

## RNA-Seq analysis for the potential targets and molecular mechanisms of 17 $\beta$ -estradiol in squamous cell lung carcinoma

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The efficacy of 17  $\beta$ -estradiol (E2) was valid in some cancers, while its effects on squamous cell lung carcinoma (SCLC) were still unclear. The aim of our study was to investigate the potential targets and molecular mechanisms of E2 in SCLC cells.

Two RNA libraries from human lung carcinoma cells (SK-MES-1) with and without E2 treatment were constructed and sequenced. The differentially expressed genes (DEGs) between cells with or without E2 treatment were identified by cuffdiff software. Hierarchical Clustering Analysis (HCA) was performed for displaying gene expression changes and classification. Furthermore, enrichment analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology Biological Process (GO BP) terms were performed through DAVID. The protein-protein interaction (PPI) network was constructed through STRING. Additionally, differentially expressed lncRNAs were also selected by cuffdiff software.

Total 129 DEGs including 58 up- and 71 down-regulated genes were obtained. Cancer-related pathways including small cell lung cancer, hypertrophic cardiomyopathy (HCM) and pathways in cancer and biological processes including regulation of phosphorus metabolic process, protein localization and nucleus organization were enriched. The PPI network with 113 nodes and 312 edges was constructed. CASP3, ITGA2, COL4A6, PML and CDC25B were identified as hub nodes which had more interactions with others in the PPI network. Furthermore, eight up-regulated and ten down-regulated lncRNAs were selected.

CASP3, ITGA2 and Lnc-DLK1-4:31 (one of down-regulated lncRNAs) might play pivotal roles in E2 treated SCNC cells by influencing cell apoptosis, angiogenesis and cell invasion respectively.

*Key words: squamous cell lung carcinoma, RNA-Seq, differentially expressed genes, lnc-RNA*

Squamous cell lung carcinoma (SCLC) is a common type of non-small-cell lung cancer (NSCLC) and the second leading cause of death related to lung cancer which causes approximately 400,000 deaths per year worldwide [1]. SCLC develops mainly in the upper airways and cigarette smokers are more susceptible to SCLC, therefore it is more common in men than in women [2]. Although various therapies such as radiation, chemotherapy, surgery and palliative care have been used for SCLC treatment, the overall therapeutic results were not optimistic [3, 4]. A more thorough understanding of the mechanism of SCLC may offer a new hope on treatment innovations and prevention strategies.

Estrogens are a group of human sex hormones which can be secreted primarily by the ovaries and placenta in women and the testes in men. The cell response to estrogens is in a concentration-dependent manner. Previous studies found that low concentration of 17  $\beta$ -estradiol (E2) promoted the

development of breast cancer and prostatic cancer, while 10 nM or higher concentration of E2 could cause cancer cell apoptosis [5-7]. The three major naturally occurring estrogens are E2, estrone and estriol, among them, E2 is the predominant and most active [8]. Meanwhile, Zhao *et al.* certified that E2 treatment could inhibit breast cell invasion and migration in an estrogen receptor  $\alpha$  (ER $\alpha$ )-independent way by decreasing MALAT-1 RNA level [9]. Coincidentally, estrogen receptors are also reported to play a key role in the progression of various types of NSCLC including adenocarcinoma (ADCA), SCLC, and large-cell carcinoma [10]. Moreover, the clinical and biological significances of estrogen metabolisms has been studied in NSCLCs and they might play critical roles in regulating lung cancer biology [11]. However, effects of E2 on the progression of NSCLC, especially for SCLC, have not been adequately studied and whether E2 could be a candidate treatment for SCLC was rarely studied.

The development of RNA-Seq has become increasingly important in cancer research and RNA-Seq has been employed in the study of certain type cancers such as prostate cancer, breast cancers and liver cancer. Some novel mechanisms of these cancers genesis and progression have been found with this method [12-15]. In the present study, we aimed to explore the targets and potential molecular mechanisms of E2 in SCLC cells using RNA-Seq and bioinformatics analysis, in order to provide theoretical basis for the treatment of SCLC by E2. Our study might also put forward some new insights for the molecular mechanism of E2 treatment on SCLC and supply some potential targets for lung cancer treatment.

## Materials and methods

**Cell line and cell culture.** The human lung carcinoma cell line SK-MES-1 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cell line was cultivated in Dulbecco's modified Eagles medium (DMEM, GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (FBS, GIBCO BRL, Gaithersburg, MD, USA), 100 IU/ml penicillin and 100 µg/mL streptomycin in a humidified 37°C incubator containing 5% CO<sub>2</sub> [16].

**Grouping and cell treatment.** Two groups were set up for the study, including E2 group and control group. The cells in E2 group were exposure to E2 (Sigma-Aldrich, St. Louis, MO, USA), which was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), at the concentration of 15 umol/L for 48 hours. Meanwhile, equal volume of DMSO was used in the control group.

**RNA isolation.** Total RNA was extracted from the cells untreated and treated with E2 using Trizol reagent (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions [17]. The integrity of RNA was detected by 1% agarose gel electrophoresis and the RNA concentration was determined by using an ultraviolet spectrophotometry (Cary 50, Varian, USA).

**RNA library construction and sequencing.** RNA-Seq libraries were prepared from a total of 5µg RNA using the NEB-Next® Ultra RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, U.S.A.) [18]. Briefly, RNA was fragmented into approximately 200 nt fragments. Then double-stranded cDNA was synthesized and the cDNA was end-repaired before ligation of Illumina-specific adaptors. Finally, PCR amplification was performed to obtain cDNA library. After assessing the library quality on a Bioanalyzer® (Agilent Technologies, Santa Clara, CA, U.S.A.), RNA sequencing was performed using an Illumina HiSeq 2500 (100 base paired ends) [19]. Data analysis was performed using the HiSeq Analysis Software (Illumina) to generate sequence data in FASTQ format.

**Mapping RNA-Seq reads to reference genome.** High quality data were obtained from raw data by removing reads with N content more than 3% and low-quality reads (Q value of ≤ 18) more than 50%. The TopHat2 software [20] was utilized to map the high quality clean reads to the reference human genome

(hg19) with default parameters. Genomics and refseq annotation files were obtained from UCSC (<http://genome.ucsc.edu/>).

**Screening differentially expressed genes (DEGs).** Cuffdiff [21] was employed for the calculation of gene expression levels of annotated genes and the screening of the DEGs between E2 and control group with cut-off criteria of  $p$ -value < 0.001 and  $|\log_2$  Fold Change (FC)| > 1. Meanwhile, heatmap was utilized to visually display the characteristic gene expression changes among different samples by Hierarchical Cluster Analysis (HCA) using gplots package in R.

**Pathway and functional enrichment analyses.** Enrichment analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology Biological Process (GO BP) terms were performed with R package Goseq [22] to identify significantly enriched pathways and the biological processes of the DEGs, using the online tool of the database for annotation, visualization and integrated discovery (DAVID, <http://david.abcc.ncifcrf.gov/>) [23] with the  $p$ -value < 0.05.

**PPI network construction.** DEGs were submitted to Search Tool for the Retrieval of Interacting Genes (STRING) [24]. All interactions in STRING were provided with a probabilistic confidence score (combined score), and only interactions with combined score > 0.9 were retained in our analysis. Then the PPI network was constructed and visualized with the Cytoscape software [25].

**Differential long non-coding RNAs (lncRNAs) analysis.** The expression values of lncRNAs were determined based on the search results on LNCipedia 3.0 database [26]. Afterwards, the differentially expressed lncRNAs were screened using Cuffdiff software [21] with the threshold of  $p$ -value < 0.01 and  $|\log$  FC| > 1.

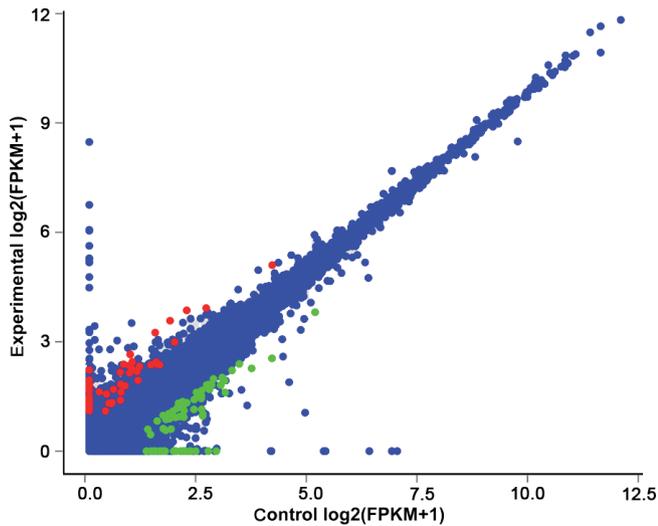
## Results

**DEG between E2 and control group.** A total of 129 DEGs were identified, including 58 up-regulated genes and 71 down-regulated genes. The scatter plot of gene expression levels is shown in Figure 1. Besides, the top 5 up-regulated genes and down-regulated genes at  $p$ -Value are listed in Table 1,

**Table 1. The top 5 up-regulated genes and down-regulated genes at  $p$ -Value**

	Gene	Log <sub>2</sub> FC	$p$ -Value
Up-regulated genes	SLC35A3	1.79769e+308	4.08E-13
	DLD	1.79769e+308	5.52E-08
	SCMH1	1.79769e+308	1.72E-07
	PLEKHG5	1.79769e+308	5.26E-07
	TLE3	1.79769e+308	6.56E-06
Down-regulated genes	SPAG9	-1.61518	3.83E-13
	SLC9A8	-1.79769e+308	3.01E-12
	PRKAA1	-1.79769e+308	1.29E-09
	JKAMP	-1.79769e+308	4.90E-08
	PAM	-1.79769e+308	8.78E-07

E2: 17 β-estradiol; FC: Fold Change



**Figure 1.** The scattergram for gene expression levels. Red, green and blue nodes represented for the up-regulated DEGs, down-regulated DEGs and non-DEGs respectively. Abscissa represented for the control group and ordinate represented for the experimental group which was treated by 17  $\beta$ -estradiol. DEGs: differentially expressed genes.

respectively. Meanwhile, HCA result also showed that the expression pattern of the characteristic gene set could well distinguish control and E2 samples (Figure 2). From the heat map, the 129 genes could be divided into two clusters. Overall, compared with control samples, the expression of genes in the cluster-1 was higher in E2 samples, whereas the expression of genes in the cluster-2 was lower in E2 samples.

**Table 2.** The enriched KEGG pathway of the differentially expressed genes

KEGG pathway	Count	<i>p</i> -Value	Genes
Small cell lung cancer	4	0.008596	LAMA4, ITGA2, COL4A6, TRAF3
Hypertrophic cardiomyopathy (HCM)	4	0.00888	DMD, CACNG6, ITGA2, PRKAA1
Pathways in cancer	6	0.023173	LAMA4, CASP3, PML, ITGA2, COL4A6, TRAF3

KEGG: Kyoto Encyclopedia of Genes and Genomes; Count stands for the number of differentially expressed genes.

**Table 3.** Gene Ontology Biological Process enrichment analysis of the differentially expressed genes (Top 10 by *p*-value)

GO ID	Term	Count	<i>p</i> -Value
GO:0051174	regulation of phosphorus metabolic process	10	0.0042178
GO:0019220	regulation of phosphate metabolic process	10	0.0042178
GO:0008104	protein localization	14	0.00445
GO:0006997	nucleus organization	4	0.0046707
GO:0006915	apoptosis	11	0.0056114
GO:0012501	programmed cell death	11	0.0062136
GO:0045859	regulation of protein kinase activity	8	0.0071658
GO:0043549	regulation of kinase activity	8	0.0085718
GO:0046907	intracellular transport	11	0.0101228
GO:0051338	regulation of transferase activity	8	0.0106039

Count stands for the number of differentially expressed genes.

**KEGG pathway and GO BP enrichment analyses.** All the enriched KEGG terms and top 10 GO BP terms are listed in Table 2 and 3. Three pathways including small cell lung cancer, hypertrophic cardiomyopathy (HCM) and pathways in cancer were enriched according to the KEGG enrichment analysis. *ITGA2* was identified in the pathway of SCLC and *CASP3* and *ITGA2* were both involved in pathways in cancer (Table 2). For the GO BP terms, processes including regulation of phosphorus metabolic process, protein localization, nucleus organization and apoptosis were significantly enriched and *CASP3* and *ITGA2* were also identified in the regulation of phosphorus metabolic process (Table 3).

**PPI network of DEGs.** The PPI network including 113 nodes and 312 interactions was constructed (Figure 2). The connectivity degree of each node in this PPI network was calculated and the top 5 nodes with degree  $\geq 10$  were *CASP3* (caspase-3), *ITGA2* (integrin alpha-2, also named collagen receptor or CD49 antigen-like family member B), *COL4A6* (collagen alpha-6(IV) chain), *PML* (promyelocytic leukemia protein) and *CDC25B* (M-phase inducer phosphatase 2).

**Differentially expressed lncRNAs.** Totally, 18 differentially expressed lncRNAs were identified between E2 and control group, including eight up-regulated lncRNAs and ten down-regulated lncRNAs. The detailed information of these differentially expressed lncRNAs is listed in Table 4.

## Discussion

Currently, SCLC is still a malignant lung tumor and seriously threatens human life. Therefore, it is urgent to explore the mechanism and develop effective prevention strategies for

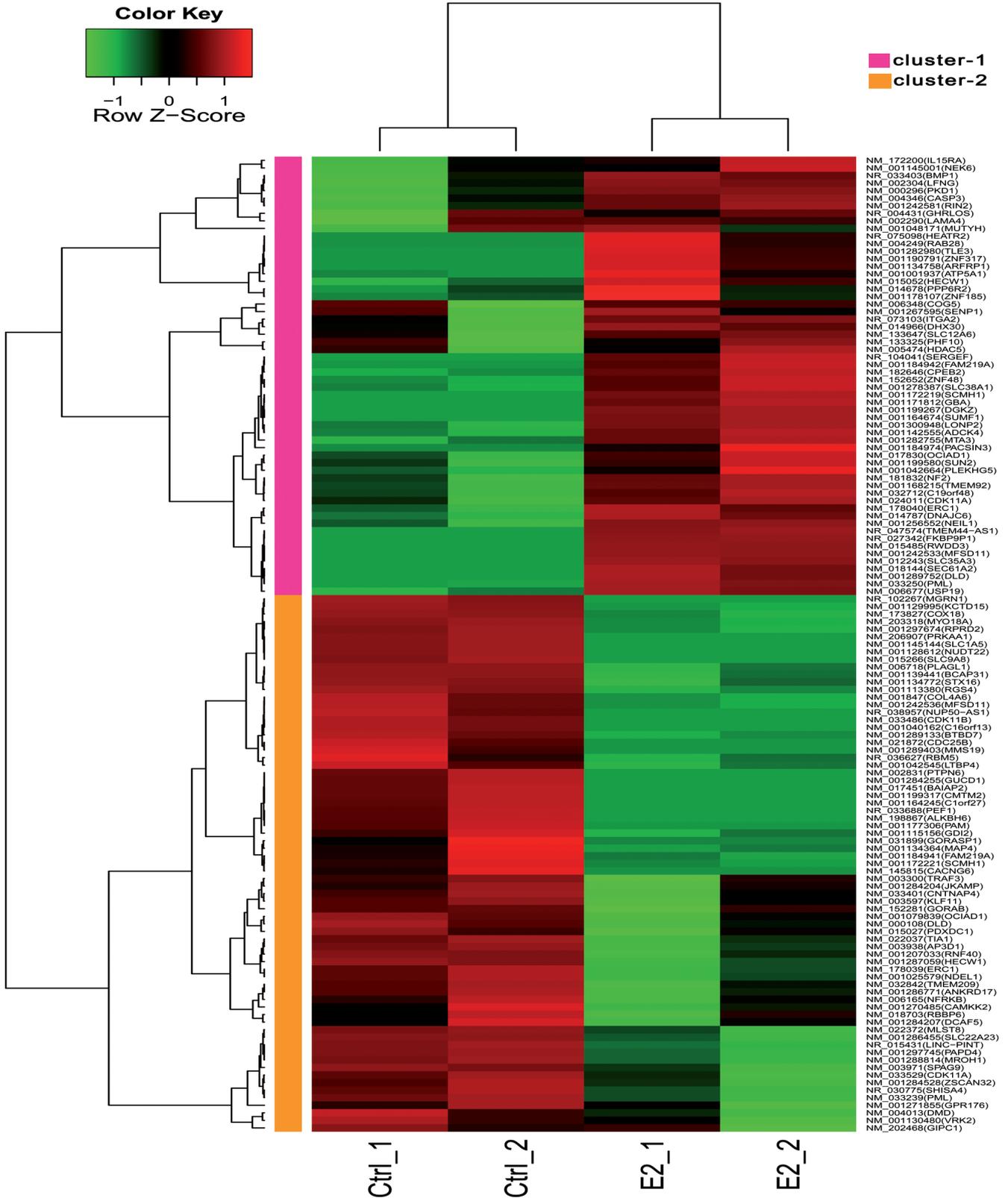
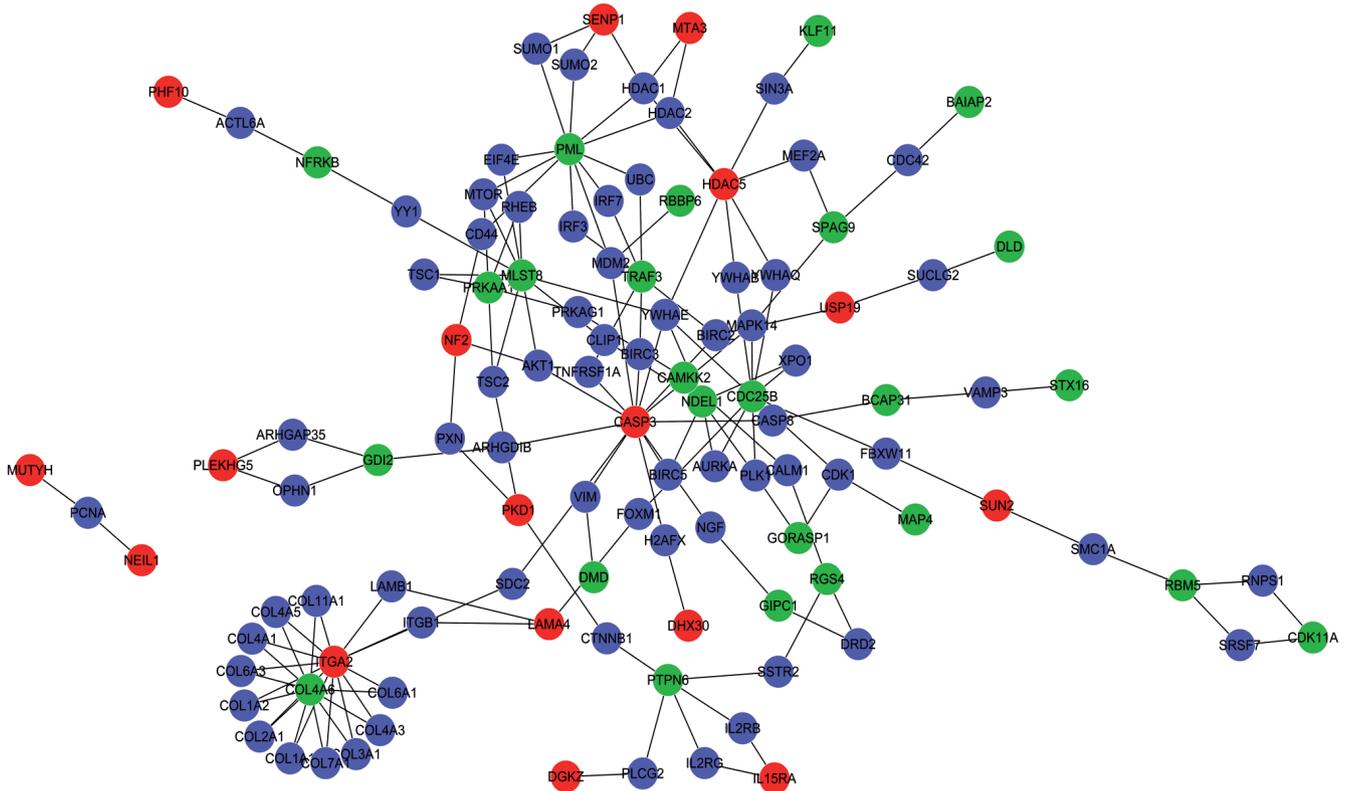


Figure 2. Hierarchical Clustering Analysis (HCA) for the differentially expressed genes (DEGs). Two clusters were marked with different colors in the left of the figure. Pink represents cluster-1 and genes in which were up-regulated in (E2) group; orange represents cluster-2 and genes in which were down-regulated in E2 group. Ctrl: control group; E2: 17  $\beta$ -estradiol group.



**Figure 3.** The protein-protein interaction network. Red, green and blue nodes represented for the up-regulated DEGs, down-regulated DEGs and non-differentially expressed genes respectively. DEGs: differentially expressed genes.

**Table 4.** Differentially expressed lncRNAs

	lncRNAs	Log <sub>2</sub> FC	p-Value
Up-regulated lncRNAs	lnc-CNN3-3:1	1.20405	0
	lnc-IL15RA-2:3	1.79769e+308	1.48E-08
	lnc-KIAA1257-3:1	3.49229	1.86E-07
	lnc-FAM138A-8:6	1.79769e+308	2.02E-05
	lnc-CD38-2:8	1.79769e+308	0.000194
	lnc-R3HDM1-1:1	1.79769e+308	0.000724
	lnc-LTC4S-2:1	1.79769e+308	0.005308
Down-regulated lncRNAs	lnc-AC091132.1-3:2	1.34725	0.002519
	lnc-AC022098.1-1:2	-1.79769e+308	2.45E-07
	lnc-IL15RA-2:2	-1.79769e+308	1.08E-06
	lnc-AC103810.1-3:1	-4.55604	0.000432
	lnc-CHD1-1:2	-1.79769e+308	0.000479
	lnc-DLK1-4:31	-1.79769e+308	0.000791
	lnc-ENOSF1-4:1	-1.03183	0.002357
	lnc-RNF39-4:12	-1.79769e+308	0.004232
	lnc-CALCOCO2-8:26	-1.79769e+308	0.004349
	lnc-SEN5-2:6	-1.79769e+308	0.007809
lnc-RP11-382B18.2.1-2:3	-1.63184	0.008066	

E2: 17  $\beta$ -estradiol; FC: Fold Change.

SCLC. E2 has been confirmed to play a critical role in many cancers including SCLC. In the present study, we studied the comprehensive regulatory mechanisms of E2 in human lung carcinoma cell line SK-MES-1 with RNA-Seq. As a result, a total of 129 DEGs between E2 and control group were screened out. In the constructed PPT network, the top 5 nodes, namely CASP3, ITGA2, COL4A6, PML and CDC25B, were selected as hub nodes with high degrees. Besides, a total of 18 differentially expressed lncRNAs were identified.

CASP3 (encoding caspase-3), as an up-regulated gene in E2 group, was enriched in pathways in cancer and some biological processes mainly including regulation of phosphorus metabolic process, regulation of phosphate metabolic process, nucleus organization and apoptosis. In addition, the PPI network in our study showed that CASP3 directly interacted with BIRC2, BIRC3 and BIRC5. CASP3 (also known as CPP32, YAMA, or apopain) is the family member of caspases which is probably the one that so far best correlates with apoptosis [27, 28]. Previous studies have shown that activation of CASP3 can be regulated by the Inhibitors of Apoptosis Proteins (IAPs) which share a common domain known as BIR (baculovirus IAP repeat), that binds to and inhibits CASP3 [29, 30]. BIRC2 (previously known as c-IAP2), BIRC3 (previously known as c-IAP1) and BIRC5 (previously known as Survivin) are family members of IAP [31]. Studies have indicated that BIRC5

contributes to tumorigenesis and progression of NSCLCs due to its apoptosis suppression functions [32, 33]. Moreover, it has been reported that E2 can cause cancer cell apoptosis [34]. Therefore, we speculated that cell apoptosis was enhanced in SCNC after E2 treatment by up-regulating expression of *CASP3* and affecting the relationship between *CASP3* and IAP members (*BIRC2*, *BIRC3* and *BIRC5*).

*ITGA2* was also a hub node and interacted with 14 proteins in the PPI network. Previous study has indicated that *ITGA2* encoding the  $\alpha$ -subunit of integrin  $\alpha_2\beta_1$ , which plays an important role in cancer development [35]. The expression of integrin  $\alpha_2\beta_1$  is associated with motility, invasiveness and cellular differentiation of a variety of tumors [36]. Additionally, the overexpression of collagen receptor integrin such as  $\alpha_2\beta_1$  in tumor cells has been reported to play a role in angiogenesis which was increased in NSCLC [37, 38]. However, controversial effects of *ITGA2* on cancer metastasis are proposed by several studies recently. Ding *et al.* has been reported that knocking down of *ITGA2* could stimulate cancer cell migration but did not affect the invasion process, and down-regulation of *ITGA2* induced primary cancer cell migration by regulating depolymerization of cytoskeleton and detaching the cell-cell adhesion [39]. There were also some literatures reported that *ITGA2* was up-regulated in human lung squamous cell carcinoma after anti-cancer drugs treatment, such as curcumin [40]. So up-regulation of *ITGA2* might be involved in the migration process in SCLC after treated by E2. The relationships between *ITGA2* and some members of collagen family (*COL6A3*, *COL6A1*, *COL3A1*, *COL2A1*, *COL1A2*, *COL1A1*, *COL4A3*, *COL4A5* and *COL7A1*) were also seen in the PPI network. Collagen structure plays a critical role in controlling tumor micro-environment which is important in the tumor angiogenesis progression according to previous researches [41]. Collagens including collagen Types I, III are found in vascular tissues and induce thrombosis in arteries and veins by influencing the angiogenesis progression [42]. Moreover, type I collagen can accelerate the angiogenesis process of hydrogel bioengineered tumors by tumor-endothelial cell co-culture in vitro [43]. Therefore, it was reasonable to suspect that the relationship between *ITGA2* and collagen family might play a pivotal role in the E2 treatment for SCNC and the angiogenesis process might be involved.

In addition, *Lnc-DLK1-4:31* was one of the significantly down-regulated lncRNAs in E2 group. Delta-Like 1 Homolog (*DLK1*) belongs to the EGF-like family and plays important roles in regulating cell differentiation [44]. Studies on *DLK1* have revealed its abnormal expression in a variety of tumors, including glioma, hepatocellular carcinoma and lung cancer [45, 46]. Increasing evidences revealed that the overexpression of *DLK1* could accelerate the proliferation of lung cancer cells and *DLK1* could promote lung cancer cell invasion depending on Notch signaling [47]. Thus, E2 might inhibit SCLC cell invasion by down-regulating *Lnc-DLK1-4:31* to suppress the expression of *DLK1*.

## Conclusion

In conclusion, *CASP3*, *ITGA2*, and *Lnc-DLK1-4:31* might play pivotal roles in E2 treated SCNC cells by influencing cell apoptosis, angiogenesis and cell invasion respectively. This study confirmed that E2 could be considered as potential treatment for SCLC and the analysis results might provide some molecular mechanisms for E2 as a treatment. However, further experiments should be conducted to confirm the expression changes of the key DEGs. For further investigating the effects of these hub nodes and lncRNAs on the SCLC treated by E2, more experiments *in vivo* and *in vitro* are required in the future.

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