Aberrant Methylation Modifications Reflect Specific Drug Responses in Small Cell Lung Cancer

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Research

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Abstract

Background: Epigenetic alterations of specific genes are closely related to chemotherapeutic efficacy in various cancers. However, few comprehensive profiles of differentially methylated genes (DMGs) in small cell lung cancer (SCLC) were available. The current study aimed to explore the association between aberrant methylation modifications of coding and non-coding genes and chemotherapy insensitivity, thus constructing lncRNA-miRNA-mRNA regulatory network in SCLC.

Methods: In this study, methylated DNA immunoprecipitation sequencing (MeDIP-Seq) and RNA sequencing (RNA-Seq) was employed to six SCLC patients who were sensitive and insensitive to first-line standard chemotherapy. Then, we verified the therapeutic predictive effects of DMGs in 62 SCLC cell lines and constructed lncRNA-miRNA-mRNA network on the basis of different cohorts and databases.

Results: Of 4552 DMGs which were selected (fold change ≥ 2 , $p < 0.01$) between chemo-sensitive and chemo-insensitive group, coding genes constituted the largest percentage (3873/4551, 85.08%), followed by lncRNAs (479/4551, 10.52%) and miRNAs (162/4552, 3.56%). Further, both two groups demonstrated two methylation peaks near transcription start site and transcription end site. Two lncRNA-miRNA-mRNA networks which based on genes that showed opposite results in genetic expression and epigenetic modifications suggested the extensive genome connection between chemotherapy efficacy-related non-coding RNAs (ncRNAs) and mRNAs. Combining four DMGs, including hsa-miR-34a, LINC00461, LINC01018, and LINC01484 could effectively predict first-line chemotherapy response in SCLC, indicating that they might play vital roles in SCLC chemotherapy insensitivity.

Conclusions: This study was the first to evaluate multiple drugs efficacy-related ncRNAs and mRNAs which were modified by methylation in SCLC. In addition, DMGs identified in our research might serve as predictors for chemotherapy effect and promising therapeutic targets to reverse drugs-insensitivity by complex lncRNA-miRNA-mRNA mechanisms in SCLC. Further comprehensive studies of these findings are worthy of expecting.

Background

Lung cancer is responsible for the higher morbidity and mortality rate than any other kinds of malignant tumor, among which small cell lung cancer (SCLC) accounts for about 10–15% (1, 2). As a highly malignant form of neuroendocrine tumor, the two-stage classification of SCLC remained widely used (3). For extensive-stage SCLC patients (ES-SCLC), the platinum-based combination regimen, including platinum plus etoposide (EP) and platinum plus irinotecan (IP), was still recommended as the first-line therapy. However, the median overall survival (OS) of the standard chemotherapy regimen for SCLC was only about one year or shorter because of the high incidence rate of primary or secondary chemotherapy insensitivity (4, 5). Moreover, about 40% clinical SCLC patients was insensitive to chemotherapeutic drugs, which affected the long-term prognosis of SCLC seriously (5–7). Consequently, exploring the

molecules and mechanisms of chemotherapy insensitivity is the key to providing new therapeutic targets and improving clinical prognosis in SCLC.

Multiple researches illustrated the great influences of epigenetic alterations in the process of progression, metastasis and chemotherapy insensitivity in SCLC (8, 9). Epigenetic mechanisms contain DNA methylation, differential expression of non-coding RNAs (ncRNAs), and chromosome remodeling, etc. Under the complex effects of epigenetic modifications, the changes of gene expression and the phenotype was heritable, without DNA sequence alterations (10). DNA methylation could modify the transcriptional activity of tumor associated genes, including oncogenes and suppressor genes, thus regulating carcinogenesis and tumor development (11–13). More specifically, the hypermethylation of promoter regions of coding genes, such as *FHIT*, *MGMT*, and *RASSF1A*, led to the gene silencing in lung cancer (13–16). On the contrary, the high levels of methylation in gene body contributed to the overexpression of genes, which was similar to the function of hypomethylation in tumor tissues (16–18). In terms of chemotherapy insensitivity, the expression of tumor suppressor genes, such as *IGFB3*, *GSTP1*, *MGMT*, and *RARβ2*, was down-regulated in non-small cell lung cancer (NSCLC) drug-resistant cell lines by promoter hypermethylation, but few data were available in SCLC (19, 20).

Other than coding RNAs, ncRNAs included long non-coding RNAs (lncRNAs) which more than 200 nucleotides in length, and microRNAs (miRNAs), the length of which was about 18–25 nucleotides, respectively (21, 22). Epigenetic modifications also occurred in lncRNAs and miRNAs. The hypermethylated status of promoter in lncRNAs and miRNAs, such as AFAP1-AS1, miR-205, miR-200, miR-196b, miR-34b/c, and miR-886-3p, was associated with the low expression of corresponding RNAs in SCLC and NSCLC, thus affecting the tumor growth and invasion (23–25). Mounting evidences investigated the significant roles of lncRNAs and miRNAs in chemotherapy insensitivity (26, 27). In SCLC drug-resistant cell lines, the aberrant expression of various lncRNAs and miRNAs was found, including lncRNA HOTTIP, lncRNA HOTAIR, miR-92a-2, miR-100, miR-134, miR-137, and miR-147, etc (28–33). However, the researches that focused on the comprehensive relationship between tumor-related genes, lncRNAs, miRNAs and the level of methylation in SCLC chemotherapy response was insufficient. The aim of this study was to identify epigenetic changes of coding and non-coding genes that influenced the efficacy of chemotherapy in SCLC by combining methylated DNA immunoprecipitation sequencing (MeDIP-Seq) and RNA sequencing (RNA-Seq). Moreover, we constructed the novel network of methylated lncRNAs, miRNAs and mRNAs, in order to better understand the complex mechanisms of chemotherapy insensitivity in SCLC. Then, we highlighted four therapeutic predictive epigenetic signatures for SCLC and verified their performances in 62 SCLC cell lines.

Methods

Patients and clinical specimens

The research involved six patients who underwent percutaneous transthoracic needle aspiration biopsy from 2018 to 2019 at Shanghai Pulmonary Hospital, China. All enrolled participants were diagnosed with

SCLC by two independent pathologists. The tumor-node-metastasis (TNM) staging system of International Association for the Study of Lung Cancer (IASLC) and the two-stage system of Veterans Administration Lung Study Group (VALG) were adopted. The experiment was approved by the ethics committee of Shanghai Pulmonary Hospital (ethical number 18151). We collected lung biopsy samples after obtaining written informed consent from all subjects. All clinical specimens were kept at temperatures of below minus 80 degrees Celsius before the process of DNA extraction.

MeDIP-seq

For MeDIP-seq, the PureLink Genomic DNA Mini Kit (K18200, Thermo) was applied to extract genomic DNA from pulmonary biopsy tissue. By means of a bioruptor (Diagenode), purified genomic DNA was sonicated to an average size within the scope of 100-500 base pair (bp). The end-repair of DNA fragments was followed by A-tailed and custom TruSeq adapters attached with the TreSeq DNA sample preparation Kit (Illumina).

Then, protein A + G magnetic beads bound to 5-methylcytidine (5mc) antibody or 5-hydroxyl-methylcytidine (5hmc) antibody was employed to the test of immunology precipitation with DNA fragments linked to adapters. After amplifying for 15 cycles by polymerase chain reaction (PCR) method, the enriched methylated DNA libraries fully sequenced on Illumina HiSeq platform with the sequencing pattern of paired-end 2x150.

Differentially methylated genes (DMGs) bioinformatic analysis

A total of raw read data from MeDIP-Seq were processed by the removal of adapter sequences using the Cutadapt tool (version 1.12) (34), and low-quality sequences with Trimmomatic (version 0.39) (35). Next, the quality control of the remaining data was performed by Fastqc (version 0.11.9) (36), which calculated the amount of clean sequence reads and the proportion of identification error among 100 or 1000 bases. Subsequently, clean data were mapped to human genome through the usage of Bowtie2 (version 2.3.5.1) (37). MASC (version 2.0) were applied for the peak calling, which identified methylated enriched areas of aligned genomes (38). The determination of differentially methylated peaks of genes and the calculation of reads per kilobase per million (RPKM) depended on the method of DESeq2 in DiffBind, a R package (39). Finally, MeDIPseeker was employed to annotate, compare and visualize differentially methylated peaks among all specimens (40). Bioinformatic analyses were conducted using R 3.5.3 software (<https://www.r-project.org/>) and Python 3.8 software (<https://www.python.org/>).

DMGs needed to be satisfied with two conditions: first, the fold change was greater than or equal to two; second, the p value less than 0.01. The genome methylation levels of different clinical samples were analyzed, thus drawing the methylated density plot of 2 kb upstream and downstream of transcriptional sites. The differentially methylated regions of coding and non-coding genes were classified as promoter, intron, 5'UTR, 3'UTR, CDS, and distal intergenic.

Signal transduction pathway analysis

A Gene Ontology (GO) blot and a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway bar-blot for DMGs among all SCLC specimens were obtained by the R package called clusterProfiler. The annotations of GO (<http://www.geneontology.org/>) provide detailed information on the three major functions of genes and proteins, including cellular components, biological processes, and molecular function. The KEGG pathway database, a systematic database that integrates genome and function information, was carried out for enrichment analysis. The q value was generated through adjusting the value of p. P value less than 0.05 and q value less than 0.01 indicated the statistically significant enrichment.

RNA-seq and differentially expressed genes (DEGs) identification

After the extraction of RNA from six SCLC samples, KAPA RiboErase Kit (Human/Mouse/Rat, HMR) was used for the removal of ribosomal RNA (rRNA). The count and quality assessment of raw RNA data was performed by spectrophotometry and electrophoresis, as we reported before (27). Several methods were used for the construction and the quality control of the RNA library, including TruSeq RNA library preparation kit (Illumina, San Diego, CA, USA), quantitative polymerase chain reaction (qPCR), electrophoresis, and so forth. Thereafter, the Illumina HiSeq™2000 platform demonstrated the RNA-seq. We employed TopHat software (version 2.1.1) and Cufflinks (version 1.2.1), which based on the human genome and annotations from the public database, for sequence alignment, fragments per kilobase per million reads calculation, and DEGs visualization. The definition of DEGs was as suggested before (27).

LncRNA-miRNA-mRNA interaction network construction

The differentially expressed (DE) lncRNAs and miRNAs in SCLC were aligned to the DMGs detected by MeDIP-Seq. The regulatory network, which involved DElncRNAs with abnormal methylated status, DE miRNAs with abnormal levels of methylation, and DE mRNAs, were constructed by Cytoscape software (version 3.7.1; <https://cytoscape.org/>) (41). The statistically significant miRNA/lncRNA-targeted genes were identified from the TarBase database (<http://www.tarbase.com/>) (42) and the ENCORI database (The Encyclopedia of RNA Interactomes; <http://starbase.sysu.edu.cn/>) (43, 44). The TarBase database was composed of the verified target genes of miRNA by experiments. The ENCORI platform, which is the latest version of starBase, provided a variety of interactome information among lncRNAs, miRNAs, and mRNAs. All selected target genes in the network were validated by reliable methods, including reported assay, western-blot, and qPCR.

Validation of four therapeutic predictive epigenetic signatures for SCLC

In order to verify therapeutic predictive values of four epigenetic signatures in SCLC, we used CellMiner database (<https://discover.nci.nih.gov/cellmineradb/>) (45). As one of cancer-related databases, CellMiner currently summarizes the pharmacogenomic data of five sources in total 118 SCLC cell lines. Detailed information that provided by CellMiner was as follows: expression levels of more than 60,000 genes, methylation levels of more than 20,000 genes, and drugs responses of more than 500 drugs, etc. Pearson's correlation coefficients range from -1 to 1. P value less than 0.05 indicated the statistically

significant difference. After downloading suitable methylation profiling and drug sensitivity dataset from CellMiner, R 3.5.3 software (<https://www.r-project.org/>) was used for visualizing differential methylation levels of corresponding genes between chemotherapy sensitive and insensitive group.

Results

Clinical characteristics of SCLC patients

The clinical pathological features of six SCLC patients enrolled in the research were summarized. All enrolled participants were male smokers, with an average age of 64.2 years (range: 54–69 years). More specifically, five out of six confirmed with SCLC patients were older than 60 years old. ES-SCLC comprised the highest proportion of the total (4/6, 66.7%), while limited-stage SCLC patients (LD-SCLC) made up 33.3% (2/6). The group of chemotherapy sensitivity consisted of three patients who achieved partial response (PR), while three members of chemo-insensitive group experienced stable disease (SD) or progressive disease (PD) after first-line standard chemotherapy (Table 1).

MeDIP-Seq analysis results

For MeDIP-Seq, a large number of raw reads were obtained from six SCLC fine-needle aspiration specimens, 82.02×10^6 ($\pm 11.94 \times 10^6$) raw reads of chemo-sensitive group, and 83.43×10^6 ($\pm 1.23 \times 10^6$) raw reads of chemo-insensitive group, respectively. The mapped ratios of the group of chemotherapy sensitivity and insensitivity were over 95%, while the unique mapped ratios of these two groups were slightly exceeding 60%.

Comparing these two datasets, no statistical differences were found ($p > 0.05$; Table 2).

DMGs profile and methylation enrichment distribution

The identification of DMGs in SCLC chemo-sensitive and chemo-insensitive tissues were performed by MeDIP-Seq. Of 4552 DMGs which met selection criterias (fold change ≥ 2 , $p < 0.01$), coding genes constituted the largest percentage (3873/4551, 85.08%), followed by lncRNAs (479/4551, 10.52%) and miRNAs (162/4552, 3.56%), while other ncRNAs occupied a relatively small proportion (38/4552, 0.83%; Figure 1A). The percentage of hypermethylated coding genes, lncRNAs, miRNAs, and ncRNAs in the group of chemotherapy sensitivity were 60.86%, 57.20%, 56.17%, 50.00%, respectively (Figure 1B). Further, both these two groups demonstrated two methylation peaks near the scopes of transcription start site (TSS) and transcription end site (TES) (Figure 1C). Specifically, the methylation enrichment of all DMGs spread over the promoter region, the intron region, and the distal intergenic region. The proportion of methylation enrichment in above three mentioned regions was around 30% among the differentially hypermethylated protein-coding genes (Figure 1D). For lncRNAs and miRNAs, the distal intergenic region shared the highest enrichment of methylation (Figure 1E and 1F). The methylation enrichment in the 5'UTR region and the downstream region was observed in protein-coding genes, but hardly discovered in lncRNAs and miRNAs.

The heatmap of systematic clustering analysis helped grasp the comprehensive picture of methylation differences in SCLC. As the Figure 2 showed, the genome methylation level of chemo-sensitive group was higher than it of chemo-insensitive group. Compared with the group of chemotherapy sensitivity, 1516 coding genes, 205 lncRNAs, and 71 miRNAs were differentially hypermethylated in the chemo-insensitive group.

GO and KEGG pathway analysis for DMGs

The functional enrichment analysis of DMGs in SCLC tumor tissues incorporated the GO and KEGG pathway analysis. The results of GO analysis indicated that biological process was principally related to the hypermethylated genes of the chemo-insensitive group, molecular function ranked the second, while cellular component was the least associated enrichment. The top 5 DMG-related terms were as follows: muscle organ development, muscle tissue development, striated muscle tissue development, synaptic membrane, and DNA-binding transcription activator activity which is RNA polymerase II-specific (Table 3 and Figure 3). According to KEGG pathway enrichment analysis, the Wnt signaling pathway was the most enriched pathways of DMGs, followed by signaling pathways regulating pluripotency of stem cells, glycerophospholipid metabolism, synaptic vesicle cycle, and so on (Table 4 and Figure 4).

Different expression patterns of genes in SCLC are associated with the levels of methylation

The genes methylation levels assessed by MeDIP-Seq and the genes expression levels evaluated by RNA-seq were combined together to fully evaluate the relationship between gene methylation and expression in SCLC chemo-insensitive tissues. Of 1516 differentially hypermethylated protein-coding genes in chemo-insensitive tissue, 1102 genes overexpressed (1102/1516, 72.7%), whereas the expression of 414 genes were decreased (414/1516, 27.3%; Figure 5A). Likewise, 931 overexpressed genes comprised 39.5% of 2357 hypomethylated protein-coding genes, while 1426 genes with low expression made up the rest (1426/2357, 60.5%; Figure 5A). The top 10 hyper- and hypo- methylated genes showing differential expression in chemo-insensitive tissues compared with chemo-sensitive tissues were presented in Table 5. For 71 hypermethylated miRNAs, only the expression of 12 miRNAs was decreased in chemo-insensitive group (12/71, 16.9%; Figure 5B). Most of the remaining hypermethylated miRNAs demonstrated high level gene expression (59/71, 83.1%). More than half of hypomethylated miRNAs expression were downregulated (53/91, 58.2%), while the expression of the rest hypomethylated miRNAs were upregulated (38/91, 41.8%). Table 6 displayed the top 10 list of miRNAs which showed opposite methylation level and gene expression across six SCLC samples. For lncRNAs, a total of 102 differentially methylated lncRNAs expressed differentially, of which 78 lncRNAs with hypomethylation and high expression, and 24 lncRNAs with hypermethylation and low expression (Figure 5C). Table 7 summarized the top 10 list of differential lncRNAs in chemo-insensitive group. The correlation between methylated status and expression level of DMGs, including coding genes, lncRNAs, and miRNAs, was statistically significant (both $p < 0.001$).

Then, the significant relationship between methylation levels and expression levels of several chemotherapy efficacy-related genes, such as *CELF2*, *CRMP1*, *FAM135B*, *PON3*, and *ZNF529*, was

validated across 65 SCLC cell lines (Figure S1). Pearson's correlation coefficients illustrated the negative correlations between the methylation levels and gene expression (both $p < 0.05$), which was consistent with our findings and further emphasized the close correlation between methylation and gene expression in SCLC.

LncRNA-miRNA-mRNA interaction network

To explore the regulatory relationship between DMGs that expressed differentially and their candidate targeted genes, lncRNA-miRNA-mRNA interaction networks were constructed. The interaction network in Figure 6 contained eight miRNAs that hyper- or hypo- methylated in SCLC by the results of MeDIP-Seq and showed up- or down- regulated expression by the results of RNA-Seq, 219 target mRNAs, and 32 lncRNAs. In particular, hsa-miR-34a owned the richest target genes, totaling 70 mRNAs and 7 lncRNAs. Apart from hsa-miR-1269a and hsa-miR-589, the connection among other miRNAs was revealed in different degree. For example, hsa-miR-34a shared some target mRNAs with hsa-miR-148a, including MET, MYC, BCL2, GAS1, TGIF2, WNT1, and MAP3K9. In addition, TP53, MYB, and BIRC5 were common target mRNAs for hsa-miR-34a and hsa-miR-150. OIP5-AS1, was the only lncRNA which interacted with three candidate miRNAs, including hsa-miR-320d, hsa-miR-148a, and hsa-miR-150.

Among the top 10 hypermethylation and low expression or hypomethylation and high expression lncRNAs list, 10 differentially methylated lncRNAs in SCLC were selected for generating the lncRNA-miRNA-mRNA interaction network (Figure 7). All enrolled lncRNAs, including LINC00461, LINC01006, LINC01018, LINC01121, LINC01194, LINC01435, LINC02298, RDH10-AS1, H1FX-AS1, and CATIP-AS1, showed the total of 44 predictive target miRNAs and 122 mRNAs. Specifically, the regulatory network of LINC01435 was the largest. We noticed that both LINC01006 and LINC02298 interacted with hsa-miR-708 and hsa-miR-28. The networks of LINC00461 and H1FX-AS1 were linked by hsa-miR-485. However, none of enrolled lncRNAs owned the common target mRNAs.

Four therapeutic predictive epigenetic signatures for SCLC

By means of literature consulting, we found methylation levels four epigenetic signatures among above top10 lists, including hsa-miR-34a, LINC01484, LINC00461, and LINC01018, were closely related to tumor development, progression, and drug resistance. Hence, we firstly evaluated the effects of methylation level of single gene on chemotherapy drugs sensitivity, including etoposide, irinotecan, carboplatin, and cisplatin (Table S1). The predictive value of single epigenetic signature on SCLC first-line chemotherapy drug response in SCLC cell lines ($N = 62$) agreed well with our findings from clinical specimens. Unfortunately, only the methylation level of LINC01484 showed significant predictive value on etoposide sensitivity ($r=0.28$, $p=0.027$). Then, to better predict drug sensitivity in SCLC, we combined four epigenetic features together and explored the corresponding predictive value (Figure 8 and Table S1). When compared with individual epigenetic feature, the analysis results illustrated better predictive value of hsa-miR-34a in combination with LINC01484, LINC00461, and LINC01018 in both etoposide, irinotecan and carboplatin response with decreased p value. Significantly, four epigenetic signatures for SCLC presented the best prediction ability on etoposide sensitivity than others ($r=0.33$, $p=0.0083$).

Discussion

Epigenetic mechanisms, which contain DNA methylation, aberrant expression of miRNAs and lncRNAs, fulfilled an important role in cancers including but not limited to SCLC (8). Moreover, a growing number of evidences emphasized the contribution of epigenetic alternation of cancer-related ncRNAs to chemotherapy insensitivity (19, 20, 28–33). In the current experiment, we explored the unique methylation patterns of protein-coding and non-coding genes between SCLC patients who experienced PR and patients who were insensitive to the standard chemotherapy. Further, the correlation between methylation levels and SCLC-related ncRNAs expression was evaluated through combining the results of MeDIP-Seq and RNA-Seq. Then, we established two lncRNA-miRNA-mRNA interaction networks which based on two types of DMGs, 8 miRNAs and 10 lncRNAs, separately. Finally, four epigenetic signatures that we developed by clinical samples and verified by 62 SCLC cell lines could effectively predict chemotherapy response in SCLC.

Given the highly coordination and diversity of epigenetic modifications in malignancy, some epigenetic regulation might be critical to the formation, progression, recurrence and resistance of tumors (46). CpG islands which are mainly located in the region of TSS of genes have the highest methylated rate (47). In the present study, we demonstrated that the highest methylated peak in both chemo-sensitive and chemo-insensitive groups were at the TES site, while another methylated peak was observed at the TSS site. Further, we found relatively high methylated rate of promoter region in the protein-coding genes and that of distal intergenic region of miRNAs and lncRNAs, revealing that different methylated patterns between coding and non-coding genes in SCLC might contribute to SCLC etiology and therapeutic efficacy.

The heat map depicted the methylation profiles of DMGs between chemo-sensitive and chemo-insensitive group. The GO analysis identified that DMGs in the chemo-insensitive group mainly enriched in the process of muscle-related development, the component of synaptic membrane, and the function of DNA-binding transcription activator activity. It was reported that muscle loss was closely related to cachexia and poor prognosis in various kinds of cancer, including lung cancer (48, 49). In SCLC, Kalari *et al.* (50) also revealed that the enrichment of DMGs were principally presented in processes of neuronal differentiation and acting as transcription factors, which was consistent with our GO results and enhanced the dependability of our research. Several studies also revealed that synuclein- γ , as one of neuronal proteins, induced drug resistance in breast cancer, lung cancer, and so on (51–53). However, little clinical studies were conducted to explore the relationship between above methylation-related GO terms and chemotherapy response in SCLC. To the best of our knowledge, we firstly linked these meaningful cellular components, biological processes, and molecular function to the chemotherapy insensitivity in SCLC. The KEGG pathway analysis of DMGs in the chemo-insensitive group highlighted the significant roles of Wnt signaling pathway, signaling pathways regulating pluripotency of stem cells, metabolism-related pathways, and synaptic vesicle cycle pathway in the insensitivity of chemotherapy in SCLC. The aberrant methylation level of *WNT5A*, *APC*, *CTNND2*, *SFRP4*, *PLCB2*, *CCND2*, *PRICKLE2*, *TCF7L1*, *SMAD3*, *RAC1*, *FZD2* were found in the Wnt signaling pathway. For relapsed SCLC patients, Wagner *et al.* (54) found that the activation of Wnt signaling pathway mediated chemoresistance. The

mechanisms of Wnt signaling pathway in chemoresistance were as follows: apoptosis-related, DNA repair-related, immune evasion-related, drug accumulation and metabolism procedure (55–57). Tumor stem cells (TSCs) possess self-renewal capacity and extensive proliferative potential, thus resulting in the maintenance, invasion, metastasis, relapses and drugs-resistance of a variety of cancers (58). In SCLC, Eramo *et al.* (59) and Sarvi *et al.* (60) reported that the higher expression of CD133 in TSCs was responsible to the drugs-resistance, including cisplatin, etoposide, paclitaxel and gemcitabine. Yu *et al.* (61) demonstrated that the overexpression of ERK, MDR and ABCG2 in $\alpha 2\delta 1(+)$ TSCs participated in the drugs-resistance in SCLC by regulating voltage-dependent calcium channel $\alpha 2\delta 1$. Given all these, we hypothesized that the enrichment of DMGs in these GO terms and KEGG pathways might promote differential expression level of corresponding genes and poorer chemotherapy efficacy by complex mechanisms, thus contributing to poorer outcome in SCLC.

Furthermore, the opposite results in genetic expression and epigenetic modifications were shown in SCLC, suggesting the potential impact of methylation level on the expression status of corresponding genes. We respectively summarized the top 10 hyper- and hypo- methylated curative effect-related genes which showing reverse expression among SCLC clinical samples. In addition, the statistically significant correlations between methylation levels and gene expression level of some corresponding were observed across SCLC cell lines. The widely connection and mutual regulation between lncRNAs and miRNAs was investigated in various cancers (62, 63). Considering both lncRNAs and miRNAs could modulate gene expression either directly or indirectly, we established lncRNA-miRNA-mRNA interaction networks of efficacy-associated DMGs in SCLC. However, the influences of most differentially methylated miRNAs and lncRNAs in chemotherapy response were not well characterized in SCLC. In our study, hsa-miR-34a which showed hypermethylation status and low expression level targeted the maximum number of genes in the differentially methylated miRNAs-based network. In multiple cancer types, many research results demonstrated that the expression level of hypermethylated hsa-miR-34a was downregulated, which further strengthened the relationship between methylation and expression level (64–66). It was reported that hsa-miR-34a, as one of tumor suppressor miRNAs, participated in the drugs-resistance network of chronic lymphocytic leukemia and NSCLC (67, 68). Regrettably, the mechanisms of hsa-miR-34a in regulating chemotherapy insensitivity were not fully illustrated in SCLC owing to the complex correlation between hsa-miR-34a and other genes, such as *TP53*, *MYC*, *EMT*, *HOTAIR*, and so on (33, 69, 70). We also observed the higher expression level and hypomethylated status of LINC00461 and LINC01484, the lower expression level and hypermethylated status of LINC01018 in the SCLC chemo-insensitive group compared with the chemo-sensitive group, indicating the underlying function of these lncRNAs in drugs insensitivity. In rectal cancer, the higher expression level of LINC00461 induced cisplatin-based resistance by regulating miR-593-5p and cyclin D1 level (71). LINC01484, which was considered as one of independent prognostic factors for bladder urothelial carcinoma, improved the metastatic potential of cancer cells (72). LINC01018, which acted as a tumor suppressor, regulated the progression of liver cancer (73). Nonetheless, little data was known for the function and the methylation profiles of these lncRNAs in SCLC. Therefore, the current study put forward new views for the methylation profiles of therapeutic efficacy-associated miRNAs and lncRNAs candidates in SCLC.

According to the Pearson's correlation coefficients, results of relationship between four key genes and drug sensitivity in SCLC cell lines were highly consistent with those concluded by using clinical samples. For example, hypomethylation levels of hsa-miR-34a, LINC01484 and LINC00461, and hypermethylation level of LINC01018 were found among clinical SCLC patients with chemotherapy-insensitivity. Similar results were also found in SCLC cell lines, thus further validating our results and enhancing the credibility. However, some conflicting findings occurred in irinotecan and platinum. Firstly, the level of LINC00461 methylation was negatively correlated with irinotecan sensitivity in cell lines, which was contrary to our clinical finding. In our view, the principal reason was that all enrolled SCLC patients underwent EP treatment (etoposide plus carboplatin/cisplatin), instead of irinotecan treatment, suggesting that methylation signatures varied from drug to drug. Secondly, both hsa-miR-34a and LINC00461 showed controversial predictive values in platinum. The combined therapy of etoposide and platinum in clinical patients might lead to the cumulative results of methylation levels of hsa-miR-34a and LINC00461. Thus, MeDIP-seq displayed the comprehensive predictive effect of corresponding genes on clinical combination therapy, instead of single drug. Rather, analysis of SCLC cell lines focused on the correlation of epigenetic features to the single drug sensitivity, which may result in opposite outcomes between clinic and laboratory. In addition, the predictive ability of single epigenetic characteristic was unsatisfied in SCLC, while encouraging improvement of predictive ability was found after combining four signatures together. In comparison with the significant outcome in etoposide response prediction, predictive differences of irinotecan and carboplatin was not statistically significant. Given all these, combining four epigenetic features together contributed to better predictive value on the efficacy of chemotherapeutic drugs in clinical practice. Moreover, further study would be needed to explore specific factors to predictive other drugs response.

Epigenetic therapies might be the promising strategy to tackle drugs-insensitivity and enhance curative effect in SCLC (8, 9). RRx-001 was the epigenetic and immunity inhibitor which could reverse platinum-resistance. A completed phase 2 clinical trial, called QUADRUPLE THREAT (NCT02489903), applied RRx-001 plus EP treatment to refractory SCLC patients and achieved cheerful efficacy for the first time (74). An ongoing phase 3 clinical trial, called REPLATINUM (NCT03699956), will further explore the application of RRx-001 in SCLC (75). Other epigenetic drugs, including DS-3201b (NCT03879798), CC-486 (oral Azacitidine, NCT02223052), Vidaza (NCT02223052), INCB059872 (NCT02712905), and SGI-110 (guadecitabine, NCT03913455) are also undergoing clinical trials in SCLC. We are looking forward the meaningful results of theses clinical trials. In addition, in consideration of the obvious restriction of our study which analyzed with a relatively small sample size, further studies with larger sample sizes are expected to fully investigate the function and epigenetic alterations of these genes in SCLC patients who insensitive to chemotherapy.

Conclusions

In this study, we compared the high-throughout sequencing results of MeDIP-seq and RNA-Seq to identify a variety of protein-coding genes, lncRNAs and miRNAs which are differentially methylated and expressed in SCLC chemo-insensitive tissues compared with chemo-sensitive tissues. Moreover, we

outlined lncRNA-miRNA-mRNA networks and highlighted some DMGs which are likely to be predictors for chemotherapy effect and promising therapeutic targets to help SCLC patients who suffer from drugs-insensitivity. The main roles, epigenetic alterations, and mutual regulation of these chemo-insensitivity-related genes in SCLC should be deeply and comprehensively illuminated in further researches.

List Of Abbreviations

bp, base pair; DE, differentially expressed; DMGs, differentially methylated genes; ES-SCLC, extensive-stage SCLC patients; EP, platinum plus etoposide; GO, Gene Ontology; IP, platinum plus irinotecan; IASLC, International Association for the Study of Lung Cancer; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNAs; LD-SCLC, limited-stage SCLC; MeDIP-Seq, methylated DNA immunoprecipitation sequencing; miRNAs, microRNAs; ncRNAs, non-coding RNAs; NSCLC, non-small cell lung cancer; OS, overall survival; PD, progressive disease; PR, partial response; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RPKM, reads per kilobase per million; RNA-Seq, RNA sequencing; SCLC, small cell lung cancer; SD, stable disease; TNM, tumor-node-metastasis; TSS, transcription start site; TES, transcription end site; VALG, Veterans Administration Lung Study Group; 5mc, 5-methylcytidine; 5hmc, 5-hydroxyl-methylcytidine.

Declarations

Ethics approval and consent to participate: 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 approved by Shanghai Pulmonary Hospital ethics committee (No. 18151). All participants gave their informed consent.

Consent for publication: All participants gave their informed consent for publication.

Availability of data and materials: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions (I) Conception and design: Peixin Chen, Yayi He; (II) Administrative support: Yayi He; (III) Provision of study materials or patients: Peixin Chen; (IV) Collection and assembly of data: Peixin

Chen; (V) Data analysis and interpretation: Peixin Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Tables

Table 1
Clinical characteristics of SCLC patients included in the study

Characteristic	N (%)	Characteristic	N (%)
Age, mean, years	64.2	T stage	
< 60	1(16.7)	1–2	2(33.3)
≥ 60	5(83.3)	3–4	4 (66.7)
Male	6 (100)	N stage	
Smoker	6(100)	0–1	2(33.3)
Chemotherapy		2–3	4 (66.7)
Etoposide plus cisplatin	1(16.7)	Metastasis	
Etoposide plus carboplatin	5(83.3)	No	2(33.3)
Response assessment		Yes	4 (66.7)
PR	3 (50.0)	TNM staging	
SD	2 (33.3)	III	2(33.3)
PD	1(16.7)	IV	4 (66.7)
VALSG staging			
LD	2(33.3)		
ED	4 (66.7)		
Abbreviation: ED, extensive disease; LD, limited disease; N, Lymph Node; PD, progressive disease; PR, partial response; SCLC, small cell lung cancer; SD, stable disease; T, Tumor; VALSG, Veterans Administration Lung Study Group.			

Table 2
MeDIP-Seq data of clinical Samples

	Chemo-sensitive group	Chemo-insensitive group	P
Total Raw Reads, M (mean ± SD)	82.02 ± 11.94	83.43 ± 1.23	0.876
Total Clean Reads, M (mean ± SD)	78.18 ± 11.26	79.68 ± 0.96	0.860
Mapped Reads, M (mean ± SD)	76.32 ± 11.32	77.23 ± 1.57	0.916
Mapped Ratio (mean)	97.57%	96.91%	0.426
Unique Mapped Reads, M (mean ± SD)	47.25 ± 5.54	49.47 ± 2.43	0.631
Unique Mapped Ratio (mean)	60.68%	62.12%	0.658
Abbreviation: MeDIP-Seq, methylated DNA immunoprecipitation sequencing; M, millions; SD, standard deviation; PR, partial response; PD, progressive disease; SD, stable disease; P, P value for whole.			

Table 3
The top5 enriched GO terms of DMGs.

GO ID	Term	Count	P
GO:0007517	muscle organ development	25	1.07×10^{-6}
GO:0060537	muscle tissue development	25	7.50×10^{-6}
GO:0014706	striated muscle tissue development	22	1.29×10^{-5}
GO:0097060	synaptic membrane	23	3.66×10^{-5}
GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	23	1.08×10^{-4}
Abbreviation: Count, the number of DMGs participated in the corresponding GO term; DMGs, differentially methylated genes; GO, Gene Ontology; P, P value for whole.			
Statistically significant data were marked with bold and underline.			

Table 4
The top5 enriched KEGG pathways of DMGs

KEGG ID	Pathway name	Count	P
hsa04310	Wnt signaling pathway	11	1.32×10^{-3}
hsa04550	Signaling pathways regulating pluripotency of stem cells	10	1.63×10^{-3}
hsa00564	Glycerophospholipid metabolism	8	2.06×10^{-3}
hsa04721	Synaptic vesicle cycle	7	2.27×10^{-3}
hsa00561	Glycerolipid metabolism	6	2.93×10^{-3}
Abbreviation: Count, the number of DMGs participated in the corresponding pathway; DMGs, differentially methylated genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; P, P value for whole.			
Statistically significant data were marked with bold and underline.			

Table 5

The top 10 hypermethylated and hypomethylated protein-coding genes showing differential expression in SCLC chemo-insensitive tissues compared with chemo-sensitive tissues.

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	Coding gene expression
	chemo-insensitive group	Chemo-sensitive group				
APBB1IP	18.91 $\pm$ 3.52	0.47 $\pm$ 0.33	572.05	7.02 $\times 10^{-20}$	Up	Down
ZNF529	6.70 $\pm$ 2.41	0.06 $\pm$ 0.02	786.88	8.51 $\times 10^{-20}$	Up	Down
TP53TG3D	15.60 $\pm$ 3.73	0.44 $\pm$ 0.45	418.77	1.03 $\times 10^{-19}$	Up	Down
CELF2	8.03 $\pm$ 1.16	0.05 $\pm$ 0.04	568.10	1.37 $\times 10^{-19}$	Up	Down
CRMP1	10.12 $\pm$ 4.80	0.13 $\pm$ 0.13	173.65	1.29 $\times 10^{-18}$	Up	Down
RNF223	5.76 $\pm$ 1.22	0.42 $\pm$ 0.35	302.33	1.04 $\times 10^{-17}$	Up	Down
BTBD11	5.12 $\pm$ 1.18	0.50 $\pm$ 0.28	227.54	1.18 $\times 10^{-17}$	Up	Down
SYNE1	8.96 $\pm$ 1.94	0.02 $\pm$ 0.02	308.69	1.21 $\times 10^{-17}$	Up	Down
Abbreviation: RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.						
Statistically significant data were marked with bold and underline.						

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	Coding gene expression
	chemo-insensitive group	Chemo-sensitive group				
TSPAN4	8.26 $\pm$ 5.06	0.21 $\pm$ 0.10	1184.45	1.44 $\times 10^{-17}$	Up	Down
VILL	7.75 $\pm$ 0.86	0.52 $\pm$ 0.55	95.01	6.46 $\times 10^{-17}$	Up	Down
NRG2	0.58 $\pm$ 0.39	15.91 $\pm$ 0.97	-487.75	1.84 $\times 10^{-36}$	Down	Up
RYR2	0.18 $\pm$ 0.09	7.07 $\pm$ 2.23	-1176.27	3.74 $\times 10^{-28}$	Down	Up
WNT10A	0.26 $\pm$ 0.30	7.32 $\pm$ 0.76	-849.22	8.29 $\times 10^{-26}$	Down	Up
FAM135B	0.49 $\pm$ 0.10	5.83 $\pm$ 2.03	-792.35	8.65 $\times 10^{-25}$	Down	Up
PON3	0.35 $\pm$ 0.20	6.49 $\pm$ 0.39	-504.95	3.82 $\times 10^{-23}$	Down	Up
KBTBD12	0.32 $\pm$ 0.22	4.56 $\pm$ 1.39	-382.68	2.99 $\times 10^{-20}$	Down	Up

Abbreviation: RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.

Statistically significant data were marked with bold and underline.

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	Coding gene expression
	chemo-insensitive group	Chemo-sensitive group				
NRG3	0.82 $\pm$ 0.29	5.66 $\pm$ 4.65	-508.46	5.29 $\times 10^{-20}$	Down	Up
CHRM3	0.10 $\pm$ 0.07	4.89 $\pm$ 2.23	-694.58	6.40 $\times 10^{-20}$	Down	Up
SLC6A11	0.46 $\pm$ 0.30	5.89 $\pm$ 1.57	-436.55	7.33 $\times 10^{-20}$	Down	Up
RIMS1	0.44 $\pm$ 0.26	9.30 $\pm$ 4.07	-424.61	3.06 $\times 10^{-18}$	Down	Up
Abbreviation: RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.						
Statistically significant data were marked with bold and underline.						

Table 6

The top 10 hyper- and hypo- methylated miRNAs expressed differentially in SCLC chemo-insensitive tissues compared with chemo-sensitive tissues

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	miRNA expression level
	chemo-insensitive group	Chemo-sensitive group				
MIR320D1	4.78 $\pm$ 2.16	0.46 $\pm$ 0.38	83.29	2.94 $\times 10^{-10}$	Up	Down
MIR4641	4.03 $\pm$ 2.95	0.36 $\pm$ 0.26	430.54	3.99 $\times 10^{-10}$	Up	Down
MIR133A2	2.14 $\pm$ 1.04	0.33 $\pm$ 0.10	82.71	3.87 $\times 10^{-6}$	Up	Down
MIR6742	21.41 $\pm$ 2.63	4.47 $\pm$ 4.91	26.54	4.63 $\times 10^{-6}$	Up	Down
MIR4666A	3.50 $\pm$ 2.74	0.02 $\pm$ 0.01	292.04	1.26 $\times 10^{-5}$	Up	Down
MIR148A	3.43 $\pm$ 2.31	0.33 $\pm$ 0.34	44.02	1.2 $\times 10^{-4}$	Up	Down
MIR1973	24.79 $\pm$ 1.33	3.00 $\pm$ 3.04	27.28	4.04 $\times 10^{-4}$	Up	Down
MIR1469	7.00 $\pm$ 2.30	2.64 $\pm$ 0.60	3.46	4.76 $\times 10^{-3}$	Up	Down
MIR1267	7.72 $\pm$ 3.99	3.98 $\pm$ 2.70	4.86	5.36 $\times 10^{-3}$	Up	Down

Abbreviation: miRNAs, microRNAs; RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.

Statistically significant data were marked with bold and underline.

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	miRNA expression level
	chemo-insensitive group	Chemo-sensitive group				
MIR1247	8.48 $\pm$ 3.81	2.75 $\pm$ 2.22	4.11	7.74 $\times 10^{-3}$	Up	Down
MIR4478	0.23 $\pm$ 0.10	8.47 $\pm$ 6.37	-837.53	1.74 $\times 10^{-11}$	Down	Up
MIR365B	2.20 $\pm$ 0.21	3.02 $\pm$ 0.62	-2.97	3.13 $\times 10^{-5}$	Down	Up
MIR378C	2.28 $\pm$ 1.00	3.11 $\pm$ 0.28	-4.92	7.87 $\times 10^{-5}$	Down	Up
MIR589	1.66 $\pm$ 0.31	2.46 $\pm$ 0.57	-3.25	2.08 $\times 10^{-4}$	Down	Up
MIR4438	1.72 $\pm$ 0.54	3.09 $\pm$ 0.63	-5.24	4.21 $\times 10^{-4}$	Down	Up
MIR4798	2.33 $\pm$ 0.25	3.89 $\pm$ 1.70	-2.43	1.32 $\times 10^{-3}$	Down	Up
MIR150	2.03 $\pm$ 0.35	3.40 $\pm$ 0.38	-2.93	1.46 $\times 10^{-3}$	Down	Up
MIR8062	2.02 $\pm$ 0.53	5.46 $\pm$ 3.21	-4.00	2.87 $\times 10^{-3}$	Down	Up
MIR34A	2.11 $\pm$ 0.32	3.12 $\pm$ 0.42	-2.81	3.20 $\times 10^{-3}$	Down	Up

Abbreviation: miRNAs, microRNAs; RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.

Statistically significant data were marked with bold and underline.

Gene symbol	RPKM (mean ± SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	miRNA expression level
	chemo-insensitive group	Chemo-sensitive group				
MIR1269A	5.34 ± 1.67	10.57 ± 4.93	-2.45	4.14 × 10 ⁻³	Down	Up
Abbreviation: miRNAs, microRNAs; RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.						
Statistically significant data were marked with bold and underline.						

Table 7

The top 10 hyper- and hypo- methylated lncRNAs expressed differentially in chemo-insensitive tissues compared with chemo-sensitive tissues

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	lncRNA expression level
	chemo-insensitive group	Chemo-sensitive group				
H1FX-AS1	7.29 $\pm$ 2.04	0.41 $\pm$ 0.44	205.07	2.12 $\times 10^{-14}$	Up	Down
LINC00354	3.18 $\pm$ 1.21	0.68 $\pm$ 0.42	63.12	2.18 $\times 10^{-11}$	Up	Down
CATIP-AS1	8.17 $\pm$ 2.21	1.34 $\pm$ 0.23	7.73	1.12 $\times 10^{-8}$	Up	Down
LINC02119	3.69 $\pm$ 1.42	0.40 $\pm$ 0.19	54.57	2.83 $\times 10^{-8}$	Up	Down
LINC01192	3.73 $\pm$ 2.19	1.00 $\pm$ 0.00	49.87	6.09 $\times 10^{-5}$	Up	Down
LINC01018	1.64 $\pm$ 0.93	0.59 $\pm$ 0.23	38.59	8.65 $\times 10^{-5}$	Up	Down
LINC01168	2.67 $\pm$ 1.77	0.11 $\pm$ 0.06	43.41	1.19 $\times 10^{-4}$	Up	Down
RDH10-AS1	2.82 $\pm$ 1.88	0.38 $\pm$ 0.47	41.36	1.79 $\times 10^{-4}$	Up	Down
LINC01194	4.37 $\pm$ 0.85	0.75 $\pm$ 0.41	9.85	5.73 $\times 10^{-4}$	Up	Down

Abbreviation: lncRNAs, long non-coding RNAs; RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.

Statistically significant data were marked with bold and underline.

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	lncRNA expression level
	chemo-insensitive group	Chemo-sensitive group				
LINC00469	4.72 $\pm$ 0.56	1.90 $\pm$ 0.19	3.32	1.23 $\times 10^{-3}$	Up	Down
LINC02298	0.41 $\pm$ 0.31	3.54 $\pm$ 2.65	-484.38	2.58 $\times 10^{-10}$	Down	Up
LINC01435	0.98 $\pm$ 0.31	3.17 $\pm$ 1.25	-28.84	1.89 $\times 10^{-8}$	Down	Up
LINC01264	0.82 $\pm$ 0.09	2.42 $\pm$ 1.26	-125.37	3.18 $\times 10^{-7}$	Down	Up
LINC01968	1.79 $\pm$ 0.14	2.31 $\pm$ 0.15	-3.16	1.70 $\times 10^{-6}$	Down	Up
LINC01121	3.18 $\pm$ 2.20	6.00 $\pm$ 0.59	-6.36	3.42 $\times 10^{-6}$	Down	Up
LINC01484	1.61 $\pm$ 0.19	6.24 $\pm$ 0.92	-7.31	4.17 $\times 10^{-6}$	Down	Up
LINC01565	1.93 $\pm$ 0.20	4.25 $\pm$ 0.81	-3.58	1.30 $\times 10^{-5}$	Down	Up
LINC01006	8.30 $\pm$ 3.93	12.83 $\pm$ 1.28	-3.68	1.77 $\times 10^{-5}$	Down	Up
LINC00461	0.08 $\pm$ 0.05	3.56 $\pm$ 3.81	-442.64	2.59 $\times 10^{-5}$	Down	Up

Abbreviation: lncRNAs, long non-coding RNAs; RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.

Statistically significant data were marked with bold and underline.

Gene symbol	RPKM (mean $\pm$ SD)		Mean fold change (chemo-insensitive/sensitive group)	P	Methylation status	lncRNA expression level
	chemo-insensitive group	Chemo-sensitive group				
LINC01143	1.80 $\pm$ 0.64	2.45 $\pm$ 0.56	-4.82	3.03 $\times 10^{-5}$	Down	Up
Abbreviation: lncRNAs, long non-coding RNAs; RPKM, reads per kilobase per million; SD, standard deviation; SCLC, small cell lung cancer; P, P value for whole.						
Statistically significant data were marked with bold and underline.						

Figures

Figure 1

DMGs profile and methylation enrichment distribution. A) The proportion of coding and non-coding RNAs in all DMGs. Of 4552 DMGs were selected (fold change ≥ 2 , $p < 0.01$), coding genes constituted the largest percentage (85.08%), followed by lncRNAs (10.52%) and miRNAs (3.56%). B) The distribution of differentially hypermethylated genes in the chemo-insensitive and chemo-sensitive group. C) Distribution of reads obtained by MeDIP-seq in the intragenic gene region, and regions 2 kb upstream and downstream 2 kb of the genes. D) Among differentially methylated protein-coding genes in two groups, the proportion of methylation enrichment in the promoter region, the intron region, and the distal intergenic region was around 30%. E-F) The methylation enrichment of all differentially methylated

miRNAs and lncRNAs. The distal intergenic region shared the highest enrichment of methylation in both lncRNAs and miRNAs. Abbreviation: DMGs, differentially methylated genes; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; ncRNAs, non-coding RNAs; TSS, transcription start site; TES, transcription end site; -2 kb, 2 kb region upstream of the TSS; +2kb, 2 kb region downstream of TES.

Figure 2

The heatmap of DMGs in small cell lung cancer. Compared with the group of chemotherapy sensitivity, 1516 coding genes, 205 lncRNAs, and 71 miRNAs were differentially hypomethylated in the chemo-insensitive group. Abbreviation: DMGs, differentially methylated genes; lncRNAs, long non-coding RNAs; miRNAs, microRNAs.

Figure 4

KEGG pathway enrichment analysis of differentially methylated genes between the chemo-insensitive and chemo-sensitive group. Dot size reflects the number of genes enriched in each signaling pathway. Dot color indicates P-value. Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 5

Different expression patterns of genes in SCLC are associated with the levels of methylation. A) The relationship between expression and methylation levels in protein-coding genes. B) The relationship between expression and methylation levels in miRNAs. C) The relationship between expression and methylation levels in lncRNAs. The correlation between methylated status and expression level of DMGs,

Figure 7

LncRNA-miRNA-mRNA interaction network for 10 lncRNAs that hyper- or hypo- methylated in SCLC and showed up- or down- regulated expression. Abbreviation: lncRNAs, long non-coding RNAs; miRNAs, microRNAs; SCLC, small cell lung cancer.

Figure 8

Four therapeutic predictive epigenetic signatures for SCLC based on cancer cell lines drug responses and methylation levels. A) The heatmap displayed the methylation levels of four epigenetic signatures, including hsa-miR-34a, LINC01484, LINC00461, and LINC01018, between SCLC etoposide sensitive and insensitive group (N = 62). B-D) Scatterplots showed the predictive value of four epigenetic signatures for single drug sensitivity, including etoposide, irinotecan, and carboplatin. Positive correlation between the methylation levels of four genes and all three drugs responses were found. The significant predictive value of four epigenetic signatures were found in etoposide therapy ($p=0.0083$). Abbreviation: SCLC, small cell lung cancer; r, Pearson's correlation coefficients; p, p value for whole.

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