Abnormal genome-wide DNA methylation induced by cisplatin may contribute to the chemo-resistance of human small cell lung cancer

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Abstract

Background: So far, the platinum-based chemotherapy (e.g. cisplatin-etoposide doublet) is still the backbone for SCLC management due to its high respond rate both in LS-SCLC and ES-SCLC. However, cisplatin treatment often results in the development of chemo-resistance, leading to therapeutic failure and becoming the main obstacle to improve the therapeutic efficacy. Currently, little has been known about the genome-wide abnormal methylation of SCLC induced by cisplatin, which might provide prospective layouts to discover the potential genes and the signal pathways related with chemo-resistance of SCLC. Results: A total of 58,401 sites was identified to be differentially methylated ($|\Delta \beta| \geq 0.20$) in H446/DDP cells compared with that of H446 cells, of which 25,991 genes were found to be hypomethylated and 32,410 genes were shown to be hypermethylated. KEGG enrichment displayed that the differentially hypomethylated genes were mainly gathered in MAPK signaling pathway, ECM-receptor interaction, and Focal adhesion, while the differentially hypermethylated genes were clustered in Neuroactive ligand-receptor interaction, Type I diabetes mellitus, Focal adhesion, Allograft rejection, ECM-receptor interaction, CAMs, Graft-versus-host disease, Intestinal immune network for IgA production, ARVC, and Viral myocarditis (KEGG enrichment, qvalue < 0.05). Among the 152 genes which were selected as the MDR-related candidate genes for qRT-PCR to testify whether the abnormal methylation regulated the expression of related genes at the mRNA level, 69 hypomethylated genes were revealed to be significantly increased and the other 54 hypermethylated genes evidently decreased in H446/DDP cells compared with that of H446 cells. Moreover, the upregulated genes with the hypomethylated sites were found to be mainly
clustered in Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, and Cell adhesion molecules (CAMs), while the downregulated genes with the hypermethylated sites were mainly clustered in MAPK signaling pathway, Pathways in cancer, Melanoma, Osteoclast differentiation, and Prostate cancer. Conclusions: Cisplatin could induce a large-scale abnormal methylation in the whole genome of SCLC. Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, and Cell adhesion molecules (CAMs) were most likely to be affected by cisplatin via methylation to contribute to the development of chemo-resistance and other malignant biological behavior of SCLC cells.

Background

Global Cancer Statistics 2018 demonstrates that lung cancer remains the most commonly diagnosed cancer with a proportion of 11.6% in the total cases and the leading cause of cancer death with 18.4% of the total cancer deaths in both sexes combined worldwide [1]. The morbidity rate will rise swiftly to reach as many as one million in 2025 if no effective measures were taken [2-4]. Globally, non-small cell lung cancer (NSCLC) comprises approximately 80-85% of all lung cancers with adenocarcinoma and squamous cell carcinoma comprising the predominant histological subtypes of NSCLC and small cell lung cancer (SCLC, also known as oat-cell carcinoma) derived from bronchial epithelial cells accounts for 13-15% of all diagnosed lung cancers [5]. Compared with the major subtypes of NSCLC, the prognosis of SCLC is extremely poor in that SCLC is an aggressive high-grade neuroendocrine tumor associated with a rapid doubling time and a high growth fraction combined with the early development of widespread metastases (most commonly to the brain, liver, or bone) resulting in a 95% mortality rate, which
makes SCLC the most lethal lung cancer subtype [6]. Additionally, SCLC has the strongest association with smoking, with only 2% of cases occurring in never-smokers [7], leading to a high load of somatic mutations induced by tobacco carcinogens [8,9].

SCLCs were classified into limited stage (LS) and extensive stage (ES) according to the Veterans Administration Lung Study Group (VALG) staging system. Current standard of treatment is concurrent chemoradiation for LS-SCLC and chemotherapy alone for ES-SCLC. In recent years, advances in the understanding of the high mutational burden of SCLC and SCLC biology have provided opportunities for therapeutic intervention and led to the development of novel experimental therapies including targeted agents and immunotherapies [10]. For instance, Poly (ADP-ribose) polymerase (PARP) inhibitor talazoparib are under clinical investigation in combination with cytotoxic therapies and inhibitors of cell-cycle checkpoints. The reported Objective Response Rate (ORR) was 9% and the clinical benefit rate at ≥16 weeks was 26% [11]. Targeting of histone-lysine N-methyltransferase enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) was found to maintain the sensitivity of SCLC xenografts to chemotherapy by preventing schlafen family member 11 (SLFN11) silencing [12]. High expression of the inhibitory Notch ligand Delta-like protein 3 (DLL3) in most SCLCs encouraged an anti-DLL3-antibody-drug conjugate for preclinical and clinical activity [13]. Additionally, though distinct from that of other solid tumors, few tumor-infiltrating lymphocytes and low levels of the immune-checkpoint protein programmed cell death 1 ligand 1 (PD-L1) were detected in SCLC, a number of clinical trials of this promising immunotherapeutic approaches, such as targeting the inhibitory immune-checkpoint proteins cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [14] and PD-1 or its ligand PD-L1
[15,16], are underway. However, generally, in contrast to the rapidly changing status of NSCLC, which has notched success after success with a spate of targeted agents and immunotherapies, SCLC has been notorious for its lack of progress as drug after drug. In fact, inhibitors of VEGF, IGFR, mTOR, EGFR, and HGF has failed and fallen by the wayside due to little or no impact on progression-free survival (PFS) or overall survival (OS) [10,17]. The median OS duration of patients with ES-SCLC is stalled, frustratingly, at < 10 months, with a discouraging 5-year OS of 1-5% [18]. The platinum-based chemotherapy (e.g. cisplatin-etoposide doublet) is still the backbone for SCLC management due to its high respond rate both in LS-SCLC and ES-SCLC [19].

However, the platinum-based chemotherapy is a double-edged sword for SCLC. Without treatment, ES-SCLC is rapidly and invariably fatal within 2 to 4 months [20]. With care of chemotherapy, responses are dramatic (approximate 90% cases are responsive to chemotherapy primitively) but sadly short-lived: SCLC inevitably relapses and the disease recurrence characterized by drug resistance is associated with a median OS often < 6 months [21,22] (the median OS for SCLC patients in the third line setting is 4.7 months [23], a survival rate which has scarcely improved over the last 40 years). Compared to the primary disease, the recurrent SCLC is more aggressive with less response to therapy (e.g. topotecan, a topoisomerase I inhibitor) [24]. So far, no effective treatment regimens have been developed for patients whose disease has progressed after first- and second-line therapy.

Cisplatin, a platinum-derivative agent, exerts anticancer effects via multiple mechanisms, of which the most prominent mode of action involves the generation of DNA lesions followed by the activation of the DNA damage response and the induction of mitochondrial apoptosis [25]. Despite a consistent rate of initial
responses, cisplatin treatment often results in the development of chemo
tolerance, leading to therapeutic failure and becoming the main obstacle to
improve the therapeutic efficacy. Over the past three decades, an intense research
has been conducted and several mechanisms that account for the cisplatin-resistant
phenotype of tumor cells were explored and classified as pre-target, on-target, post-
target, and off-target resistance [26]. The known mechanisms explain the cisplatin
resistance at the molecular level to a certain extent, however, regretfully, the
therapeutic regimens developed from these reported mechanisms have failed to
achieve improved outcomes in SCLC patients [27,28]. Therefore, to explore the
other potential chemo-resistant mechanisms of SCLC is of great importance to
discover novel chemotherapy agents and improve the efficacy of chemotherapy
treatment.

The previous findings that DNA methylation is far more vulnerable than DNA
sequence to external factors gave us clue that the epigenetic modification might
play a pivotal role in the development of the acquired chemo-resistance of SCLC
[29,30]. Actually, DNA methylation status changes have been reported to be the
propelling factor in the acquired multidrug resistance (MDR) in glioma cell line SGH-
44/ADM [31], chronic myeloid leukemia cells [32], human epithelial ovarian cancer
cells [33], and NSCLC [34,35]. Additionally, histone deacetylation of ATP binding
cassette subfamily B member 1 (ABCB1) promoter was found to be a potential
routine for MDR induction in SCLC [36]. Currently, little has been known about the
genome-wide methylation frameworks of the chemo-resistant cells of SCLC, which
might provide prospective layouts to discover the potential genes and the signal
pathways related with chemo-resistance of SCLC. Thus, this research reported for
the first time the genome-wide abnormal methylation pattern of chemo-resistant
H446/DDP cells of human SCLC induced by the cisplatin. The analysis revealed that Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, and Cell adhesion molecules (CAMs) might most likely be regulated by methylation induced by cisplatin to contribute to the development of chemo-resistance and other malignant biological behavior of SCLC cells. Targeting these pathways might provide new treatment options and strategies to improve therapeutic effect to extend survival of patients with SCLC.

Methods

Cell lines and culture

The human SCLC cell line H446 was purchased from the Institute of Biochemistry and Biology, Chinese Academy of Sciences (Shanghai, China). The cisplatin resistant H446/DDP cells was established as previously described [37]. In brief, the progenitor H446 cells were treated with first shock of high-dose cisplatin and then maintaining in lower dose cisplatin. The induced cells were verified to be cross-resistant to hydroxycamptothecin, vincristine, and 5-fluorouracil. Both H446 and H446/DDP cells were cultured in RPMI-1640 medium (Hyclone) containing 10% FBS and 1% streptomycin/penicillin at 37°C in 5% CO₂. Cells were passaged using 0.25% trypsin with 0.1% EDTA (Hyclone) when attaining to 90% confluence or harvested in logarithmic phase of growth for all experiments described below.

Sensitivity to chemotherapeutic agents in vitro

To validate the chemo-resistance of H446/DDP cells to chemotherapeutic drugs with different pharmacological mechanism, the 50% inhibitory concentration (IC₅₀) to cisplatin, etoposide, gemcitabine, paclitaxel, docetaxel, and pemetrexed were compared between H446/DDP and H446 cells. Briefly, different concentration of
cisplatin (0, 0.625, 1.25, 2.50, 5.00, and 10.00 μg/ml), etoposide (0, 15.00, 30.00, 60.00, 120.00, and 240.00 μg/ml), gemcitabine (0, 3.29, 6.58, 13.16, 26.32, and 52.64 μg/ml), paclitaxel (0, 7.50, 15.00, 30.00, 60.00, and 120.00 μg/ml), docetaxel (0, 5.00, 10.00, 20.00, 40.00, and 80.00 μg/ml), and pemetrexed (0, 3.00, 6.00, 12.00 24.00, and 48.00 μg/ml) was added respectively into 96-well plates seeded with 5×10³ cells and treated for 48 hrs. Then the cells of each well were incubated with 100 μl fresh culture medium containing 10 μl CCK-8 solution for 1.5 hrs (Cat: C0038, Beyotime, Shanghai, China). The absorbance value at the wavelength of 450 nm was measured. Cells incubated without chemotherapeutic agents were treated as negative controls. IC₅₀ was calculated using GraphPad Prism 5.0 software.

**Detection of MMP, ATP, and ROS**

To further validate the chemo-resistance of H446/DDP cells to cisplatin, the mitochondrial membrane potential (MMP), the intracellular ATP levels, and levels of intracellular reactive oxygen species (ROS) were analyzed between H446/DDP and H446 cells after treated with cisplatin. To detect MMP, cells were plated in 6-well plates and allowed to adhere overnight before treatment with cisplatin at 0, 0.5, 1.0, and 2.0 μg/ml, respectively. After treated by cisplatin for 24 hrs, the cells was incubated with 5 μmol/L fluorescent dye JC-1 dimly for 30 min at 37°C (Cat: C2006, Beyotime, Shanghai, China), washed with PBS to remove the excess dye, and then observed using fluorescence microscopy (Olympus BX51, Japan).

To assay the intracellular ATP level, after seeded into 96-well plates at the density of 5×10³ cells/well for 24 hrs, the cells were treated with cisplatin (0, 0.5, 1.0, and 2.0 μg/ml, respectively) for 48 hrs. Then the intracellular ATP levels were detected using a commercial ATP assay kit in accordance with the manual (Cat: S0026,
Beyotime, Shanghai, China). In brief, the assay buffer was gently mixed with the substrate at room temperature. The mixed reagent (100 μl) was added into each well and incubated with shaking for 15 min at room temperature. Then the luminescence was measured using a microplate reader (Beckman Coulter SP-Max2300A2).

Levels of intracellular ROS were detected with an oxidation sensitive fluorescent dye DCFH-DA (Cat: S0033, Beyotime, Shanghai, China). In brief, 5×10^5 cells were plated in 12-well plates and allowed to adhere overnight before incubation with different concentrations of cisplatin (0, 1.0, and 2.0 μg/ml, respectively) for 24 hrs. Cells were washed twice with ice-cold PBS to remove medium. Serum free medium of 1 ml with 1 μl DCFH-DA (10 mM) was added to each tube and incubated at 37℃ for 20 min. Subsequently, the DCF fluorescence picture was captured every five other minutes by fluorescence microscopy (Olympus BX51, Japan).

**Illumina Infinium Human Methylation 450K bead chip and data analysis**

DNA was isolated from H446 and H446/DDP cells with DNeasy Cell and Tissue Kit (Qiagen) in accordance with the standard protocols by Shanghai Sinomics Corporation (Shanghai, China), and subsequently, the estimation of sample purity and concentration was conducted with Nanodrop 2000 (ThermoScientific). According to the manufacturer’s standard protocol, as much as 500 ng of genomic DNA from each sample was used for sodium bisulfite conversion with the EZ DNA methylation Gold Kit (Zymo Research, USA). Genome-wide DNA methylation was assessed using the Illumina Infinium Human Methylation 450K BeadChip (Illumina Inc, USA) following the manufacturer’s instructions. Methylation level was obtained by analyzing the array data (.IDAT files) with ChAMP package in R. The methylation status of all the probes was signified as β value, i.e. the ratio of the methylated
probe intensity to the overall probe intensity (sum of methylated and unmethylated
probe intensities plus constant α, where α = 100). CpG sites with |Δβ| ≥ 0.20 (in
H446/DDP vs H446) and adjusted p value ≤ 0.05 were considered as differentially
methylated sites. A CpG was considered hypermethylated if Δβ ≥ 0.20 or
hypomethylated if Δβ ≤ -0.20. Average β value of promoters and CpG islands (CGIs)
were compared between H446/DDP and H446 cells. Promoters and CGIs with |Δβ| ≥
0.20 and adjusted P value ≤ 0.05 were considered for further analysis. String 10.5
and Cytoscape 3.3.0 were applied to predict the relationship among the genes with
differentially methylated sites (i.e. promoters and CGIs) and construct the
interaction network, respectively.

Gene Expression determined by Quantitative real-time PCR (qRT-PCR)

Total RNA of the cells was isolated using TRIzol in compliance with the
manufacturer’s recommendation (Invitrogen, Carlsbad, CA, USA). According to the
manual instruction, after the RNA concentration was estimated using the Nanodrop
2000 (ThermoScientific), two milligrams aliquots were reverse transcribed using
PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Cat: RR047B,
Takara, Dalian, China). Then, the SYBR green-based qRT-PCR was then performed in
triplicate using an ABI Step-One-Plus Real-Time quantitative PCR Systems (Applied
Biosystems, Foster, CA, USA) and the level of gene-expression was normalized by
glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR cycling conditions
consisted of 5 min at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C, 30
s of annealing at 55°C and 30 s of extension at 72°C. The relative expression values
were computed by the △△Ct method. The primers for qRT-PCR were list in Table S1.

Statistical Analysis

The statistical differences of between-groups were estimated by two-tailed
Student’s t-test. All statistical analyses were performed using the Statistical Package for Social Science 15 for Windows (SPSS Inc., Chicago, IL, USA). A statistical difference was accepted as significant if $p < 0.05$. Each experiment was repeated at least three times.

Results

**H446/DDP cells exhibited MDR phenotype relative to H446 cells**

To validate the resistance of H446/DDP cells to chemotherapeutic drugs, $IC_{50}$ (to cisplatin, etoposide, gemcitabine, paclitaxel, docetaxel, and pemetrexed, respectively), ROS, MMP, and ATP production were compared between H446/DDP and H446 cells. As shown in Fig.1A, $IC_{50}$ of H446/DDP cells to cisplatin (3.21 vs. 0.998, $p = 0.0210$), etoposide (125.10 vs. 28.99, $p = 0.0001$), gemcitabine (33.08 vs. 17.37, $p = 0.0048$), paclitaxel (63.92 vs. 37.30, $p = 0.0002$), docetaxel (18.33 vs. 12.46, $p = 0.0109$), and pemetrexed (20.03 vs. 12.12, $p = 0.0110$) was significantly higher than that of H446 cells. Additionally, both MMP (Fig.1B) and ATP concentration (Fig.1C) were strikingly higher while ROS (Fig.1D) were absolutely lower in H446/DDP cells compared with that of H446 cells after treatment with cisplatin, strongly demonstrating that H446/DDP cells exhibited MDR phenotype relative to H446 cells.

**Genome-wide methylation patterns of H446/DDP cells relative to H446 cells**

The comparison of the genome-wide methylation data between H446/DDP and H446 cells revealed that a total of 58,401 sites was identified to be differentially methylated ($|\Delta \beta| \geq 0.20$) in H446/DDP cells compared with that of H446 cells, of which 25,991 genes were found to be hypomethylated and 32,410 genes were shown to be hypermethylated, indicating that cisplatin could induce a large-scale
abnormal methylation in the whole genome of H446/DDP cells. As shown in Fig.2A, most of the methylated sites were distributed in the intervals with lower $|\Delta\beta|$, especially $|\Delta\beta| \leq 0.4$. The less methylated sites were detected with the $|\Delta\beta|$ increased. There is no distribution bias of the hypomethylated and hypermethylated sites in each intervals stratified by the different $|\Delta\beta|$ (Fig.2A). To uncover whether the genes with the differentially methylated sites were preferent or clustered on certain chromosomes, the differentially methylated genes were localized on each chromosome of H446/DDP and H446 cells. The localization displayed that there was no distribution preference or cluster of the hypomethylated or hypermethylated genes on each chromosome (Fig.2B), displaying that the methylated sites distributed evenly across the genome.

**GO and KEGG enrichment analysis**

To investigate whether the genes with the differentially methylated sites were clustered functionally, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of these genes with $|\Delta\beta| \geq 0.4$ were analyzed according to the latest GO (http://geneontology.org/) or KEGG database (http://www.genome.jp/kegg/), which disclosed that the differentially methylated genes were involved in many important biological functions. When enrich factor $\geq 5.0$ was utilized as cut-point for GO enrichment, the genes with hypomethylated sites were found to be mainly clustered in collagen type IV, glossopharyngeal nerve development, trigeminal nerve structural organization, regulation of prostatic bud formation, regulation of extracellular matrix assembly, wound healing involved in inflammatory response, cerebellar granule cell differentiation, epithelial cell fate commitment, metanephric S-shaped body morphogenesis, and positive regulation of smooth muscle cell differentiation (top 10 of GO enrichment, Fig.2C and Table 1),
while the hypermethylated genes were enriched in interleukin-1 receptor activity, flavone metabolic process, structural molecule activity conferring elasticity, detection of mechanical stimulus involved in sensory perception of pain, cell body fiber, regulation of osteoclast development, negative regulation of synaptic transmission, glutamatergic, and G-protein coupled glutamate receptor signaling pathway (Fig.2D and Table 2). Glomerulus morphogenesis was the only one common term of GO enrichment between the hypomethylated genes and the hypermethylated genes (Fig.2C, 2D, Table 1, and Table 2).

When enrich factor $\geq 2.0$ was utilized as cut-point for KEGG enrichment analysis, the hypomethylated genes were revealed to be enriched in Glycosaminoglycan biosynthesis, African trypanosomiasis, Maturity onset diabetes of the young, Dorsal-ventral axis formation, Biosynthesis of unsaturated fatty acids, Hedgehog signaling pathway, Basal cell carcinoma, and Hypertrophic cardiomyopathy (HCM) (Fig.2E and Table 3), while the hypermethylated genes were enriched in Fatty acid biosynthesis, Allograft rejection, Type I diabetes mellitus, Graft-versus-host disease, Mucin type O-Glycan biosynthesis, Asthma, Intestinal immune network for IgA production, Pantothenate and CoA biosynthesis, Thyroid cancer, Autoimmune thyroid disease, Viral myocarditis, Steroid hormone biosynthesis, Complement and coagulation cascades, Staphylococcus aureus infection, and CAMs (Fig.2F and Table 4).

Aldosterone-regulated sodium reabsorption, Amoebiasis, Arrhythmogenic right ventricular cardiomyopathy (ARVC), ECM-receptor interaction, Focal adhesion, Glycosaminoglycan biosynthesis, Hypertrophic cardiomyopathy (HCM), and Sulfur relay system were the common terms of KEGG enrichment between the hypomethylated genes and the hypermethylated genes. The GO and KEGG enrichment indicated that many important signal pathways were affected by
cisplatin, causing dramatically changes in morphology, structure, function, physiology, and others.

To identify which signal pathway was significantly influenced by cisplatin through methylation, qvalue < 0.05 was used as the cut-point for KEGG enrichment. As shown in Table 5, the differentially hypomethylated genes was mainly clustered in ECM-receptor interaction, MAPK signaling pathway, and Focal adhesion. Differently, the differentially hypermethylated genes was clustered in Neuroactive ligand-receptor interaction, Type I diabetes mellitus, Focal adhesion, Allograft rejection, ECM-receptor interaction, CAMs, Graft-versus-host disease, Intestinal immune network for IgA production, Arrhythmogenic right ventricular cardiomyopathy (ARVC), and Viral myocarditis (Table 6). ECM-receptor interaction and Focal adhesion were the two common KEGG enrichment terms between the hypomethylated genes and the hypermethylated genes with qvalue < 0.05.

**Network diagram of the differentially methylated genes**

The relationship of each gene with the other genes with the differential methylation was analyzed by constructing the network using String 10.5 and Cytoscape 3.3.0. The purple and red circle represented the hypomethylated and hypermethylated genes, respectively (Fig.3 and Fig.4). The deeper color displayed the greater difference. The line represented the relationship between genes. Degree indicated the number of genes associated with the other genes. For example, degree = 10 represents that the gene interacts with the other 10 genes. The larger the degree, the more genes that interact with it. As shown in Fig.5A, among the hypomethylated genes, *POTEF, ESR1, RAC2, PRKCA, NOTCH1, ARPM1, EFCAB3, BCL2, CACNA1C*, and *HDAC4* were revealed to be associated with more than the other 10 genes. In contrast, of the hypermethylated genes, *HACE1, LRGUK, FYN, ACACB, AR, PIK3CG,*
ACTBL2, SMAD3, ERBB4, and RUNX1 were revealed to be associated with more than the other 10 genes (Fig.5B).

**Expression of 152 genes with the differential methylated sites by qRT-PCR**

To testify whether the gene expression was regulated by the methylation at the mRNA level, 152 genes were selected for qRT-PCR based on the beta difference (|Δβ| ≥ 0.7) and the numbers of the methylation sites on each gene. Among the 152 genes, 69 hypomethylated genes significantly upregulated and the other 54 hypermethylated genes evidently downregulated in H446/DDP cells compared with that of H446 cells (Fig.6). It is worth noting that three hypomethylated genes (ESR1, RAC2, and ABCB1) downregulated in H446/DDP cells relative to that of H446 cells (all p < 0.05). The loci of the differentially methylated sites on the three genes were list in Table 7. No significant differences of the other 26 genes including 16 hypomethylated genes (LHX9, IL1A, KRTAP8-1, FBLN7, CXCL1, CSMD1, CRABP2, CCDC11, WNT10B, BMP8B, BMPR1B, NCAM2, NGFR, MAPT, TNFSF12, and SDC3) and 10 hypermethylated genes (TSPAN5, FAR2, STYXL1, ZNF518B, CST11, EHF, TBXAS1, HLA-DPB1, HLA-DPA1, and FYN) were observed between H446/DDP and H446 cells (data not shown).

Among the 69 upregulated genes, foldchanges (FCs) of 19 hypomethylated genes were greater than or equal to 5.0 (CNTN1, COL4A6, CXCL3, DAPK1, FGFR1, FGFR2, FLNC, IL1B, IL20RB, IL7, KIT, KLHDC1, KRTAP24-1, LYPD1, MAPRE2, RARB, RASSF5, RGS6, and TOX3). The upregulated genes with the hypomethylated sites were mainly clustered in Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, and CAMs (Table 8). Among the 54 downregulated genes, FCs of 14 hypermethylated genes were greater than or equal to 5.0 (CACNA2D1, CACNG6, CSF1, EGF, GLI1, HLA-DQA1, HLA-DQB1, HLA-DRB1, LIF, MMP2, PDGFD,
PECAM1, EDAR, and RBM47). The downregulated genes with the hypermethylated sites were mainly clustered in MAPK signaling pathway, Pathways in cancer, Melanoma, Osteoclast differentiation, Prostate cancer, and Glioma (Table 8). Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, CAMs, Focal adhesion, and Regulation of actin cytoskeleton were the six common pathways affected by the upregulated genes with the hypomethylated sites and the downregulated genes with the hypermethylated sites.

Discussion

Currently, little has been known about the genome-wide methylation frameworks of the chemo-resistant cells of SCLC, which might provide prospective layouts to discover the potential genes and the signal pathways related with chemo-resistance of SCLC. Hence, we compared the genome-wide methylation profiles between chemo-resistant H446/DDP cells of human SCLC with its progenitor H446 cells in the study. The comparison displayed that a total of 58,401 sites (i.e. promoters and CGIs) was identified to be differentially methylated in H446/DDP cells compared with that of H446 cells, of which 25,991 genes were found to be hypomethylated and 32,410 genes were shown to be hypermethylated and there was no distribution preference or cluster of the hypomethylated or hypermethylated genes on each chromosome, strongly suggesting that cisplatin could cause a genome-wide DNA methylation and the abnormal DNA methylation, one of the most frequent epigenetic alteration, might contribute to the development of chemo-resistance of SCLC.

GO and KEGG enrichment of the differentially methylated genes ($|\Delta \beta| \geq 0.40$) revealed that many important biological process, cellular components, molecular
function of cells, and signal pathways were affected by the epigenetic alteration (Table 1-4, Fig.2), displaying that chemo-resistance phenotype of SCLC was determined by a very complicated cellular and molecular network. Once the tumor cells developed a chemo-resistant phenotype, the morphology, components, metabolism, and biological process of cells altered accordingly. Notably, among the signal pathways enriched on the basis of enrich factor ≥ 2.0 for KEGG enrichment, Aldosterone-regulated sodium reabsorption, Arrhythmogenic right ventricular cardiomyopathy (ARVC), and ECM-receptor interaction were disclosed to be the common KEGG enrichment terms between the hypomethylated genes and the hypermethylated genes (Fig.2E, 2F, Table 3, and Table 4). Aldosterone, a steroid hormone, regulates renal Na\(^+\) reabsorption and, therefore plays an important role in the maintenance of salt and water balance [38]. Arrhythmic right ventricular cardiomyopathy (ARVC), also known as arrhythmic right ventricular dysplasia, is an inherited disease characterized by progressive replacement of the myocardium by adipose and fibrous tissue that predisposes to development of ventricular tachycardia (VT) and to sudden cardiac death (SCD) [39]. The abnormal methylation of genes related with Aldosterone-regulated sodium reabsorption and ARVC induced by cisplatin might explain some of the clinical manifestations that the cancer patients often suffer the edema (excess water accumulated in the body), palpitation and shortness of breath (cardiac dysfunction) after treated with cisplatin. Extracellular matrix (ECM) is a three-dimensional, non-cellular structure that constitutes a complex network to regulate the occurrence of tissue, support and connect tissue, and the physiological activities of cells, especially the abscission, adhesion, degradation, migration, and proliferation, the whole process of erosion and metastasis of malignant tumors [40]. The findings that ECM-receptor interaction
was the common KEGG enrichment terms between the hypomethylated genes and
the hypermethylated genes suggested that the abnormally methylated genes
related with ECM-receptor interaction might contribute to the development of
cisplatin-resistance of SCLC.

Additionally, Glycosaminoglycan (GAGs) biosynthesis presented the highest
enrichment factor among the KEGG enrichment terms of the hypomethylated genes
(Fig.2E and Table 3). GAGs are charged, unbranched polysaccharides consisting of
repeating disaccharide units and play roles in various biological events, including
cell growth, cytokinesis, and differentiation via. binding to and coordinating the
activity of proteins involved in cell attachment, migration and differentiation,
neuronal plasticity, blood coagulation, lipid metabolism, and pathogen infectivity. In
addition, GAGs carry out mechanical and rheological functions in synovial tissues
and fluid [41]. Appreciably different from the hypomethylated genes, Fatty acid
biosynthesis presented the highest enrichment factor among the KEGG enrichment
terms of the hypermethylated genes (Fig.2F and Table 4). Fatty acids (FAs), a
diverse class of molecules consisting of hydrocarbon chains of different lengths and
degrees of desaturation, are used to synthesize many lipids, which are used in
energy metabolism and storage and have important roles as signaling molecules.
FAs form the hydrophobic tails of phospholipids and glycolipids, which, together
with cholesterol, represent major components of biological membranes.
Additionally, FAs are assembled into triacylglycerides (TAGs), nonpolar lipids that
are synthesized and stored during high nutrient availability and that release ample
energy when broken down [42]. Tumors have a high rate of glucose uptake and
perform glucose fermentation independently of oxygen availability. Tumor cells
generate almost all their cellular FAs through de novo synthesis which almost
accounted for more than 93% of the FAs biosynthesis and fatty acid synthase (FASN) was identified as the tumor antigen OA-519 in aggressive breast cancer [43,44]. Since then, numerous studies have confirmed the importance of FA biosynthesis for cancer cell growth and survival [42,45]. The GAGs with the highest enrichment factor among the KEGG enrichment terms of the hypomethylated genes and Fatty acid biosynthesis with the highest enrichment factor among the KEGG enrichment terms of the hypermethylated genes indicated that both GAGs and Fatty acid biosynthesis were regulated by the abnormal DNA methylation induced by cisplatin. How GAGS and Fatty acid biosynthesis were involved in the chemo resistance of SCLC is worth exploring.

To further identify which signal pathway was dominantly influenced by cisplatin through methylation, qvalue < 0.05 was used as the cut-point for KEGG enrichment. Among the enriched signal pathways of genes with the hypomethylated loci, MAPK signaling pathway, ECM-receptor interaction, and Focal adhesion were highlighted with qvalue < 0.05 (Table 5), showing that the three pathways were most likely to be affected by DNA hypomethylation induced by cisplatin, which were confirmed by qRT-PCR analysis (Fig.5). The findings gave us clue that the three signal pathway might play vital roles in the chemo-resistance of SCLC. In contrast, Neuroactive ligand-receptor interaction, Focal adhesion, Type I diabetes mellitus, Allograft rejection, ECM-receptor interaction, Cell adhesion molecules (CAMs), Graft-versus-host disease, Intestinal immune network for IgA production, Arrhythmogenic right ventricular cardiomyopathy (ARVC), and Viral myocarditis were dominant with qvalue < 0.05 among the enriched signal pathways of genes with the hypermethylated loci (Table 6). It's worth noting that Neuroactive ligand-receptor interaction was highlighted with the most counts of hypermethylated genes (54/614)
and the smallest q-value among the enriched signal pathways of genes with hypermethylated sites (q-value = 0.004), indicating that Neuroactive ligand-receptor interaction might be inhibited in the chemo-resistant cells, which might interpret the clinical phenomenon that the cancer patients often suffer the headache, numbness and pain of hands and feet (peripheral nerve disorder), and neurology and depression, after treated with cisplatin.

Moreover, the genes with the hypomethylated sites and the genes with the hypermethylated sites formed two complicated networks (Fig.3 and Fig.4). POTE ankyrin domain family member F (POTEF) and HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1) were relatively located at the center of the network constructed by the hypomethylated genes and the hypermethylated genes, respectively (Fig.3 and Fig.4). POTEF belongs to the POTE membrane protein family, which is primate specific and includes 13 paralogs dispersed among eight chromosomes. The POTE proteins were considered to be cancer-testis antigens because they were expressed in many cancers but restricted to only a few normal tissues in the reproductive system [46,47]. Recently, POTEF was found among the top driver oncogenic genes of breast cancer, with a mutation prevalence of over 5% [48]. Additionally, POTEF was identified as a binding partner of Ricinus communis agglutinin I, which may play a critical role in triple-negative breast cancer metastasis [49]. Moreover, POTEF-AS1 was revealed to promote cell growth, repress genes related to the Toll-like receptor signaling and apoptosis pathways, and inhibited apoptosis in docetaxel-treated LNCaP cells, suggesting that POTEF-AS1 would play a key role in the progression of prostate cancer by repressing Toll-like receptor signaling [50]. HACE1 belongs to the HECT family of ubiquitin ligases (HECT E3), which have intrinsic catalytic activity and specificity for
substrates involved in the regulation of growth and apoptosis [51]. HACE1 was identified as a tumor suppressor gene involved in the spontaneous tumorigenesis of several cancers in vivo, including lymphoma [52]. The downregulated HACE1 was found to be associated with neuroblastoma progression and poor patient OS [53]. Furthermore, HACE1 is downregulated in Wilm’s tumor patients, and the alteration is mediated through hypermethylation of the cytosine phosphate guanine (CpG) island 177 (CpG-177), which is located upstream of the transcription startsite (TSS) [51]. Hypermethylation of CpG-177 in the HACE1 promoter is frequently observed in colorectal and gastric carcinomas, and hypermethylation of HACE1 is associated with the severity of clinic pathological findings, especially lymph node metastasis, in colorectal carcinomas [54-56]. Thus, HACE1 was demonstrated to be a tumor suppressor gene in natural killer cell malignancies and to be down-regulated through a combination of deletion and cyto-sine phosphate guanine island hypermethylation [57]. Therefor, the prior reports on POTEF and HACE1 indicated that the two abnormally methylated genes might be involved in the chemo-resistance of SCLC. The underlying mechanisms are worthy investigating.

The verification of relationship between gene expression and methylation by qRT-PCR revealed that the expression of 69 hypomethylated genes significantly increased and the other 54 hypermethylated genes evidently decreased in H446/DDP cells compared with that of H446 cells, providing evidences that the expression of the most of genes were regulated by DNA methylation. Interestingly, the upregulated genes with the hypomethylated sites were found to be mainly clustered in Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, and Cell adhesion molecules (CAMs), while the downregulated genes with the hypermethylated sites were mainly clustered in MAPK signaling
Pathway, Pathways in cancer, Melanoma, Osteoclast differentiation, and Prostate cancer, suggesting that these pathways were most likely to be affected by cisplatin via methylation. Additionally, Pathways in cancer was the commonly enriched term between the upregulated genes and the downregulated genes, strongly hinting that cisplatin might cause the remodeling of cancer-related genes, which might enhance the malignant biological behavior of chemo-resistant cells of human SCLC.

Conclusions

This research reported for the first time the comprehensive genome-wide abnormal methylation profile of chemo-resistant cells of human SCLC induced by the cisplatin. The abnormal methylation of genes related with Aldosterone-regulated sodium reabsorption, ARVC, and Neuroactive ligand-receptor interaction induced by cisplatin might explain the clinical phenomenon that the cancer patients often suffer the edema (excess water accumulated in the body), palpitation and shortness of breath (cardiac dysfunction), headache, numbness and pain of hands and feet (peripheral nerve disorder), and neurology and depression after treated with cisplatin, to a certain extent. Additionally, Pathways in cancer, MAPK signaling pathway, Cytokine-cytokine receptor interaction, and Cell adhesion molecules (CAMs) could be regulated by cisplatin via hypomethylation to contribute to the development of chemo-resistance and other malignant biological behavior of SCLC cells. Targeting the related pathways might provide new treatment options and strategies to improve therapeutic effect to extend survival of patients with SCLC. Future researches will focus on the function exploration of the related pathways and genes in the development of chemo-resistance and other malignant biological behavior of SCLC cells.
Abbreviations

NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; LS: limited stage; ES: extensive stage; VALG: Veterans Administration Lung Study Group; PARP: Poly (ADP-ribose) polymerase; SLFN11: schlafen family member 11; EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit; DLL: delta like canonical Notch ligand; PD-L1: Programmed cell death 1 ligand 1; PD-1: Programmed cell death 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; PFS: progression-free survival; OS: overall survival; MDR: multidrug resistance; ABCB1: ATP binding cassette subfamily B member 1; MAPK: mitogen-activated protein kinase; CAMs: cell adhesion molecules; ECM: extracellular matrix; IC₅₀: 50% inhibitory concentration; MMP: mitochondrial membrane potential; ROS: reactive oxygen species; CGIs: CpG islands; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; ARVC: Arrhythmogenic right ventricular cardiomyopathy; HCM: Hypertrophic cardiomyopathy.

Declarations

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Availability of data and materials

The original data from Illumina Infinium Human Methylation 450K bead chip were available in NCBI (GEO accession number: GSE140743). The data sets supporting
the results of this study are included in the manuscript and its additional files.

**Authors’ contributions**

FYJ, SLY, and XHL conceived and designed the experiments. YW, SLY, JL, and RLG performed the data analysis and performed the qRT-PCR experiments. LYS, CWJ, RJZ, RLG, and YC induced the MDR H446/DDP cells and maintained the MDR cells and its progenitor cells. YW and SLY wrote the manuscript. FYJ revised the manuscript. All authors reviewed and approved the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Tables
Table 1 GO enrichment of the hypomethylated genes with enrich factor $\geq 5.0$
| GO_ID     | Description                                           | Type                  | GeneRatio | BgRatio | pvalue  |
|-----------|-------------------------------------------------------|-----------------------|-----------|---------|---------|
| GO:0005587| collagen type IV                                      | cellular_component    | 5/1978    | 6/18668 | 0.000857|
| GO:0021563| glossopharyngeal nerve development                    | biological_process    | 4/1978    | 5/18668 | 0.002689|
| GO:0021637| trigeminal nerve structural organization              | biological_process    | 4/1978    | 5/18668 | 0.002689|
| GO:0060685| regulation of prostatic bud formation                 | biological_process    | 4/1978    | 5/18668 | 0.002689|
| GO:0072148| epithelial cell fate commitment                       | biological_process    | 12/1978   | 18/18668| 0.000004|
| GO:0021559| trigeminal nerve development                          | biological_process    | 6/1978    | 9/18668 | 0.000775|
| GO:0021707| cerebellar granule cell differentiation              | biological_process    | 6/1978    | 9/18668 | 0.000775|
| GO:0035581| sequestering of extracellular ligand from receptor    | biological_process    | 5/1978    | 8/18668 | 0.000775|
| GO:0060837| blood vessel endothelial cell differentiation         | biological_process    | 5/1978    | 8/18668 | 0.000775|
| GO:0072050| S-shaped body morphogenesis                           | biological_process    | 5/1978    | 8/18668 | 0.000775|
| GO:0072102| glomerulus morphogenesis                              | biological_process    | 5/1978    | 8/18668 | 0.000775|
| GO:0097152| mesenchymal cell apoptotic process                    | biological_process    | 8/1978    | 13/18668| 0.000775|
| GO:0021785| branchiomotor neuron axon guidance                    | biological_process    | 6/1978    | 10/18668| 0.000775|
| GO:2001054| negative regulation of mesenchymal cell apoptotic process| biological_process    | 6/1978    | 10/18668| 0.000775|
| GO:0007440| foregut morphogenesis                                 | biological_process    | 6/1978    | 11/18668| 0.000775|
| GO:0008331| high voltage-gated calcium channel activity           | molecular_function    | 6/1978    | 11/18668| 0.000775|
| GO:0014051| gamma-aminobutyric acid secretion                    | biological_process    | 6/1978    | 11/18668| 0.000775|
| GO:0021683| cerebellar granular layer morphogenesis              | biological_process    | 6/1978    | 11/18668| 0.000775|
| GO:0032460| negative regulation of protein oligomerization       | biological_process    | 6/1978    | 11/18668| 0.000775|
| GO:0048672| positive regulation of collateral sprouting          | biological_process    | 6/1978    | 11/18668| 0.000775|
| GO:0090128| regulation of synapse maturation                     | biological_process    | 7/1978    | 13/18668| 0.000775|

Table 2 GO enrichment of the hypermethylated genes with enrich factor ≥ 5.0
| GO_ID     | Description                                                                 | TYPE                  | GeneRatio | BgRatio | pvalue  |
|-----------|------------------------------------------------------------------------------|-----------------------|-----------|---------|---------|
| GO:0004908 | interleukin-1 receptor activity                                              | molecular_function    | 6/2005    | 7/18668 | 0.000299 |
| GO:0051552 | flavone metabolic process                                                    | biological_process    | 4/2005    | 6/18668 | 0.004798 |
| GO:0097493 | structural molecule activity conferring elasticity                          | molecular_function    | 4/2005    | 6/18668 | 0.004798 |
| GO:0050966 | detection of mechanical stimulus involved in sensory perception of pain     | biological_process    | 4/2005    | 7/18668 | 0.007571 |
| GO:0070852 | cell body fiber                                                             | cellular_component    | 4/2005    | 7/18668 | 0.007571 |
| GO:2001204 | regulation of osteoclast development                                         | biological_process    | 4/2005    | 7/18668 | 0.007571 |
| GO:0051967 | negative regulation of synaptic transmission, glutamatergic                 | biological_process    | 5/2005    | 9/18668 | 0.003839 |
| GO:0007216 | G-protein coupled glutamate receptor signaling pathway                       | biological_process    | 7/2005    | 13/18668| 0.000996 |

**Table 3** KEGG enrichment of genes with the hypomethylated loci with enrich factor \( \geq 2.0 \)

| Pathway ID | Description                          | pvalue     | Count | genes                                                                 |
|------------|--------------------------------------|------------|-------|----------------------------------------------------------------------|
| hsa00532   | Glycosaminoglycan biosynthesis       | 0.001352   | 8     | DSE CHST11 CSGALNACT1 CSGALNACT1 HBA1 PRKCA APOA1 PRKCB HBA          |
| hsa04512   | ECM-receptor interaction             | 0.000135   | 22    | RELN ITGA7 COL6A2 LAMC SDC3 SDC1 COL4A4 ITGA1                       |
| hsa05143   | African trypanosomiasis             | 0.009444   | 9     | PKLR MAFA NR5A2 PAX6 F                                  |
| hsa04950   | Maturity onset diabetes of the young | 0.036190   | 6     | PIWIL2 FNM2 ETS1 CPEB1                                          |
| hsa04320   | Dorso-ventral axis formation         | 0.036190   | 6     | COLL1B2 RELN ITGA7 COL6A2 LAMC SDC3 SDC1 COL4A4 ITGA1             |
| hsa01040   | Biosynthesis of unsaturated fatty acids | 0.050480  | 5     | SDC3 SDC1 COL4A4 ITGA1 CSGALNACT1 DSE                              |
| hsa04960   | Aldosterone-regulated sodium reabsorption | 0.011820 | 10    | HSD11B2 PRKCA SLC9A3R                                              |
| hsa04340   | Hedgehog signaling pathway           | 0.006325   | 13    | WNT3 GLI2 SHH WNT3A T                                             |
| hsa05412   | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 0.002485 | 17    | ACTN3 CACNA1F ITGA7 C A CACNA2D2 DMD CACNB4 WNT3 GLI2 SHH WNT3A T    |
| hsa05217   | Basal cell carcinoma                 | 0.013500   | 12    | WNT3 GLI2 SHH WNT3A T                                             |
| hsa05410   | Hypertrophic cardiomyopathy (HCM)    | 0.003654   | 18    | CACNA1F ITGA7 PRKAA2 C CACNB4 PRKAG2 ITGA5 T                         |

**Table 4** KEGG enrichment of genes with the hypermethylated loci with enrich factor \( \geq 2.0 \)
| Pathway ID | Description                                      | pvalue        | Count | genes                                                                 |
|------------|--------------------------------------------------|---------------|-------|----------------------------------------------------------------------|
| hsa00061   | Fatty acid biosynthesis                          | 0.051680      | 2     | ACACB FAS                                                             |
| hsa05330   | Allograft rejection                              | 0.000196      | 13    | FASLG CD28 HLA-DMB HD OB HLA-G CD40LG                                 |
| hsa04940   | Type I diabetes mellitus                         | 0.000074      | 15    | FASLG GAD1 CD28 HLA-l DPB1 CD86 HLA-DOB HL                           |
| hsa05332   | Graft-versus-host disease                        | 0.000523      | 13    | FASLG CD28 HLA-DMB K CD86 HLA-DOB HLA-G GALTNL4 GCNT4 GALNT3          |
| hsa000512  | Mucin type O-Glycan biosynthesis                 | 0.003111      | 9     | FCER1A HLA-DMB RNAS3T IL15RA CD28 HLA-DMB C ITGA4 CD40LG             |
| hsa05310   | Asthma                                           | 0.003935      | 9     | FCER1A HLA-DMB RNAS3T IL15RA CD28 HLA-DMB C ITGA4 CD40LG             |
| hsa04960   | Aldosterone-regulated sodium reabsorption        | 0.011420      | 10    | SGK1 PRKCA KINJ1 ATP1 CYP3A4 HSD3B2 UGT2B- B UGT1A7 SRD5A2 UGT1A7   |
| hsa05140   | Steroid hormone biosynthesis                     | 0.007065      | 13    | CYP3A4 HSD3B2 UGT2B- B UGT1A7 SRD5A2 UGT1A7 KNG1 CFB MASP1 CFI C8 C8B|
| hsa04610   | Complement and coagulation cascades              | 0.006678      | 15    | CYP3A4 HSD3B2 UGT2B- B UGT1A7 SRD5A2 UGT1A7 KNG1 CFB MASP1 CFI C8 C8B|
| hsa05150   | Staphylococcus aureus infection                  | 0.014960      | 12    | CFB HLA-DMB MASP1 CF SDC2 NRXN3 CADM1 NR NLGN3 CLDN14 SELL HL          |
| hsa04514   | Cell adhesion molecules (CAMs)                   | 0.000413      | 29    | SDC2 NRXN3 CADM1 NR NLGN3 CLDN14 SELL HL CD86 ITGA4 CD4H4 HLA-DMB    |

Table 5 KEGG pathways of the differentially hypomethylated genes with qvalue < 0.05
**Table 6** KEGG pathways of the differentially hypermethylated genes with qvalue < 0.05

| Pathway ID | Description                                                                 | GeneRatio | BgRatio | pvalue   | qvalue   | genes                                                                 |
|------------|-----------------------------------------------------------------------------|-----------|---------|----------|----------|-----------------------------------------------------------------------|
| hsa04080   | Neuroactive ligand-receptor interaction                                      | 54/614    | 272/5894| 0.000021 | 0.004404 | GALR1 LPAR3 LDRD2 LDRD5 P2RY10 GABRA: FYN CO SHC4 K COL4A1 MYLK3 ITGA4 E |
| hsa04510   | Focal adhesion                                                              | 41/614    | 200/5894| 0.000087 | 0.006060 | FYN CO SHC4 K COL4A1 MYLK3 ITGA4 E                                    |
| hsa04940   | Type I diabetes mellitus                                                    | 15/614    | 45/5894 | 0.000074 | 0.007695 | FASLG HLA-DMB HLA-DOA HLA-DPA1 HLA-DRA HLA-DPB1 HLA-DOB HLA-G          |
| hsa05330   | Allograft rejection                                                         | 13/614    | 39/5894 | 0.000196 | 0.010250 | FASLG HLA-DMB HLA-DOA HLA-DPA1 HLA-DRA HLA-DPB1 HLA-DOB HLA-G          |
| hsa04512   | ECM-receptor interaction                                                    | 21/614    | 85/5894 | 0.000327 | 0.013670 | SDC2 COL6A1 RELN LAM2 COL5A3 COL4A6 THBS2 RASGRF1                      |
| hsa04514   | Cell adhesion molecules (CAMs)                                               | 29/614    | 136/5894| 0.000413 | 0.014390 | SDC2 NRXN3 CADM1 NRCAM PDCD1LG2 JAM2 CLDN10 CD28 CNTNAP2 CADM3          |
| hsa05332   | Graft-versus-host disease                                                   | 13/614    | 43/5894 | 0.000523 | 0.015620 | FASLG HLA-DMB HLA-DOA HLA-DPA1 HLA-DRA HLA-DPB1 HLA-DOB HLA-G          |
| hsa04672   | Intestinal immune network for IgA production                                | 14/614    | 49/5894 | 0.000614 | 0.016040 | FASLG HLA-DMB HLA-DOA HLA-DPA1 HLA-DRA HLA-DPB1 HLA-DOB HLA-G          |
| hsa05412   | Arrhythmogenic right ventricular cardiomyopathy (ARVC)                       | 18/614    | 74/5894 | 0.000946 | 0.021970 | CACNG2 CACNA2 DMD DES SGCG ITGA11 CTNNA2 LEF1 ITGA4 CACNB2 SGCB          |
| hsa05416   | Viral myocarditis                                                           | 17/614    | 72/5894 | 0.001751 | 0.036600 | CD40LG CD80 IL15RA CD80                                                                                                                                                           |

**Table 7** Distribution of the methylated sites on the abnormally expressed genes
| UCSC_REFGENE_NAME | Foldchanges | beta_difference | UCSC_REFGENE_GROUP |
|------------------|-------------|----------------|-------------------|
| ESR1             | -6.06       | 0.38478629     | 5'UTR             |
|                  |             | 0.38029742     | 5'UTR             |
|                  |             | 0.2084509      | Body; Body; Body; Body |
|                  |             | 0.28957789     | Body; Body; Body; Body |
|                  |             | 0.37846598     | Body; Body; Body; Body |
|                  |             | -0.31019632    | 5'UTR             |
|                  |             | -0.41098191    | 5'UTR; TSS1500    |
|                  |             | -0.3243587     | 5'UTR; TSS1500    |
|                  |             | -0.31535468    | 5'UTR; TSS1500    |
|                  |             | -0.2732013     | 5'UTR; TSS1500    |
|                  |             | -0.21284801    | 5'UTR; TSS1500    |
|                  |             | -0.21207078    | 1stExon; 5'UTR    |
|                  |             | -0.20579793    | 5'UTR; 1stExon; 5'UTR |
|                  |             | -0.39979892    | 3'UTR; 3'UTR; 3'UTR; 3'UTR |
| RAC2             | -10.34      | -0.31084445    | TSS200            |
|                  |             | 0.39540156     | TSS1500           |
|                  |             | -0.56920735    | 1stExon; 5'UTR    |
| ABCB1            | -2.27       | 0.26705929     | Body              |
|                  |             | 0.26412243     | Body              |
|                  |             | 0.24597528     | Body; Body; TSS1500; Body |
|                  |             | -0.21206685    | 5'UTR; TSS200; TSS200; TSS200 |
|                  |             | -0.2342092     | 5'UTR; TSS200; TSS200; TSS200 |
|                  |             | -0.24716201    | 5'UTR             |
|                  |             | -0.26582916    | 5'UTR             |
|                  |             | -0.36261124    | 1stExon; 5'UTR; 1stExon; 5'UTR; 5'UTR |
|                  |             | -0.36606718    | 1stExon; 5'UTR; 1stExon; 5'UTR; 5'UTR |
|                  |             | -0.3957685     | TSS1500; TSS1500; 5'UTR; TSS1500 |
|                  |             | -0.4373359     | 5'UTR             |

Table 8 Signal pathways of the upregulated and downregulated genes of H446/DDP
| Expression     | Methylation status | Description                                      | Pathways                                                                 |
|---------------|-------------------|--------------------------------------------------|--------------------------------------------------------------------------|
| Upregulated   | Hypomethylated    | Pathways in cancer                               | BMP4 FGFR2 KIT RARB TF       |
|               |                   | MAPK signaling pathway                            | FGFR2 FGFR1 FLNC CAC         |
|               |                   | Cytokine-cytokine receptor interaction            | KIT IL7 TNFRSF19 TNFSF11     |
|               |                   | Cell adhesion molecules (CAMs)                    | CNTN1 ALCAM CLDN1 H          |
|               |                   | Small cell lung cancer                            | RARB TOX3 ITGA2 LAMA3        |
|               |                   | ECM-receptor interaction                          | TOX3 ITGA2 LAMA3             |
|               |                   | Focal adhesion                                    | TOX3 ITGA2 LAMA3             |
|               |                   | Regulation of actin cytoskeleton                  | FGFR1 FGFR2 ITGA2            |
|               |                   | ABC transporters                                  | KIT IL7 ITGA2                |
|               |                   | Acute myeloid leukemia                            | CCNA1 KIT RUNX1              |
|               |                   | Endocytosis                                       | FGFR2 KIT HSPA1A             |
| Downregulated | Hypermethylated   | MAPK signaling pathway                            | CACNA2D1 CACNG6 EGF          |
|               |                   | Pathways in cancer                               | EGF PIK3R1 TGFA ETS1         |
|               |                   | Melanoma                                          | EGF PDGFD PIK3R1 FGF         |
|               |                   | Osteoclast differentiation                        | IL1R1 PIK3R1 TNFSF11         |
|               |                   | Prostate cancer                                   | EGF PIK3R1 IGF1 TGFAP        |
|               |                   | Glioma                                            | EGFI PIK3R1 IGF1 TGFAP       |
|               |                   | Cell adhesion molecules (CAMs)                    | NLGN3 NRCAM SDC2             |
|               |                   | Focal adhesion                                    | EGF PDFGD PI3R1 BCLC          |
|               |                   | Hypertrophic cardiomyopathy (HCM)                 | CACNA2D1 CACNG6 IGF          |
|               |                   | Dilated cardiomyopathy                            | CACNA2D1 CACNG6 IGF          |
|               |                   | Non-small cell lung cancer                        | EGF PIK3R1 TGFA              |
|               |                   | ErbB signaling pathway                            | EGF PIK3R1 TGFA              |
|               |                   | Pancreatic cancer                                 | EGF PIK3R1 TGFA              |
|               |                   | Cytokine-cytokine receptor interaction            | EGF IL1R1 TNFRSF11A          |
|               |                   | Renal cell carcinoma                              | ETS1 PIK3R1 TGFA             |
|               |                   | Regulation of actin cytoskeleton                  | EGF MRAS PDGFD              |