Identification of key genes and pathway related to chemoresistance of small cell lung cancer through an integrative bioinformatics analysis

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Background: Small cell lung cancer (SCLC), the most malignant of all the lung cancer subtypes, is characterized by drug resistance. This study sought to explore the key genes and pathways associated with the chemoresistance of SCLC.

Methods: The drug sensitivity of chemosensitive and chemoresistance SCLC cell lines was measured by Cell Counting Kit-8 assays. The total RNA from chemosensitive cell line H69 and chemoresistance cell line H69AR cells was extracted and subjected to messenger RNA (mRNA) and long non-coding RNA (lncRNA) microarray analyses. The differentially expressed genes (DEGs) and the differentially expressed lncRNAs (DELs) were screened out with a threshold of a |log fold change| ≥1 and an adjusted P value <0.05. A protein-protein interaction network was constructed, and hub genes were screened out. A lncRNA-mRNA co-expression network was also constructed. Gene Ontology and Kyoto Encyclopedia of Genes, Genomes enrichment analyses and Cis-regulatory element analyses were conducted on the DEGs and the top 10 upregulated DEL-co-expressed DEGs. The expression of the key genes was further analyzed in the GSE149507 data set and validated in H69/H69AR and H446/H446DDP cells by quantitative polymerase chain reaction assays.

Results: The microarray results showed that a total of 609 mRNAs and 394 lncRNAs were differentially expressed in the chemoresistant SCLC cells. The mammalian target of rapamycin (mTOR) signaling pathway was enriched among the DEGs, the top 10 upregulated DEL-co-expressed DEGs, and the *NCRNA00173*-co-expressed DEGs, which included *IGF1*, *INS*, *WNT6*, *WNT11*, *WNT2B*, and *SESN2*. *IGF1*, *WNT2B*, and *SESN2* were downregulated, and *WNT11* was upregulated in the SCLC tumor tissues in the GSE149507 data set. Further, *IGF1*, *WNT6*, *WNT1*1, and *WNT2B* were lowlier expressed and *SESN2* and *NCRNA00173* were more highly expressed in the chemoresistant cells than sensitive cells.

Conclusions: The top 10 upregulated DELs containing *NCRNA00173* may be involved in the regulation of drug resistance in SCLC. These DELs may regulate the genes related to the mTOR signaling pathway. These genes may also be biomarkers and potential targets for the treatment of SCLC.

Keywords: Small cell lung cancer (SCLC); chemoresistance; mTOR pathway; long non-coding RNA (lncRNA); microarray analysis

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Introduction

In the global cancer statistics for 2020, lung cancer ranked 2nd in terms of new cases and deaths (1). Thus, lung cancer represents a serious threat to human health and survival. Lung cancer is divided into 2 categories; that is, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (2). SCLC accounts for only 15% of all lung cancers (2). Conventional SCLC treatments include chemotherapy, radiation therapy, and surgery, among which chemotherapy is the primary treatment approach (3). The traditional 1st-line chemotherapy regimen comprises etoposide (VP-16) and cisplatin (CDDP) (4). However, due to chemoresistance, SCLC is characterized by high recurrence and metastasis rates after 1st-line chemotherapy and low efficiency and short remission periods after 2nd-line chemotherapy; thus, the 5-year survival rate of SCLC patients is low, and their prognosis is poor (5). The identification of key genes in the pathogenesis of SCLC would provide an important theoretical basis for discovering possible diagnostic or prognostic biomarkers.

Protein-coding genes play an important role in the development of SCLC; however, the sequencing of the human genome has shown that <3% of genomes can encode proteins (6). Long non-coding RNA (lncRNA) is a non-coding RNA molecule >200 nucleotides in length (7). Its function has received increasing attention in cancer research, including in SCLC (8). An increasing number of reports suggest that lncRNAs are involved in the development and chemotherapy resistance of SCLC (9). For example, *MCM3AP-AS1* could promote cell invasion and migration by sponging *miR-148a* (10). *ZFPM2-AS1* could facilitate cell proliferation and invasion by sponging *miR-3612* (11). However, the underlying mechanisms need to be explored further.

In recent years, gene microarray technology and bioinformatics analyses have been widely used in genomics research. However, the high aggressiveness and short survival of SCLC have resulted in limited relevant gene microarray data and limited drug resistance-related data sets. We previously performed microarray-based messenger RNA (mRNA) and lncRNA expression profiling using the SCLC sensitive cell line, H69, and the SCLC chemoresistant cell line, H69AR. Among the differentially expressed genes (DEGs), some have important significance in SCLC. For example, according to our previous studies, *HOTTIP* (12) and *NCRNA00173* (13) appear to induce SCLC chemoresistance by upregulating *BCL-2* and *Etk* expression, respectively. However, this microarray the microarray-based mRNA and lncRNA expression profiling of H69 and H69AR has not been comprehensively analyzed.

In this study, we conducted an integrative analysis of the lncRNA-mRNA expression profiles and functional networks involved in the chemoresistance of SCLC. By searching for the DEGs and differentially expressed lncRNAs (DELs) using bioinformatics techniques, performing a functional analysis, constructing protein-protein interaction (PPI) networks, conducting a co-expression analysis and a cis-regulation analysis, integrating the SCLC microarray data set GSE149507 from the Gene Expression Omnibus (GEO) database, and validating the gene expression *in vitro*, we sought to improve understandings of the molecular mechanisms of SCLC chemoresistance. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3642/rc).

Methods

Cell culture

The human SCLC cell lines NCI-H69 and NCI-H446, and the chemoresistant cell line NCI-H69AR were purchased from the American Type Culture Collection (USA). The CDDP-resistant NCI-H446DDP cell line was constructed in our laboratory (14). These cell lines were all cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) at 37 ℃ with 5% carbon dioxide.

CCK-8 assays

At a density of 5×10^3 cells per well, the H69, H69AR, H446, and H446DDP cells were inoculated in 96-well plates and treated for 24 h with the chemotherapy drugs of CDDP (Shandong, China), VP-16 (Jiangshu, China), or Adriamycin (ADM; Jiangshu, China). Next, the cells were incubated with 10 μL of Cell Counting Kit-8 (CCK-8; C0038, Beyotime, China) solution for 2 h, and measured for the absorbance at 450 nm, which were used. The value of absorbance at 450 nm were used to calculate the IC_{50} values of each chemotherapeutic drug after 3 independent experiments. The data described biological replicates.

LncRNA/mRNA expression profiling

The total RNA from each sample was extracted and

quantified by the NanoDrop ND-1000. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. For the microarray analysis, Human LncRNA Array v2.0 (8 x 60K, Arraystar, USA) was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. The arrays were scanned by the Agilent Scanner G2505B. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.0 software package (Agilent Technologies, USA). After the quantile normalization of the raw data, lncRNAs and mRNAs for which at least 3 out of the 6 samples had flags in the Present or Marginal ("All Targets Value") were chosen for further data analysis.

Identification of the DEGs and DELs

R package limma (version 3.40.6) (15) was used to identify the DEGs and DELs between the H69 group and H69AR group based on the following screening criteria: a |log fold change (FC)| ≥1 and an adjusted P value <0.05, as shown by volcano plot filtering. Hierarchical clustering was performed to show the distinguishable DEG and DEL expression patterns among the samples.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

ID Conversion was performed via org.Hs.eg.db package (3.10.0). GO and KEGG enrichment analyses were performed by the clusterProfiler package (3.14.3) (16) against the background of Homo sapiens. The top 10 enriched terms with the smallest adjusted P values were visualized.

PPI network construction

The DEGs were uploaded onto the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (11.5) for the PPI network construction with an interaction score of 0.7. The top 10 hub genes were analyzed in terms of betweenness, closeness, and degree. The overlapping genes were identified as hub genes.

The co-expressed DEGs of the top 10 upregulated DELs and the hub genes

The correlated genes of the top 10 DELs were screened by a Spearman analysis, with the threshold of a |c| ≥0.9 and a P value <0.05. A PPI network based on the correlated genes was constructed with an interaction score of 0.4, and the hub genes were screened out using the degree algorithm.

Cis-regulation prediction

The DEGs that were situated within 100 kbp range upstream and downstream of the selected lncRNAs and correlated with the selected lncRNAs (P<0.05) were predicted as the cis-regulated DEGs by the top 10 DELs.

Gene expression validation in GSE149507

The DEGs in the GSE149507 data set from the GEO database were analyzed using limma R package. The expression of each gene was merged based on the median expression value of their multiple probes, and then measured by both a Wilcoxon rank-sum test and paired *t*-test. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

qRT-PCR validation

The total RNA was extracted from the H69, H69AR, H446, and H446DDP cells by TRIzol (Invitrogen, Grand Island, NY, USA), reverse transcribed into complementary DNA according to the instructions of the AMV reverse transcription kit (Invitrogen, USA), and amplified according to the instructions of the SYBR green PCR Master kit (TaKaRa, Dalian Baosheng Bio, China) on a real-time polymerase chain reaction (PCR) instrument according to the manufacturer's instructions. GAPDH served as the internal reference. The quantitative PCR (qPCR) primers used are listed in Table S1. The PCR amplification conditions were: 95 ℃ for 10 min, 40 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. The relative expression levels of the genes were quantified using the $2^{-\Delta\Delta Ct}$ method after 3 independent

experiments. The data described biological replicates.

Statistical analysis

The data analyses were performed using R language (3.6.3) and GraphPad Prism (9.0.0, GraphPad Software, Inc.). The IC_{50} values of the sensitive and chemoresistance cells were compared, and the DEGs in the 2 pairs of the sensitive and chemoresistance SCLC cell lines were tested by *t*-tests. The bioinformatics analysis visualization was achieved mainly through ggplot2 (3.6.3) package. A P value <0.05 indicated a statistically significant difference.

Results

The chemosensitivity and mRNA/lncRNA expression profiles of the sensitive and chemoresistant SCLC cells

The sensitive cell line H69 and chemoresistant cell line H69AR were incubated with chemotherapeutic drugs (i.e., ADM, CDDP, and VP-16), and their cell IC_{50} values were measured by CCK-8 assays. The results showed that the H69AR cells had higher IC_{50} values than the H69 cells (see *Figure 1A*).

We collected the H69 and H69AR cells, extracted the total RNA, and conducted a microarray analysis. We screened out the DEGs and DELs using the following criteria: a $|\log FC| \geq 1$, and an adjusted P value <0.05. The volcanao maps of mRNA and lncRNAs showed that we identified 609 DEGs, of which 276 were upregulated and 333 were downregulated (see *Figure 1B*), and 394 DELs, of which 270 were upregulated and 124 were downregulated (see *Figure 1C*). The results above were also demonstrated by heat maps (*Figure 1D,1E*). The top 10 mRNAs were *FAM167A*, *NM_014621*, *HOXB9*, *NEUROG2*, *KCNQ2*, *COL23A1*, *C2orf70*, *FAM38A*, *LNPEP*, and *C3orf70* (see *Table 1*). The top 10 lncRNAs were *BC084573*, *AC009336.24*, *RP11-153F5.1*, *AK093987*, *lincRNA-EN2-1*, *RP11-513G11.1*, *RP11-520D19.2*, BC047481, *NCRNA00173*, and *CR936677* (see *Table 2*).

Enrichment analysis and PPI network construction of the DEGs

The enrichment analysis of the DEGs showed that the DEGs were enriched in 363 kinds of biological processes (BPs), 21 kinds of cellular components (CCs), and 18 kinds of molecular functions (MFs) (see https://cdn.amegroups.cn/static/public/atm-22-3642-1.docx). The top 10 BP terms were urogenital system development, mesenchyme development, response to metal ion, mesenchymal cell differentiation, mesonephros development, kidney epithelium development, kidney development, semaphorin-plexin signaling pathway, ureteric bud development, and response to calcium ion (see *Figure 2A*). The top 10 CC terms were costamere, collagen-containing extracellular matrix, perikaryon, contractile fiber part, transcription factor complex, neuronal cell body, contractile fiber, axon part, distal axon, and Z disc (see *Figure 2B*). The top 10 MF terms were receptor ligand activity, chemorepellent activity, core promoter binding, core promoter sequence-specific DNA binding, semaphorin receptor binding, DNA-binding transcription activator activity, RNA polymerase II-specific, G protein-coupled receptor binding, cell adhesion molecule binding, collagen binding, and S100 protein binding (see *Figure 2C*).

A total of 26 kinds of KEGG pathways were enriched (see Table S2), of which the most enriched were basal cell carcinoma, the cytokine-cytokine receptor interaction, the p53 signaling pathway, the FoxO signaling pathway, growth hormone synthesis, secretion and action, breast cancer, the mitogen-activated protein kinase (MAPK) signaling pathway, prolactin signaling pathway, mammalian target of rapamycin (mTOR) signaling pathway, and the phosphatidylinositol-3-kinase and protein kinase b (PI3K-Akt) signaling pathway (see *Figure 2D*).

In the PPI network of the DEGs (see *Figure 3A*), we screened out the top 10 hub genes based on the following measures of centrality: betweenness (see *Figure 3B*), closeness (see *Figure 3C*), and degree (see *Figure 3D*). By intersecting these hub genes, we identified 5 hub genes; that is, *JUN*, *INS*, *STAT1*, *CHRM1*, and *WNT1* (see *Figure 3E*), whose expressions in our microarray profile are shown in *Figure 3F*.

The roles of the top 10 DELs

To characterize the roles of the top 10 DELs (see *Figure 4A*), we screened out the DEGs co-expressed with the top 10 DELs and constructed a lncRNA-mRNA co-expression network (see *Figure 4B*). Next, the co-expressed DEGs were further used to construct a PPI (see *Figure 5A*), and the hub genes were screened out using the degree algorithm (see *Figure 5B*). We also performed an enrichment analysis of the co-expressed DEGs (see Figure S1). The results revealed that the mTOR signaling pathway was enriched.

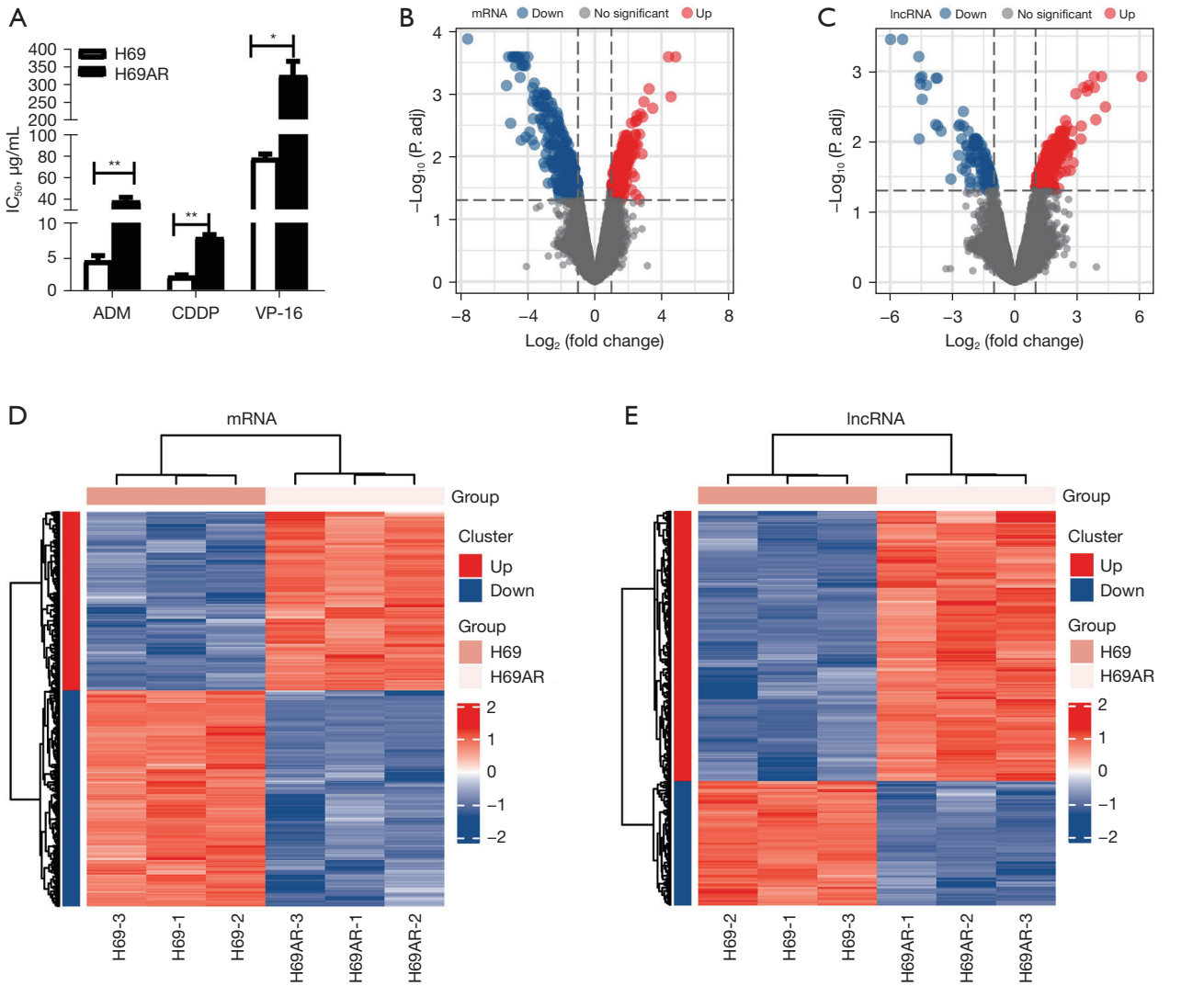

Figure 1 Chemoresistance and mRNA/lncRNA expression profiles in the H69 cells and H69AR cells. (A) CCK-8 assays were used to determine the IC_{50} values of chemotherapeutic agents [i.e., Cisplatin (CDDP), Etoposide (VP-16), and Adriamycin (ADM)] in the sensitive cell line H69 and the chemoresistant cell line H69AR. (B,C) Volcano maps of mRNAs (B) and lncRNAs (C) in the H69 cells and H69AR cells, respectively. (D,E) Heat maps of mRNAs (D) and lncRNAs (E) in the H69 cells and H69AR cells, respectively. The data are shown as the mean ± standard deviation from 3 independent experiments. *, P<0.05; **, P<0.01. mRNA, messenger RNA; lncRNA, long non-coding RNA.

Given that *NCRNA00173* is involved in the development of many tumors (17-21) and our previous study indicated that *NCRNA00173* is also involved in the chemoresistance of SCLC (13), we specifically extracted the DEGs co-expressed with *NCRNA00173* and found that they were also enriched in many cancer-related pathways, such as proteoglycans in cancer, the Wnt signaling pathway, the Hippo signaling pathway, and the mTOR signaling pathway (see *Figure 5C,5D*), which suggests that the mTOR signaling pathway might be a key pathway involved in SCLC development and chemoresistance. Additionally, we identified 4 pairs of differentially expressed lncRNA-mRNA neighbor relationships (see *Table 3*). *AC009336.24* and *BC047481* were predicted to cis-regulate *NM_014621*. *RP11-513G11.1* was predicted to cis-regulate *LRRC15*. *RP11-153F5.1* was predicted to cis-regulate *KRT8*.

Table 1 The characteristics of the top 10 mRNAs with the largest fold change

Gene name	LogFC	Regulation
FAM167A	4.8416287	Up
NM_014621	4.5614647	Up
HOXB9	4.4114865	Up
NEUROG2	3.4677203	Up
KCNQ2	3.253462333	Up
COL23A1	2.9453295	Up
C2orf70	2.868844433	Up
FAM38A	2.827368867	Up
LNPEP	2.746439533	Up
C3orf70	2.680554733	Up

LogFC, Log fold change.

Table 2 The characteristics of the top 10 lncRNAs with the largest fold change

Gene name	LogFC	Regulation
BC084573	6.112119233	Up
AC009336.24	4.360588	Up
RP11-153F5.1	4.175123267	Up
AK093987	3.898287567	Up
lincRNA-EN2-1	3.829717533	Up
RP11-513G11.1	3.820596633	Up
RP11-520D19.2	3.5520305	Up
BC047481	3.4735432	Up
NCRNA00173	3.320569967	Up
CR936677	3.199325267	Up

LogFC, Log fold change.

Data set analysis of key genes

Given the importance of the mTOR signaling pathway, we focused on the co-expressed DEGs of the lncRNA-mRNA co-expression network enriched in the mTOR signaling pathway and identified *IGF1/INS* (among the hub genes in the top 10 DEL-co-expressed DEGs) and *WNT6/WNT11/WNT2B/SESN2* (among the *NCRNA00173*-co-expressed DEGs). The expressions of these genes in our microarray analysis are shown in *Figure 6A*. Further, in the GEO database, we searched the SCLC data set GSE149507 (see *Figure 6B*) and noted the genes that were differently expressed (see *Figure 6C*). The paired *t*-test implied that the expression of *NCRNA00173*, *INS*, and *WNT6* did not differ significantly between the 2 groups. However, *IGF1*, *WNT2B*, and *SESN2* were under-expressed in the SCLC tumor tissues, while *WNT11* was overexpressed in the tumor tissues (see *Figure 6D-6J*). We also compared the expression of the hub genes in the PPI analysis of the DEGs, and found that *JUN* and *CHRM1* were downregulated in the tumor tissues, and *STAT1* was upregulated in the tumor tissues (see Figure S2).

The results showed that *IGF1*, *WNT2B*, and *JUN* which were under-expressed in the SCLC chemoresistant cell line compared to the chemosensitive cell line, were also under-expressed in the SCLC tissues compared to the normal tissues. These results suggest that the low expression of *IGF1*, *WNT2B*, and *JUN* may not only be associated with chemoresistance but may also be related to the tumorigenesis of SCLC.

Validation of the key genes enriched in the mTOR signaling pathway

We further verified the expression of the key genes enriched in the mTOR signaling pathway. We performed qPCR on the H69 and H69AR cells. The results showed that *IGF1*, *WNT6*, *WNT2B* and *WNT11* were downregulated, while *INS*, *SESN2* and *NCRNA00173* were upregulated in the H69AR cells (see *Figure* 7). These results were consistent with those of the microarray analysis, and thus confirmed the reliability of the expression profiling. The expression of the key genes was also validated in the other SCLC sensitive cell line H446 and the chemoresistant cell line H446DDP, the drug sensitivities of which were also analyzed by CCK8 assays (see Figure S3). Except for *INS*, the expression difference among the other genes was consistent with that of the H69 and H69AR cells.

Discussion

As a neuroendocrine tumor, SCLC is characterized by aggressiveness, susceptibility to early hematologic and lymphatic metastasis, and high mortality (22), which is mainly attributed to the rapid development of patients' resistance to chemotherapy. In this study, we performed a microarray analysis of H69 cells and H69AR cells to obtain the DEGs and DELs. The DEGs and DEGs co-expressed with the top 10 DELs were both enriched in the

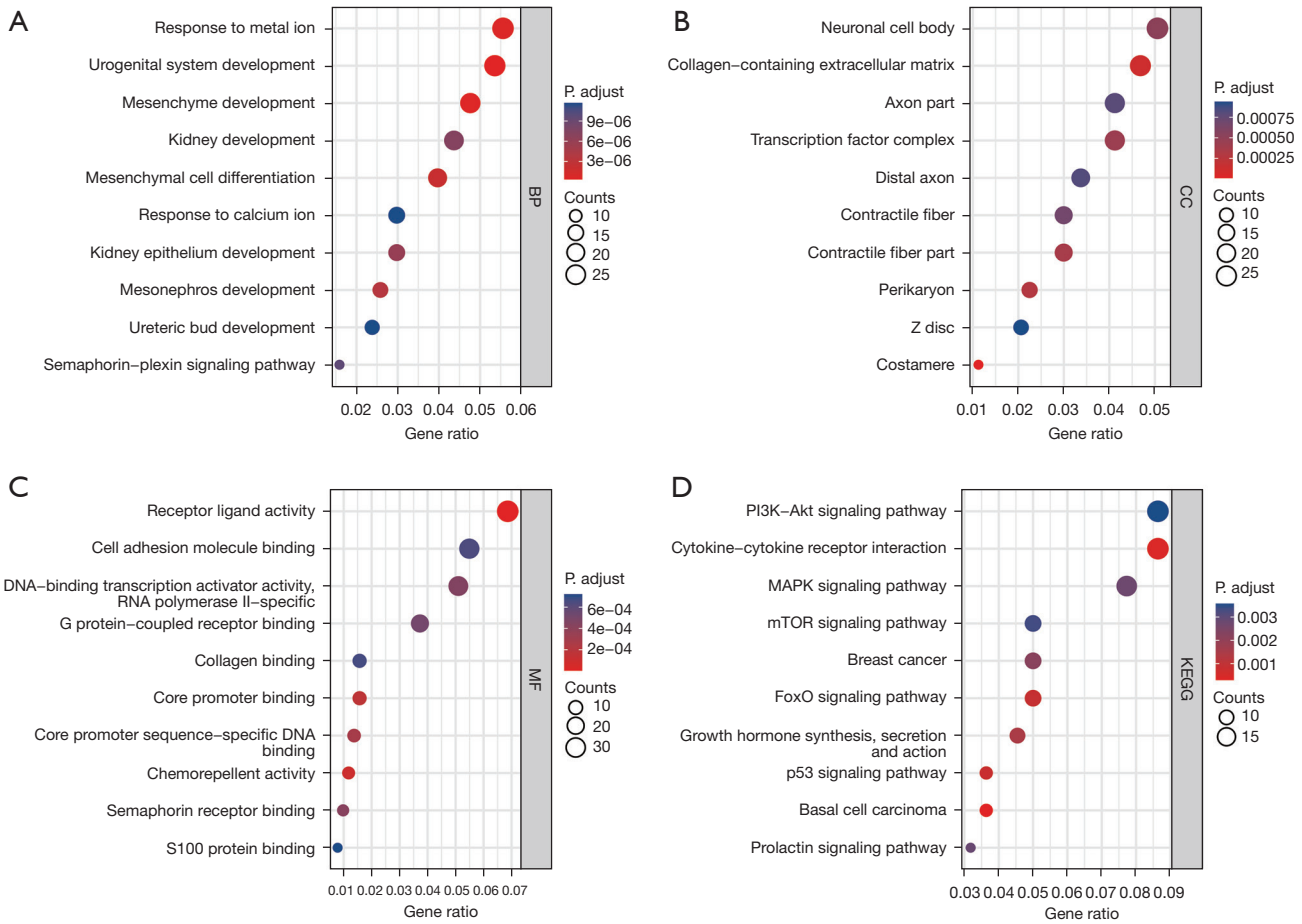

Figure 2 GO and KEGG enrichment analyses of the DEGs. (A) GO-BP enrichment analysis of the DEGs. (B) GO-CC enrichment analysis of the DEGs. (C) GO-MF enrichment analysis of the DEGs. (D) KEGG enrichment analysis of the DEGs. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; DEG, differentially expressed gene.

mTOR signaling pathway. Among the top 10 lncRNAs, *NCRNA00173* has been reported in many tumors, and is involved in several cancer-related pathways, including the mTOR signaling pathway. Further, we found that in the enriched mTOR signaling pathway, *IGF1* and *INS* were the hub genes in the top 10 DEL-co-expressed DEGs, and *WNT6*, *WNT11*, *WNT2B*, and *SESN2* were the *NCRNA00173*-co-expressed DEGs. The key genes enriched in the mTOR signaling pathway were further analyzed using a data set from the GEO database and validated by 2 pairs of chemosensitive and chemoresistant SCLC cells by qPCR assays.

Through the KEGG enrichment analysis, we noted that the DEGs were enriched in various cancer-related pathways, including the PI3K-Akt signaling pathway, MAPK signaling pathway, mTOR signaling pathway, p53 signaling pathway, and FoxO signaling pathway. Additionally, some hub genes in the PPI network of the DEGs, such as *JUN*, *INS*, *STAT1*, *CHRM1*, and *WNT1*, were involved in the top 10 significant pathways. Among them, *JUN* was involved in the MAPK signaling pathway, *INS* in the FoxO/MAPK/mTOR/PI3K-Akt signaling pathways, *STAT1* and *CHRM1* in growth hormone synthesis, secretion, and action, and *WNT1* in basal cell carcinoma and the mTOR signaling pathway. Recently, the activation of the MAPK signaling pathway (23) and the growth hormone-releasing hormone (24) were reported to be factors driving SCLC development. Further, the PI3K/Akt/mTOR pathway has been shown to play an important role in the progression and metastasis of SCLC (25). The activation of the PI3K/

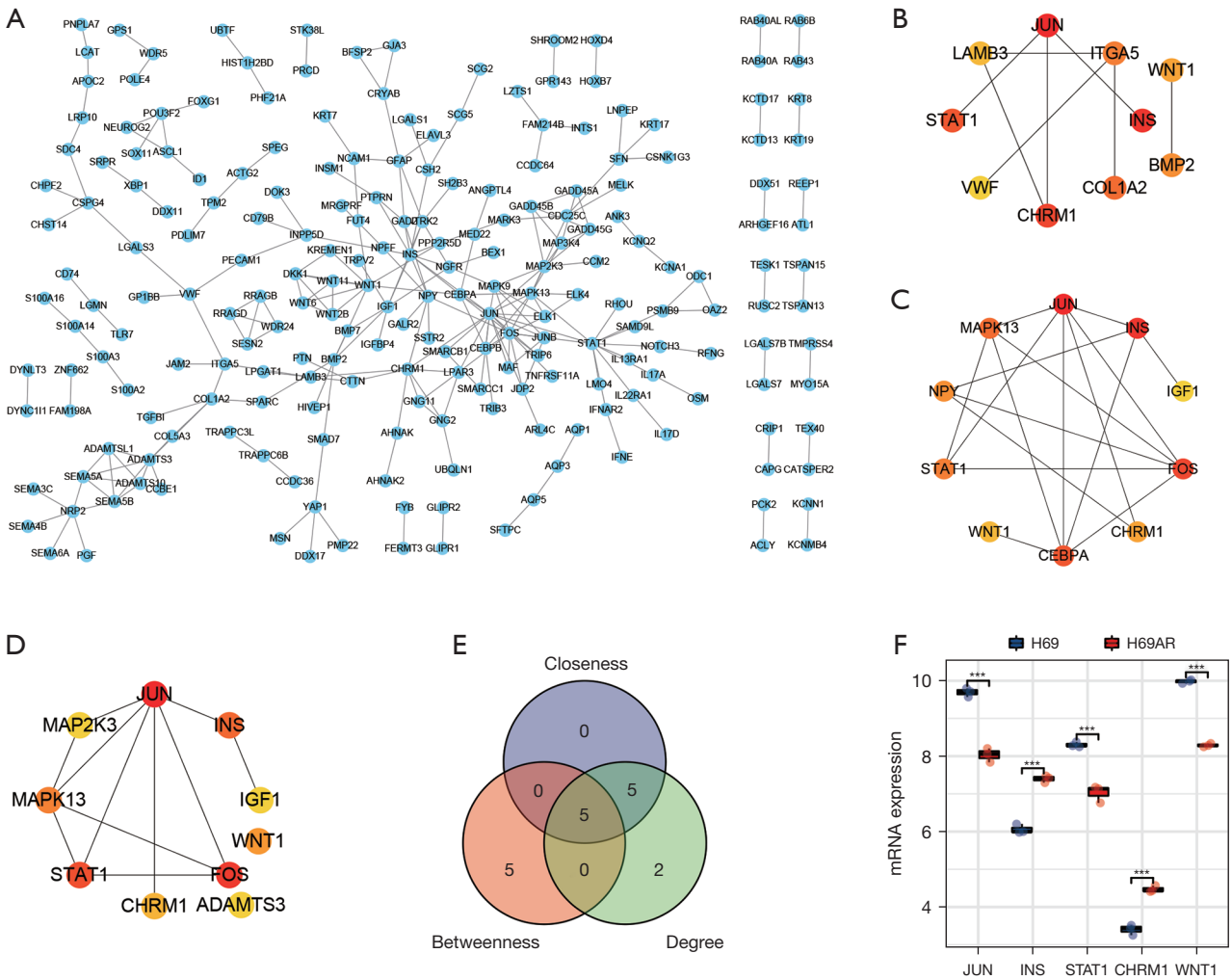

Figure 3 Hub gene screening and expression. (A) PPI network of the DEGs. (B) The top 10 hub genes calculated by the betweenness algorithm. (C) The top 10 hub genes calculated by the closeness algorithm. (D) The top 10 hub genes calculated by the degree algorithm. (E) Intersection of the 10 hub genes calculated by the 3 algorithms. (F) Hub genes' expression in our microarray dataset. ***, P<0.001. PPI, protein-protein interaction; DEG, differentially expressed gene.

Akt/mTOR pathway contributes to the chemoresistance of SCLC cells; however, this chemoresistance could be largely overcome by combining chemotherapy with PI3K/Akt/mTOR pathway inhibitors (26). As a conserved serine/threonine protein kinase, *mTOR* is a key gene for upstream pathways that regulate cell growth, proliferation, motility, survival, and autophagy (27). Autophagy is a cellular degradation process conserved in biological evolution (28,29). Recent studies have shown that autophagy maintains cellular self-balance and provides energy and synthetic materials for cells to meet the metabolic needs of normal cells and even tumor cells and are essential in maintaining the balance of cellular bio-metabolism and the tumor microenvironment (30). Recently, the interaction between autophagy and drug resistance has become a topical issue (31,32).

As we know, mRNA expression profiles are regulated by lncRNAs at the epigenetic level. LncRNA, a general term for a class of RNA molecules >200 nucleotides, is one of the hottest frontier research areas in molecular biology today (33). Through the limma analysis, we identified the top 10 DELs. Among them, *NCRNA00173* was found to be involved in the progression, of various tumors, such as lung squamous cell carcinoma (20), prostate cancer (21),

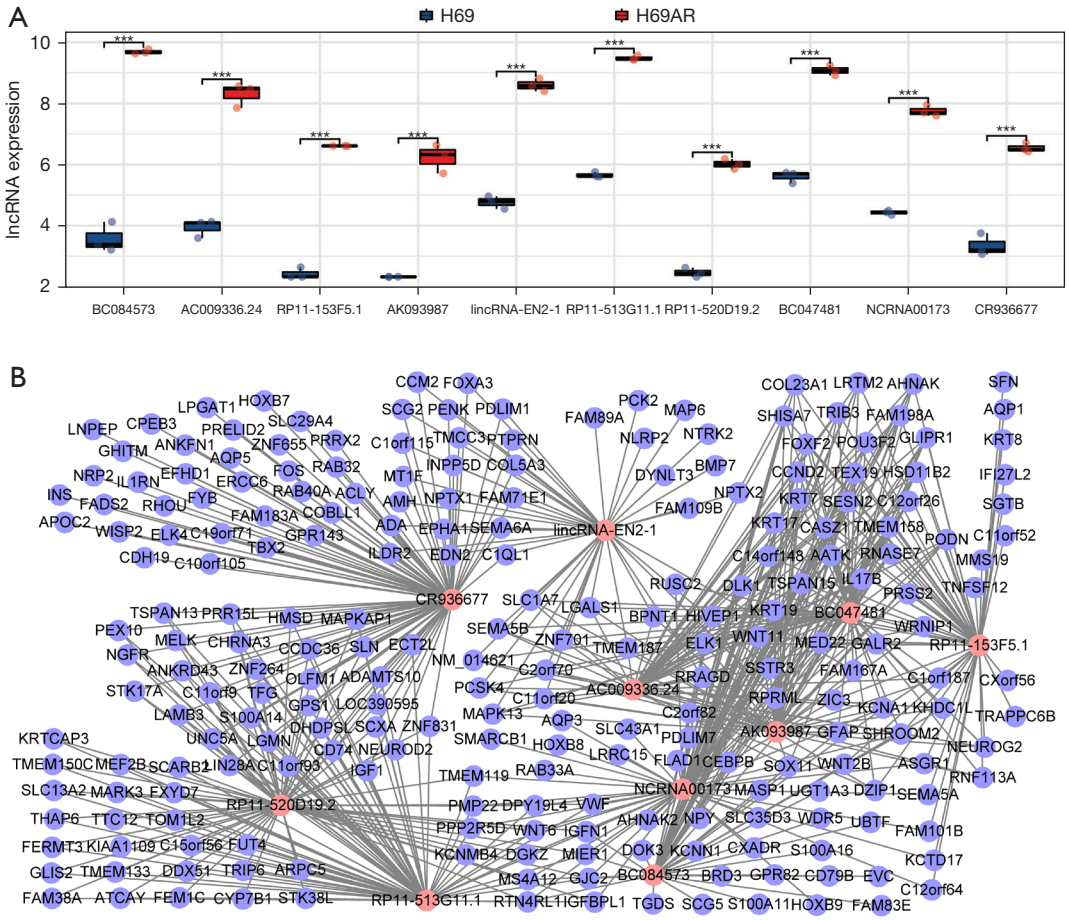

Figure 4 The top 10 upregulated DELs and their co-expressed DEGs. (A) Expression of the top 10 upregulated DELs in our microarray data set. (B) The lncRNA-mRNA co-expression network. The DEGs with absolute value of Spearman's correlation coefficients >0.9 and a P value <0.05 were considered the co-expressed DEGs of the top 10 upregulated DELs. ***, P<0.001. DEG, differentially expressed gene; DEL, differentially expressed lncRNA.

glioma (34), and melanoma (35). In our previous study, we found that *NCRNA00173* also contributes to the chemoresistance of colorectal cancer (19) and SCLC by acting as competing endogenous RNA (13). Tang *et al.* observed high levels of *RP11-513G11.1* in the peripheral blood of diffuse large B-cell lymphoma patients and found that *RP11-513G11.1* was correlated with a poor prognosis (36). To date, no research has examined the role of *BC084573*, *AC009336.24*, *RP11-153F5.1*, *AK093987*, *lincRNA-EN2-1*, *RP11-520D19.2*, *BC047481*, and *CR936677* in cancers, which also need to be explored in relation to SCLC. As far as we know, this was the first study to conduct a microarray analysis to explore the roles of lncRNAs in the chemoresistance of SCLC.

LncRNA regulates adjacent or distant mRNAs in cis or trans ways (37). Our results revealed that 4 lncRNAs may cis-regulate adjacent mRNAs. Further, we predicted the trans-regulatory functions of the top 10 lncRNAs by constructing a lncRNA-mRNA co-expression network and performing enrichment analyses of all the co-expressed DEGs. We found that most of the co-expressed DEGs were enriched in the mTOR signaling pathway. To clarify the hub genes regulated by the top 10 DELs, a PPI analysis was performed, and we found that the hub genes of *IGF1* and *INS* participate in the mTOR signaling pathway. Numerous reports have described the close association between these 2 genes and tumors (38-40). To further predict the functions of the top 10 lncRNAs and determine which lncRNA is most likely to regulate the mTOR signaling pathway, GO and KEGG analyses were performed on the co-expressed

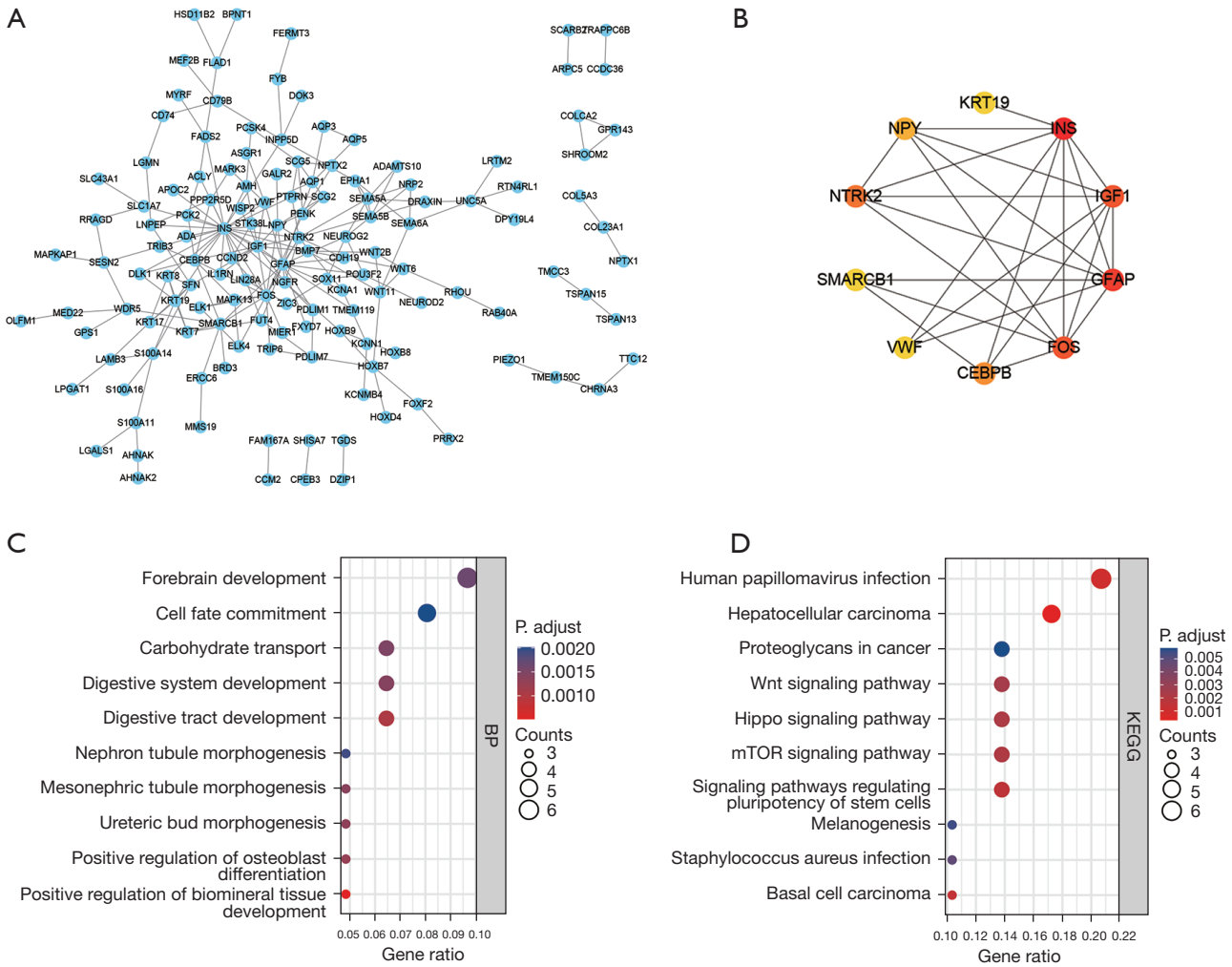

Figure 5 The hub genes in the co-expressed DEGs of the top 10 upregulated DELs and the enrichment of the NCRNA00173-co-expressed DEGs. (A) The PPI network of the co-expressed DEGs of the top 10 upregulated DELs. (B) The top 10 hub genes calculated by the degree algorithm. (C) GO-BP enrichment analysis of the NCRNA00173-co-expressed DEGs. (D) KEGG enrichment analysis of the NCRNA00173-co-expressed DEGs. DEG, differentially expressed gene; DEL, differentially expressed lncRNA; PPI, protein-protein interaction; GO, Gene Ontology; BP, biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 3 The cis-regulation analysis results showed 4 pairs of differentially expressed lncRNA-mRNA neighbor relationships

DELs	Chr	Start	End	Strand	DEGs	Chr	Start	End	Strand	Up/down_ stream	Distance (bp)
AC009336.24	chr2	177042908	177043737	+	NM_014621	chr2	177017335	177017947	+	UPSTREAM	24,960
BC047481	chr2	177037925	177042841	–	NM_014621	chr2	177017335	177017947	+	UPSTREAM	19,977
RP11-513G11.1	chr3	194014253	194030493	–	LRRC15	chr3	194075976	194090472	–	DOWNSTREAM	45,482
RP11-153F5.1	chr12	53279212	53280169	+	KRT8	chr12	53290970	53298868	–	UPSTREAM	10,800

DELs, differentially expressed lncRNAs; Chr, Chromosome; DEGs, differentially expressed genes.

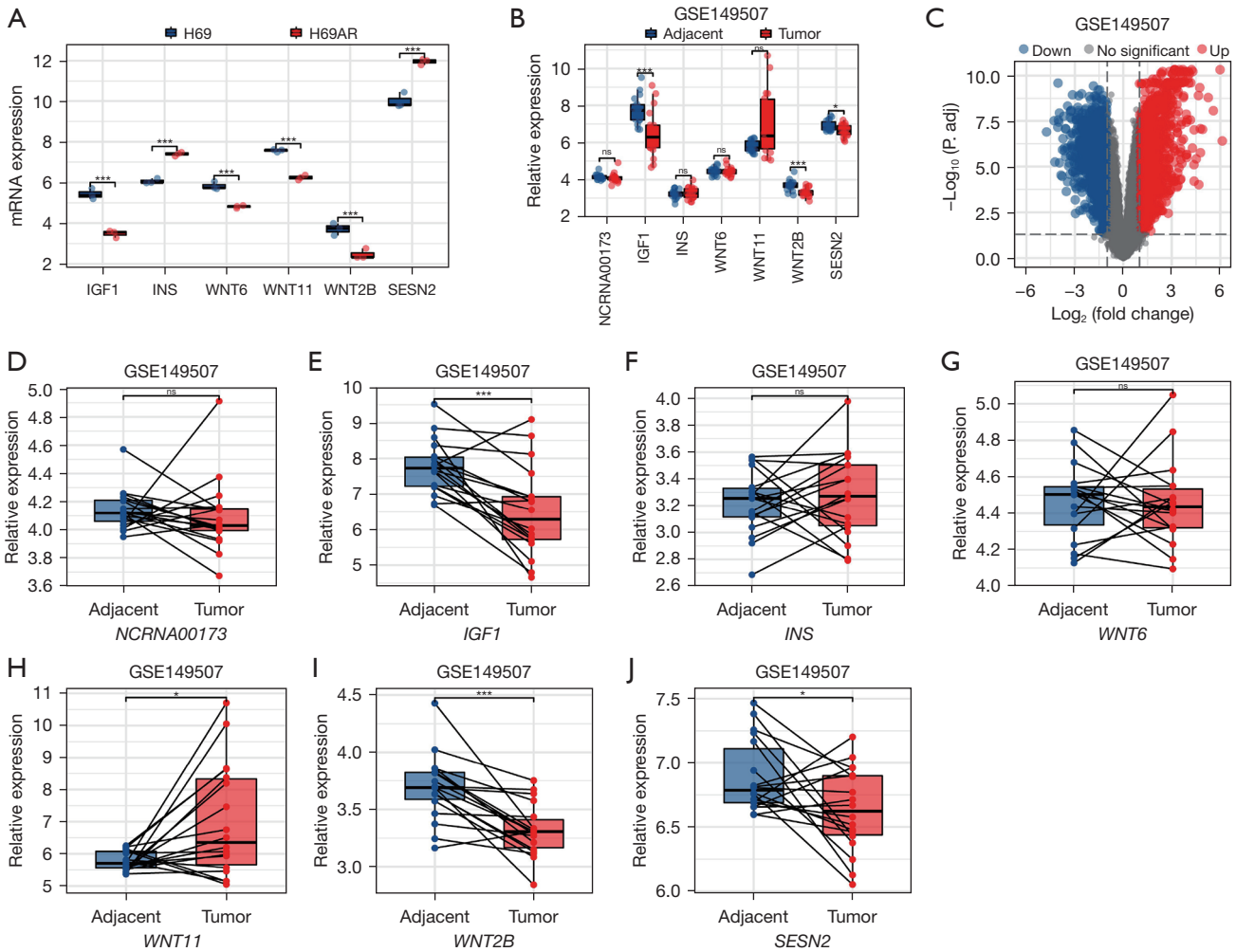

Figure 6 mTOR-related gene expression in GSE149507. (A) *NCRNA00173*, *IGF1*, *INS*, *WNT6*, *WNT11*, *WNT2B*, and *SESN2* gene expression in our microarray data set. (B) *NCRNA00173*, *IGF1*, *INS*, *WNT6*, *WNT11*, *WNT2B*, and *SESN2* expression in the adjacent tissues and tumor tissues in GSE149507. (C) Volcano maps of genes in GSE149507. (C-I) The expression of *NCRNA00173* (D), *IGF1* (E), *INS* (F), *WNT6* (G), *WNT11* (H), *WNT2B* (I), and *SESN2* (J) in the matched adjacent tissues and tumor tissues in GSE149507. ns, P>0.05. *, P<0.05. ***, P<0.001.

DEGs of each of the top 10 lncRNAs, and we found that the co-expressed DEGs of *NCRNA00173* or *RP11-153F5.1* were enriched in multiple tumor-related pathways, including the mTOR pathway.

Our results imply that these lncRNAs may promote SCLC chemoresistance by regulating the mTOR signaling pathway. We paid particular attention to *NCRNA00173*, as it has been reported to play important roles in several tumors (13,17-21). It has also been reported that lncRNAs modulate autophagy or drug resistance of SCLC; for example, the knockdown of *HIF1A-AS2* increases the autophagy-related drug sensitivity of SCLC cells (41). Cancer-associated fibroblasts release exosomes capsuled with *MEG3* to enhance CDDP chemoresistance in SCLC. Our previous studies suggest that *KCNQ1OT1* inhibition induces apoptosis and drug sensitivity by inhibiting *transforming growth factor beta 1* (42) or by activating the JAK2/STAT3 signaling pathway (43). *TUG1* promotes the transcription of *LIMK2b* by binding to *EZH2* to enhance cell growth and the chemoresistance of SCLC (14). *HOTTIP* (12) and *NCRNA00173* (13) induce SCLC chemoresistance by promoting *BCL-2* and *Etk* expression, respectively, by sponging microRNA. However, little is known about the relationship between

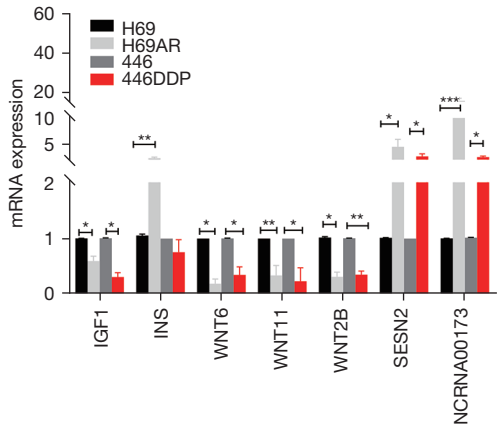

Figure 7 QPCR assays were performed to confirm gene expression. The data are shown as the mean ± standard deviation from 3 independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. QPCR, quantitative polymerase chain reaction.

NCRNA00173 and the mTOR signaling pathway and this needs to be further investigated in SCLC.

The expression of genes involved in the mTOR signaling pathway (i.e., *NCRNA00173*, *IGF1*, *INS*, *WNT6*, *WNT11*, *WNT2B*, and *SESN2*) and the hub genes of the DEGs PPI network (i.e., *JUN*, *STAT1*, *CHRM1*, and *WNT1*) were analyzed in the SCLC data set. We did not screen out chemoresistance-related data sets; however, we found that some of these genes differed in the normal and SCLC tissues in the GSE149507 data set. According to the results of the data set analysis, the low expression of *IGF1*, *WNT2B*, and *JUN* may be a biological characteristic of the occurrence and chemoresistance of SCLC. Finally, we verified the key genes enriched in the mTOR signaling pathway *in vitro*, and found that *IGF1*, *WNT6*, *WNT11* and *WNT2B* were more lowly expressed, while *SESN2*, and *NCRNA00173* were more highly expressed in the 2 chemoresistant cell lines than the chemosensitive cell lines; thus, *NCRNA00173* may induce chemoresistance in SCLC by downregulating *WNT6*, *WNT11*, and *WNT2B*, or by upregulating *SESN2*, which means the *NCRNA00173* and *SESN2* inhibitors or the *WNT6*, *WNT11*, and *WNT2B* promoters would benefit for the treatment of SCLC. These genes might be used as chemoresistance biomarkers in SCLC, and further studies on their biological functions and mechanisms need to be conducted.

There are several limitations in our study that should be noted. Firstly, we analyzed lncRNA-mRNAs only indirectly using bioinformatics analysis and the key genes were verified by qPT-PCR in SCLC cell lines, further studies on the biological functions and mechanisms of the key genes need to be conducted *in vitro* and *in vivo*. Secondly, the DEGs and the DELs were screened out and lncRNA-mRNA co-expression network was also constructed in this study. Our research group had previously performed miRNA microarray (44) which could be combined with this study to construct the ceRNA network by integrating the relationships between lncRNAs and miRNAs, miRNAs and target genes, and the co-expression between differential mRNAs and lncRNAs (positive co-expression relationship) using the database such as TargetScan. The ceRNA analysis should be further performed. Thirdly, in this study , we conducted mRNA-lncRNA microarray analysis which is an important method to screen possible lncRNAs and mRNAs associated with specific diseases. Whole exome sequencing has high coverage and high accuracy. A recent study by Wagner *et al.* revealed that WNT pathway was activated in relapsed SCLC samples by whole-exome sequencing of paired SCLC tumor samples (45). The whole exome sequencing analysis should be further performed in the SCLC chemoresistant cell lines or clinical tissues to get more accurate information about SCLC chemoresistance.

Conclusions

Through a combination of bioinformatic analyses, we identified the top 10 DELs, including *NCRNA00173*, that may be involved in drug resistance in SCLC by regulating the genes related to the mTOR signaling pathway. Combining the results of microarray and data set analyses, we found that the low expression of *IGF1*, *WNT2B*, and *JUN* may not only indicate drug resistance, but may also indicate development of SCLC. Through qPCR assays of 2 pairs of sensitive and chemoresistant SCLC cell lines, we verified that *IGF1*, *WNT6*, *WNT11*, *WNT2B*, *SESN2*, and *NCRNA00173* may serve as biomarkers of drug resistance in SCLC.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3642/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3642/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3642/coif). All authors report that this work was supported by the National Natural Science Foundation of China (No. 81902311), the Natural Science Foundation of Guangdong Province (No. 2020A1515011454), the Project of the Featured Clinical Technique of Guangzhou (No. 2019TS28), and the Characteristic Innovation Project of Guangdong Universities (Natural Science) (No. 2021KTSCX093). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Supplementary

Table S1 Primer sequences for RT-qPCR

Primer	Sequences (5'-3')
IGF	F: CAACATCTCCCATCTCTCTGGA
	R: TTGGGTTGGAAGACTGCTGA
INS	F: CAGAAGAGGCCATCAAGCAGA
	R: TTCACAAAGGCTGCGGCT
GAPDH	F: ATGGTTTACATGTTCCAATATGA
	R: TTACTCCTTGGAGGCCATGTGG
WNT6	F: AACAGGACATTCGGGAGACG
	R: CAGCTCGCCCATAGAACAGG
WNT11	F: CCGACATGCGCTGGAACT
	R: GGTCCCTCTCTCCAGGTCAA
WNT2B	F: TGGAGTGGTAGCCATAAGCA
	R: CGCTGACTGTGTAGGTATGC
SESN	F: ACAACCTCTTCTGGAGGCACT
	R: CATGTAGCGGGTGATGGCAC
NCRNA00173	F: GCATCCAGCTACCCAGACTC
	R: CCTGCAGCACGCAATTAGAC

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Table S2 KEGG enrichment of DEGs

ONTOLOGY	ID	Description	Gene Ratio	Background ratio	P value	p. adjust	q value	Count
KEGG	hsa05217	Basal cell carcinoma	8/220	63/8076	0.000284565	0.000284565	0.047869655	8
KEGG	hsa04060	Cytokine-cytokine receptor interaction	19/220	295/8076	0.000421076	0.000421076	0.047869655	19
KEGG	hsa04115	p53 signaling pathway	8/220	73/8076	0.000782327	0.000782327	0.048931026	8
KEGG	hsa04068	FoxO signaling pathway	11/220	131/8076	0.000860824	0.000860824	0.048931026	11
KEGG	hsa04935	Growth hormone synthesis, secretion and action	10/220	119/8076	0.001462265	0.001462265	0.066494562	10
KEGG	hsa05224	Breast cancer	11/220	147/8076	0.002195372	0.002195372	0.06960978	11
KEGG	hsa04010	MAPK signaling pathway	17/220	294/8076	0.002710854	0.002710854	0.06960978	17
KEGG	hsa04917	Prolactin signaling pathway	7/220	70/8076	0.002826702	0.002826702	0.06960978	7
KEGG	hsa04150	mTOR signaling pathway	11/220	155/8076	0.003320642	0.003320642	0.06960978	11
KEGG	hsa04151	PI3K-Akt signaling pathway	19/220	354/8076	0.003575144	0.003575144	0.06960978	19
KEGG	hsa04390	Hippo signaling pathway	11/220	157/8076	0.003664239	0.003664239	0.06960978	11
KEGG	hsa05205	Proteoglycans in cancer	13/220	205/8076	0.003901214	0.003901214	0.06960978	13
KEGG	hsa04657	IL-17 signaling pathway	8/220	94/8076	0.003980004	0.003980004	0.06960978	8
KEGG	hsa04630	JAK-STAT signaling pathway	11/220	162/8076	0.004648847	0.004648847	0.075500066	11
KEGG	hsa04620	Toll-like receptor signaling pathway	8/220	104/8076	0.007311119	0.007311119	0.110821174	8
KEGG	hsa04380	Osteoclast differentiation	9/220	128/8076	0.0081084	0.0081084	0.115224638	9
KEGG	hsa04512	ECM-receptor interaction	7/220	88/8076	0.009929541	0.009929541	0.132803773	7
KEGG	hsa04080	Neuroactive ligand-receptor interaction	17/220	341/8076	0.011635834	0.011635834	0.146978959	17
KEGG	hsa04658	Th1 and Th2 cell differentiation	7/220	92/8076	0.01251336	0.01251336	0.149744358	7
KEGG	hsa05162	Measles	9/220	139/8076	0.013502328	0.013502328	0.15350015	9
KEGG	hsa05202	Transcriptional misregulation in cancer	11/220	192/8076	0.015709759	0.015709759	0.15926526	11
KEGG	hsa04550	Signaling pathways regulating pluripotency of stem cells	9/220	143/8076	0.016008749	0.016008749	0.15926526	9
KEGG	hsa05225	Hepatocellular carcinoma	10/220	168/8076	0.016353571	0.016353571	0.15926526	10
KEGG	hsa04960	Aldosterone-regulated sodium reabsorption	4/220	37/8076	0.017511805	0.017511805	0.15926526	4
KEGG	hsa05216	Thyroid cancer	4/220	37/8076	0.017511805	0.017511805	0.15926526	4
KEGG	hsa05169	Epstein-Barr virus infection	11/220	202/8076	0.022044079	0.022044079	0.192774133	11

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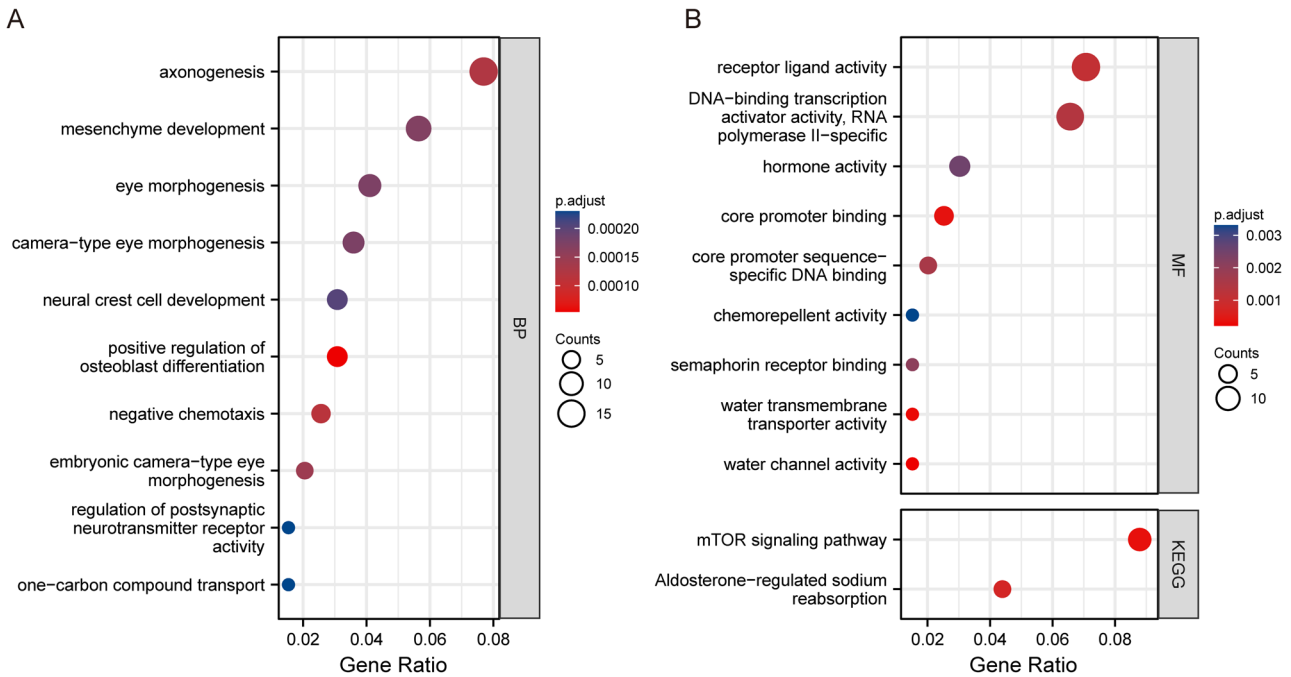

Figure S1 Enrichment of the co-expressed DEGs of the top 10 upregulated DELs. (A) GO-BP enrichment of the co-expressed DEGs of the top 10 upregulated DELs. (B) GO-MF and KEGG enrichment of the co-expressed DEGs of the top 10 upregulated DELs. BP, biological process. MF, molecular function.

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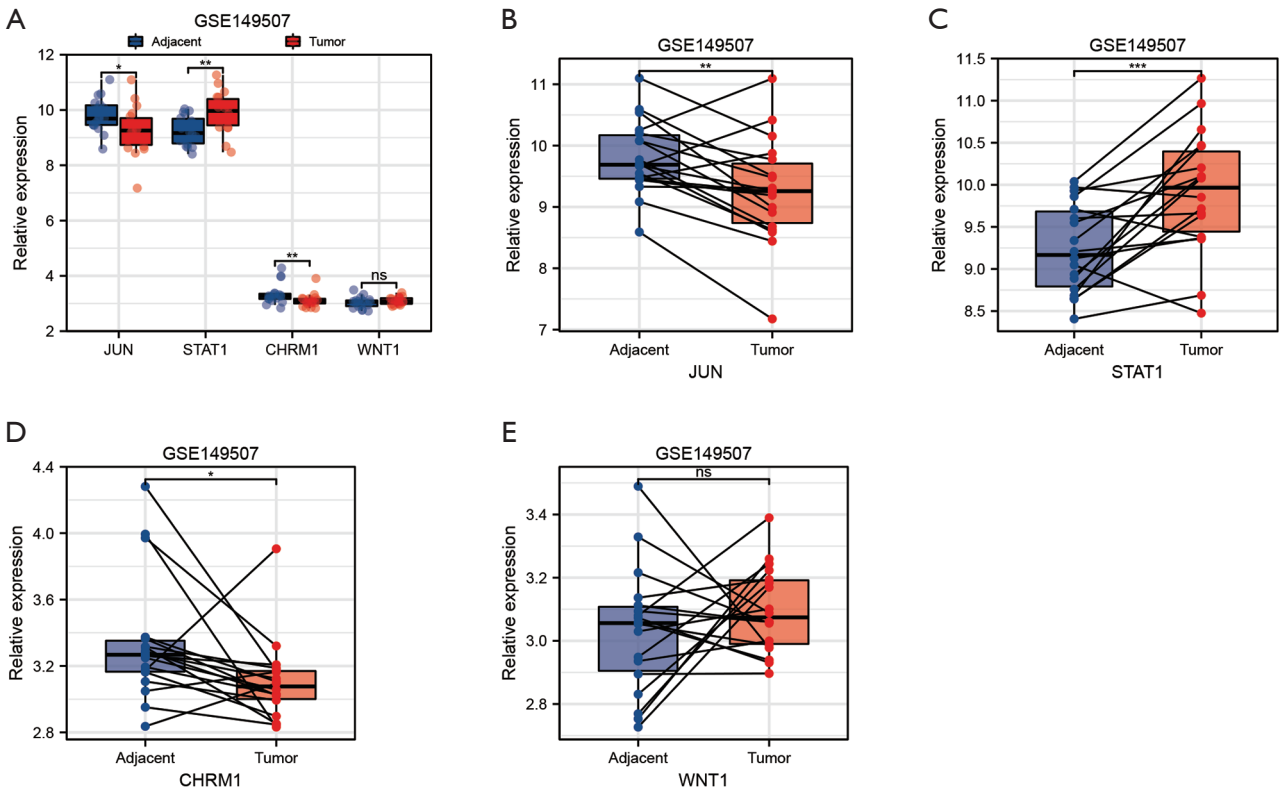

Figure S2 The expression of the hub genes from the DEGs of our microarray data was analyzed in the GSE149507 data set. (A) JUN, STAT1, CHRM1, and WNT1 expression in the adjacent tissues and tumor tissues in GSE149507. (B-E) The expression of JUN (B), STAT1 (C), CHRM1 (D), and WNT1 (E) in the matched adjacent tissues and tumor tissues in GSE149507. ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001.

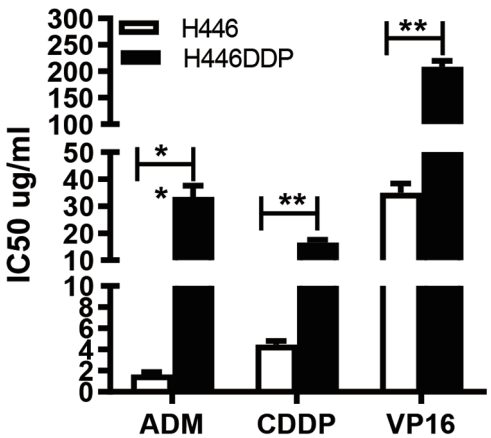

Figure S3 Chemoresistance of H446 cells and H446DDP cells to cisplatin (CDDP), etoposide (VP-16), and Adriamycin (ADM). The data are shown as the mean ± standard deviation from 3 independent experiments. **, P<0.01.

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