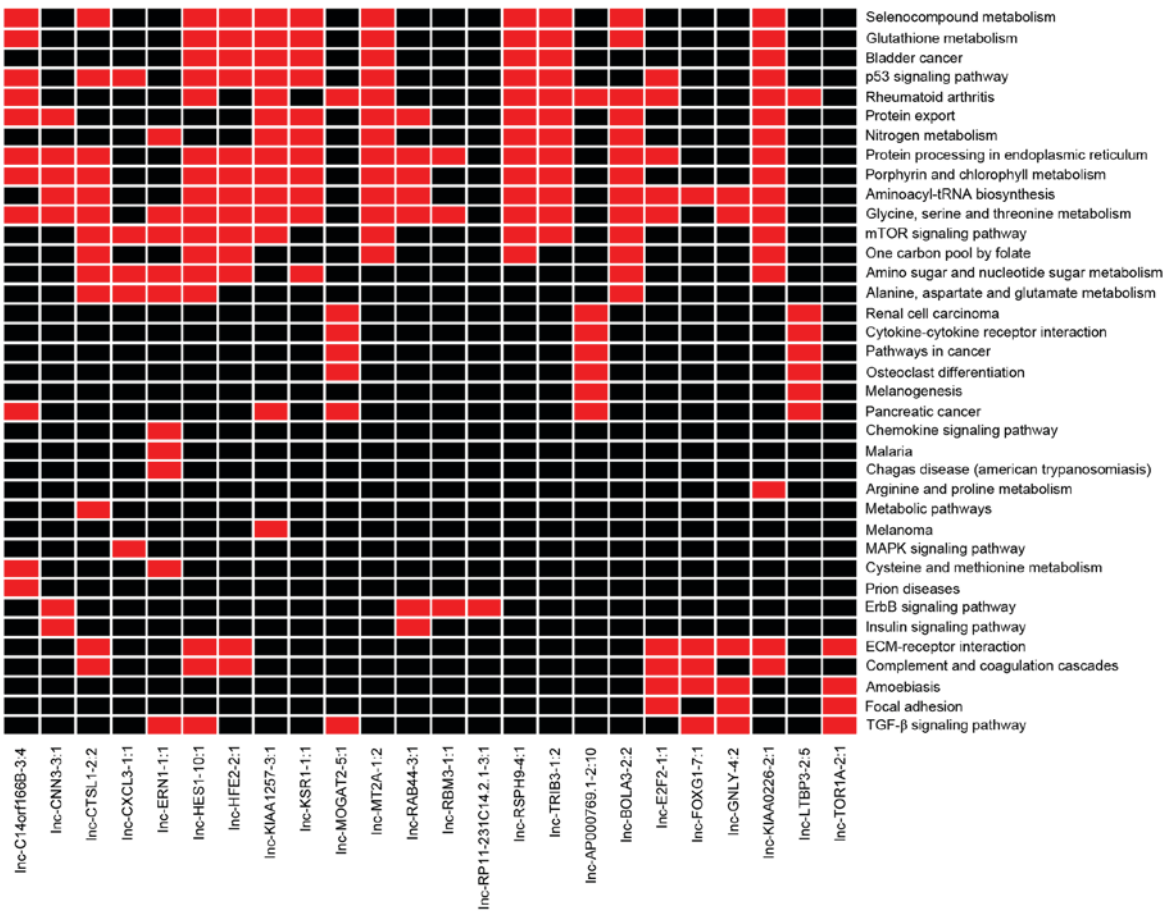

Figure 3. Enriched pathways of the co-expressed lncRNAs. Red indicates that the lncRNA was enriched in a specific pathway. Rows indicate pathways and columns indicate lncRNAs. lncRNA, long noncoding RNA.

performed using a SYBR Green kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a ViiA7 PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 1 cycle of 50˚C for 3 min and 95˚C for 3 min; followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. GAPDH was used as the internal control during expression analysis, and gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (30). Primer sequences are provided in Table I.

Statistical analysis. DEGs were screened using the limma package in R with the thresholds of $q<0.05$ and $|\log_2(FC)|>0.58$; DE-LNRs were screened using Cufflinks software with the same thresholds. Continuous variables are presented as the mean ± standard deviation, and difference between groups was calculated using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DEGs and DE-LNRs identification. According to the aforementioned criteria, a set of 261 upregulated and 56 downregulated DEGs were identified in celecoxib-treated group, compared with the untreated Control cells. Gene expressions in each group are presented in a scatter plot of FPKM (Fig. 1). Based on the predefined selection criterion, 17 lncRNAs were upregulated and 8 lncRNAs were downregulated in celecoxib-treated group compared with the untreated Control cells (Table II).

Pathway enrichment analysis of DEGs. KEGG pathway analysis indicated that the upregulated DEGs were significantly enriched in 12 pathway categories, including Aminoacyl-tRNA biosynthesis, protein processing in endoplasmic reticulum (ER), protein export, amino sugar and nucleotide sugar metabolism and mammalian target of rapamycin (mTOR) signaling pathway. The downregulated DEGs were enriched in 17 pathways, such as 'transforming growth factor (TGF)-β signaling pathway', 'extracellular matrix (ECM)-receptor interaction', 'cytokine-cytokine receptor interaction' and 'p53 signaling pathway' (Table III).

PPI network of DEGs. In the established PPI network (Fig. 2), several genes were highlighted that had high degrees; that is, a high number of connections between one gene and the others, such as VEGFA (degree=23), activating transcription factor (ATF)-4 (degree=19), FN1 (degree=16), urokinase-type plasminogen activator PLAU (degree=13), ATF3 (degree=11) and serpin E1 (SERPINE1; degree=10).

Co-expressed DE-LNRs and DEGs and their enriched functions. Using the criterion of $|CC|>0.98$, the co-expressed DE-LNRs and DEGs were screened out. As presented in Table IV, *SERPINE1* was co-expressed with lnc-CTSL1-2:2 (CC=0.998577) and lnc-CXCL3-1:1 (CC=0.986928); *VEGFA* was linked with lnc-HES1-10:1 (CC=0.98906) and lnc-AP000769.1-2:10 (CC=-0.99227); lnc-HFE2-2:1 was co-expressed with *ATF4* (CC=0.996159) and *FN1* (CC=-0.98714); *ATF3* was co-expressed with lnc-KIAA1257-3:1 (CC=0.990212), lnc-KSR1-1:1 (CC=0.99655) and

Table IV. The most highly correlated co-expressed DE-LNRs and DEGs.

DE-LNR	DEG	CC
lnc-C14orf166B-3:4	VEGFA	0.999351
	CLDN11	-0.98538
lnc-CNN3-3:1	ACOT8	0.994477
	ATAD2	-0.99951
lnc-CTSL1-2:2	SERPINE1	0.998577
	AMER1	-0.99246
lnc-CXCL3-1:1	SERPINE1	0.986928
	AXIN2	-0.9893
lnc-ENTPD6-2:1	ACVR1	0.995922
	ASPN	-0.98602
lnc-ERN1-1:1	ABTB2	0.996183
	AXIN2	-0.98832
lnc-HES1-10:1	VEGFA	0.98906
	AMER1	-0.99383
lnc-HFE2-2:1	ATF4	0.996159
	FN1	-0.98714
lnc-KIAA1257-3:1	ATF3	0.990212
	LRFN1	-0.98734
lnc-KSR1-1:1	ATF3	0.99655
	CCDC80	-0.9997
lnc-MOGAT2-5:1	ACVR1	0.986953
	ASPN	-0.9935
lnc-MT2A-1:2	AASR	0.991019
	ATAD2	-0.99032
lnc-RAB44-3:1	ACOT8	0.997679
	ATAD2	-0.99846
lnc-RBM3-1:1	ACOT8	0.990015
	ATAD2	-0.99475
lnc-RP11-231C14.2.1-3:1	ATF3	0.993415
	CCDC80	-0.98512
lnc-RSPH9-4:1	ACOT8	0.990293
	CCNF	-0.988
lnc-TRIB3-1:2	ACOT8	
	ATAD2	-0.98366
lnc-AP000769.1-2:10	ASPN	0.987202
	VEGFA	-0.99227
lnc-BOLA3-2:2	CCNF	0.985247
	PLAU	-0.99288
lnc-E2F2-1:1	AMER1	0.987329
	AARS	-0.98601
lnc-FOXG1-7:1	AMER1	0.988729
	ANTXR2	-0.9897
lnc-GNLY-4:2	COL1A1	0.989359
	CDH13	-0.99856
lnc-KIAA0226-2:1	FN1	0.981655
	AARS	-0.99647
lnc-LTBP3-2:5	ASPN	0.993248
	BTG1	-0.993
lnc-TOR1A-2:1	ATOH8	0.98542
	CDH13	-0.99533

CC, correlation coefficient; DEG, differentially expressed gene; DE-LNR, differentially expressed long noncoding RNA.

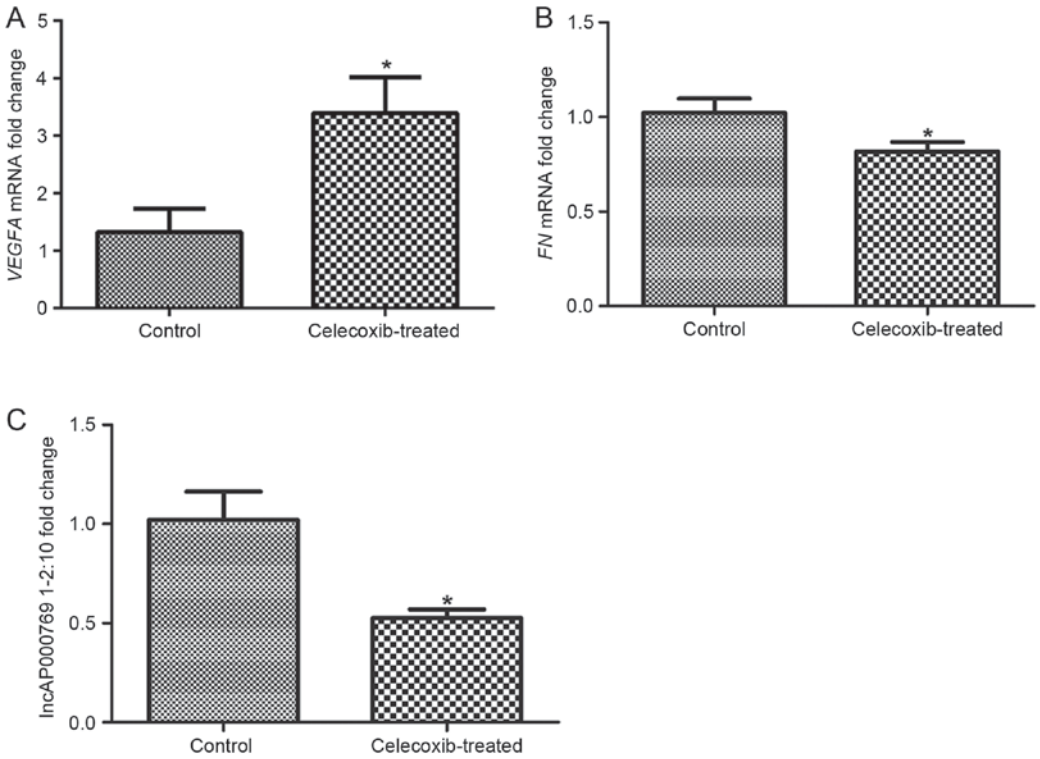

Figure 4. Expression levels of (A) VEGFA, (B) FN and (C) lncAP000769 1-2:10 were determined in SK-MES-1 cells using reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. Control. FN1, fibronectin 1; lnc, long noncoding; VEGFA, vascular endothelial growth factor A.

lnc-RP11-231C14.2.1-3:1 (CC= 0.993415); lnc-BOLA3-2:2 was linked with *PLAU* (CC= -0.99288); and lnc-KIAA0226-2:1 was co-expressed with *FN1* (CC=0.981655).

According to the enrichment analysis results of the co-expressed DEGs, the co-expressed DE-LNRs were mainly enriched in the 'p53 signaling pathway' (for example, lnc-HES1-10:1, lnc-HFE2-2:1, lnc-KIAA1257-3:1 and lnc-KSR1-1:1; Fig. 3) and the 'mTOR signaling pathway' (for example, lnc-CTSL1-2:2, lnc-CXCL3-1:1, lnc-ERN1-1:1, lnc-HES1-10:1 and lnc-HFE2-2:1; Fig. 3).

Validations of DEGs and lncRNA. To verify the identification of possible DEGs and DE-LNRs, the expression levels of *VEGFA*, *FN1* and lnc-AP000769.1-2:10 were analyzed by RT-qPCR. The results revealed that the expression level of *VEGFA* was significantly increased in celecoxib-treated SK-MES-1 cells compared with untreated Control cells (Fig. 4A). However, the expression levels of *FN1* and lnc-AP000769.1-2:10 were significantly decreased in the celecoxib-treated group compared with the Control group (Fig. 4B and C, respectively). These results were consistent with the aforementioned bioinformatics analytical results.

Discussion

The present study identified a number of genes and lncRNAs that exhibited differential expression levels in celecoxib-treated human LSQCC SK-MES-1 cells, compared with untreated cells, including the genes *VEGFA*, *ATF4* and *FN1*, and the lncRNAs lnc-AP000769.1-2:10 and lnc-HFE2-2:1. Notably, many of the identified DEGs and DE-LNRs were significantly enriched in pathways like 'protein processing in endoplasmic reticulum', 'mTOR signaling pathway' and 'ECM-receptor interaction'.

VEGFA is an essential growth factor for stimulating angiogenesis, which often accompanies tumoral growth (31). A previous study reported that by upregulating the expression of FLJ10540, VEGFA activates the phosphatidylinositol 3-kinase/AKT signaling pathway, subsequently promoting cell invasion and migration in lung cancer (32). mTOR is located downstream of AKT signaling and functions in the control of angiogenesis and cell proliferation during tumor progression (33). *VEGFA* was also reported to activate the downstream mTOR signaling pathway to promote cancer growth (34). Notably, several therapeutic drugs for lung cancer have been reported to function through this pathway. 2-(^{18}F)-fluoro-2-deoxy-d-glucose (^{18}F-FDG) is a radiolabelled sugar molecule that is commonly used to monitor the therapeutic effects of chemotherapy for many malignant tumors, and the accumulation of ^{18}F-FDG is regulated by the activation of mTOR signaling in NSCLC (35). *Cis*-diamminedichloroplatinum (II) (CDDP; also known as cisplatin) is an effective drug for the treatment of many cancers (36). However, resistance to this drug limited its potential use in lung cancer treatment. It was previously reported that overexpression of AKT activates the mTOR signaling pathway, which induces CDDP resistance in lung cancer cells (37). Therefore, inhibitors of the AKT/mTOR signaling pathway may provide a promising therapeutic target. In lung cancer, targeting of mTOR signaling was suggested as an effective method in developing therapeutic drugs (38). Curcumin, a natural extract of turmeric, is considered as an antitumoral agent, which was reported to enhance the anti-cancer ability of chemotherapy in

LSQCC by regulating multiple pathways such as VEGF signaling (39). Notably, *VEGFA* was also indicated to be enriched in the mTOR signaling pathway (36). In the present study, *VEGFA* was identified as an upregulated DEG in celecoxib-treated LSQCC cells, and was demonstrated to be significantly enriched in the mTOR signaling pathway. These data suggested that *VEGFA* may be a sensitive gene in response to celecoxib, and the increased expression may inhibit the activation of mTOR signaling, which may improve the anti-tumor effects of celecoxib on LSQCC cells.

In addition, lncRNAs may also serve crucial roles in the amplification of anti-tumor effects. Nuclear paraspeckle assembly transcript 1 (Neat1; ENST00000501122.2) is a factor required for the assembly of paraspeckle compartments in the cell (40). Neat1-containing paraspeckles were reported to be responsible for the regulation of chemosensitivity and may be induced by p53 (41). The biofunction of Neat1 is similar to lnc-AP000769.1-2:3. However, no information about lnc-AP000769.1-2:10 has yet been reported. In the present study, lnc-AP000769.1-2:10 was closely correlated with *VEGFA*, which was enriched in the mTOR signaling pathway following celecoxib treatment. In addition, the expression of lnc-AP000769.1-2:10 was significantly decreased in celecoxib treated SK-MES-1 cells. Therefore, the present study hypothesized that this lncRNA may regulate *VEGFA* gene expression in the mTOR signaling pathway, which may facilitate to the enhancement of anti-tumor effect of celecoxib for LSQCC treatment.

ATF4 was reported to be associated with cisplatin sensitivity in lung cancer cell lines (42). Celecoxib has been demonstrated to induce the expression of DR5 (43). In addition, C/EBP homologous protein (CHOP) was revealed to serve a crucial role in celecoxib-induced DR5 expression and may also be upregulated by celecoxib (44). Inhibition of *ATF4* expression by small interfering RNAs was able to abolish CHOP induction, which indicated the involvement of ATF4 in celecoxib-induced apoptosis (45). The ER is an essential site for protein processing. In many types cancer, the ER serves an important role in the structural maintenance of proteins in pivotal signaling pathways (46). Control of these proteins may offer promising target therapies. In the present study, *ATF4* was indicated as enriched in the protein processing in ER pathway, which suggested that it may influence the sensitivity of celecoxib in LSQCC through the regulation of protein processing.

The FN1 protein may be involved in several cellular activities, such as cell adhesion and migration (47,48). In lung cancer, knockdown of *FN1* was previously reported to increase the chemosensitivity of cisplatin and promote apoptosis in tumor cells (49). Notably, in the present study, *FN1* expression was downregulated in the celecoxib-treated LSQCC cells, which suggested that the reduced expression of this gene may also enhance the sensitivity of tumor cells in response to celecoxib. In addition, *FN1* has been implicated in pathways such as the ECM-receptor interaction pathway in many types cancer (50,51). Consistent with these results, *FN1* was significantly enriched in the ECM-receptor interaction pathway in the present study, which indicated that *FN1* may exert its function through its involvement in this pathway. Results from the present study also predicted that both *FN1* and *ATF4* were targets of lnc-HFE2-2:1, which suggested that the two genes may be regulated by this lncRNA in LSQCC cells following celecoxib treatment. However, there is still little information available about this lncRNA. Based on the present results, lnc-HFE2-2:1 may be a novel target to predict sensitivity of celecoxib for the treatment of LSQCC, through the regulation of *FN1* and *ATF4* expressions.

There were some limitations to the present study. First, although some genes and lncRNA had been validated in this study, the regulatory relationships between lncRNAs and DEGs have yet to be confirmed with *in vitro* experiments. Second, as LSQCC may develop from a number of lung cell dysfunctions, SK-MES-1 cells may not reflect the wider results of celecoxib. Therefore, different types of LSQCC cell lines should be used in future studies, and the intersection of DEGs and lncRNAs may be focused. Finally, an appropriate animal model should be used to confirm the identified DEGs and DE-LNRs, including investigations on the predicted signaling pathways.

In conclusion, genes (such as *VEGFA*, *ATF4* and *FN1*), and lncRNAs (such as lnc-AP000769.1-2:10 and lnc-HFE2-2:1) may be crucial molecules to enhance the anti-cancer effects of celecoxib treatment on LSQCC, and may be used as predictors for chemosensitivity of celecoxib. However, additional validation experiments are required in further studies.

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