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 ($|\Delta\beta| \geqq 0.20$) 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 resistance, 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℃ in 5% CO_2. 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 (IC_{50}) 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 5×10^3 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. IC_{50} 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℃ (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 5×10^3 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 (ThermoScietific). 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 $|\Delta\beta| \geqq 0.20$ (in H446/DDP vs H446) and adjusted *p* value ≦ 0.05 were considered as differentially methylated sites. A CpG was considered hypermethylated if $\Delta\beta \geqq 0.20$ or hypomethylated if $\Delta\beta \leqq -0.20$. Average β value of promoters and CpG islands (CGIs) were compared between H446/DDP and H446 cells. Promoters and CGIs with $|\Delta\beta| \geqq 0.20$ 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 (ThermoScietific), 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℃, followed by 40 cycles of 15 s of denaturation at 95℃, 30 s of annealing at 55℃ and 30 s of extension at 72℃. The relative expression values were computed by the $^{\triangle\triangle}$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 ($|\Delta\beta| \geqq 0.20$) 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 $|\Delta\beta| \leqq 0.4$. 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 $|\Delta\beta| \geqq 0.4$ 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 $\geqq 5.0$ 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 $\geqq 2.0$ 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, Dorso-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 ($|\Delta\beta| \geqq 0.7$) 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 ($|\Delta\beta| \geqq 0.40$) 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 qvalue among the enriched signal pathways of genes with hypermethylated sites (qvalue = 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 tobe 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; IC_{50}: 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 ≧ 5.0

GO_ID	Description	Type	GeneRatio	BgRatio	pvalue	Count	genes	enrich_factor
GO:0005587	collagen type IV	cellular_component	5/1978	6/18668	0.000857	5	COL4A5 COL4A2 COL4A3 COL4A6 COL4A4	7.86
GO:0021563	glossopharyngeal nerve development	biological_process	4/1978	5/18668	0.002689	4	PLXNA4 NAV2 HOXB3 HOXA3	7.55
GO:0021637	trigeminal nerve structural organization	biological_process	4/1978	5/18668	0.002689	4	PLXNA4 SEMA3F NRP1 SEMA3A	7.55
GO:0060685	regulation of prostatic bud formation	biological_process	4/1978	5/18668	0.002689	4	BMP4 BMP7 WNT5A SHH	7.55
GO:0072148	epithelial cell fate commitment	biological_process	12/1978	18/18668	0.000004	12	NOTCH1 ARX GATA4 PROX1 ATOH1 JAG2 FGFR3 DLL1 HES5 NRP1 NR2F2 HOXA13	6.29
GO:0021559	trigeminal nerve development	biological_process	6/1978	9/18668	0.000775	6	ISL1 PLXNA4 DRGX SEMA3F NRP1 SEMA3A	6.29
GO:0021707	cerebellar granule cell differentiation	biological_process	6/1978	9/18668	0.000775	6	KNDC1 GRID2 PROX1 NRXN1 MTPN NFIX	6.29
GO:0035581	sequestering of extracellular	biological_process	5/1978	8/18668	0.002351	5	LTBP1 NBL1 FBN2	5.9

	ligand from receptor						GREM2 FBN1	
GO:0060837	blood vessel endothelial cell differentiation	biological_process	5/1978	8/18668	0.002351	5	NOTCH1 NRP1 DLL1 TMEM100 HEY1	5.9
GO:0072050	S-shaped body morphogenesis	biological_process	5/1978	8/18668	0.002351	5	LHX1 WT1 HES5 BMP4 PDGFRB	5.9
GO:0072102	glomerulus morphogenesis	biological_process	5/1978	8/18668	0.002351	5	BMP4 PDGFRA KIRREL3 PDGFRB MEF2C	5.9
GO:0097152	mesenchymal cell apoptotic process	biological_process	8/1978	13/18668	0.000211	8	MSX2 HIF1A TBX1 BMP7 PAX2 SHH HNF1B HOXA13	5.81
GO:0021785	branchiomotor neuron axon guidance	biological_process	6/1978	10/18668	0.001208	6	PLXNA4 PLXNC1 SEMA3F PLXND1 NRP1 SEMA3A	5.66
GO:2001054	negative regulation of mesenchymal cell apoptotic process	biological_process	6/1978	10/18668	0.001208	6	HIF1A TBX1 BMP7 PAX2 SHH HNF1B	5.66
GO:0007440	foregut morphogenesis	biological_process	6/1978	11/18668	0.001814	6	EPB41L5 SMAD3 NOTCH1 GATA4 SHH NCKAP1	5.15
GO:0008331	high voltage-gated calcium channel activity	molecular_function	6/1978	11/18668	0.001814	6	CACNA1F CACNA1C CACNB2 CACNA1A	5.15

							CACNA1S CACNB4	
GO:0014051	gamma-aminobutyric acid secretion	biological_process	6/1978	11/18668	0.001814	6	NF1 NTSR1 CACNA1A SLC6A1 CACNB4 GRIK1	5.15
GO:0021683	cerebellar granular layer morphogenesis	biological_process	6/1978	11/18668	0.001814	6	KNDC1 GRID2 PROX1 NRXN1 MTPN NFIX	5.15
GO:0032460	negative regulation of protein oligomerization	biological_process	6/1978	11/18668	0.001814	6	PEX5 TMC8 CLU OPRD1 INS BCL11A	5.15
GO:0048672	positive regulation of collateral sprouting	biological_process	6/1978	11/18668	0.001814	6	RND2 WNT3 CRABP2 WNT3A NGF BCL11A	5.15
GO:0090128	regulation of synapse maturation	biological_process	7/1978	13/18668	0.000916	7	DISC1 CAMK2B RELN NEUROD2 NFATC4 NRXN1 NEURL	5.08

Table 2 GO enrichment of the hypermethylated genes with enrich factor ≧ 5.0

GO_ID	Description	TYPE	GeneRatio	BgRatio	pvalue	Count	genes	enrich_factor
GO:0004908	interleukin-1 receptor activity	molecular_function	6/2005	7/18668	0.000299	6	IL1R1 IL1RL2 IL1RL1 IL18R1 IL1RAPL2 IL1R2	7.98
GO:0051552	flavone metabolic process	biological_process	4/2005	6/18668	0.004798	4	UGT1A1 UGT1A7 PPARGC1A UGT1A10	6.21
GO:0097493	structural molecule activity conferring elasticity	molecular_function	4/2005	6/18668	0.004798	4	COL4A1 TTN FBN2 EMILIN2	6.21
GO:0050966	detection of mechanical stimulus involved in sensory perception of pain	biological_process	4/2005	7/18668	0.007571	4	FYN TRPA1 HTR2A NTRK1	5.32
GO:0070852	cell body fiber	cellular_component	4/2005	7/18668	0.007571	4	SRD5A2 SACS SPTBN4 HTR2A	5.32
GO:2001204	regulation of osteoclast development	biological_process	4/2005	7/18668	0.007571	4	LILRB1 SIGLEC15 TNFSF11 LTF	5.32
GO:0051967	negative regulation of synaptic transmission, glutamatergic	biological_process	5/2005	9/18668	0.003839	5	DRD2 NPY2R ATAD1 GRIK1 HTR2A	5.17
GO:0007216	G-protein coupled glutamate receptor signaling pathway	biological_process	7/2005	13/18668	0.000996	7	GRM1 HOMER1 TRPM1 GRM3 GRM8 GRM5 GRM2	5.01

Table 3 KEGG enrichment of genes with the hypomethylated loci with enrich factor ≧ 2.0

Pathway ID	Description	pvalue	Count	genes	enrich_factor
hsa00532	Glycosaminoglycan biosynthesis	0.001352	8	DSE CHST11 CSGALNACT1 B3GAT1 XYLT1 UST CHST7 CHPF	3.47
hsa04512	ECM-receptor interaction	0.000135	22	RELN ITGA7 COL6A2 LAMC1 COL4A6 COL2A1 ITGA2 CD44 TNXB COL4A5 LAMA3 COL1A1 SDC3 SDC1 COL4A4 ITGA5 VWF THBS4 COL4A2 COL11A2 SV2C THBS1	2.47
hsa05143	African trypanosomiasis	0.009444	9	PRKCA APOA1 PRKCB HBA1 ICAM1 GNAQ LAMA3 PRKCG IL12B	2.46
hsa04950	Maturity onset diabetes of the young	0.036190	6	PKLR MAFA NR5A2 PAX6 HNF1B INS	2.29
hsa04320	Dorso-ventral axis formation	0.036190	6	PIWIL2 FMN2 ETS1 CPEB1 NOTCH4 NOTCH1	2.29
hsa01040	Biosynthesis of unsaturated fatty acids	0.050480	5	SCD5 FADS2 ELOVL6 ACOT7 ACOX3	2.27
hsa04960	Aldosterone-regulated sodium reabsorption	0.011820	10	HSD11B2 PRKCA SLC9A3R2 PRKCB INS PRKCG SCNN1A PDPK1 IRS4 NR3C2	2.27
hsa04340	Hedgehog signaling pathway	0.006325	13	WNT3 GLI2 SHH WNT3A WNT6 BMP7 DHH WNT5A LRP2 WNT10A BMP6 WNT1 BMP4	2.22
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.002485	17	ACTN3 CACNA1F ITGA7 CACNA1C TCF7L1 CACNA2D1 CACNG8 CACNA1S TCF4 ITGA2 CACNA2D2 DMD CACNB4 ITGA5 CACNG4 CACNB2 RYR2	2.19
hsa05217	Basal cell carcinoma	0.013500	12	WNT3 GLI2 SHH WNT3A TCF7L1 WNT6 FZD9 TCF4 WNT5A WNT10A WNT1 BMP4	2.08
hsa05410	Hypertrophic cardiomyopathy (HCM)	0.003654	18	CACNA1F ITGA7 PRKAA2 CACNA1C CACNA2D1 CACNG8 CACNA1S ITGA2 CACNA2D2 DMD CACNB4 PRKAG2 ITGA5 TGFB1 CACNG4 TNNT2 CACNB2 RYR2	2.07

Table 4 KEGG enrichment of genes with the hypermethylated loci with enrich factor ≧ 2.0

Pathway ID	Description	pvalue	Count	genes	enrich_factor
hsa00061	Fatty acid biosynthesis	0.051680	2	ACACB FAS	3.20
hsa05330	Allograft rejection	0.000196	13	FASLG CD28 HLA-DMB HLA-DOA HLA-DPA1 IL12B HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G CD40LG	3.20
hsa04940	Type I diabetes mellitus	0.000074	15	FASLG GAD1 CD28 HLA-DMB LTA PTPRN2 HLA-DOA HLA-DPA1 IL12B HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G	3.20
hsa05332	Graft-versus-host disease	0.000523	13	FASLG CD28 HLA-DMB KLRD1 KIR2DL3 HLA-DOA HLA-DPA1 HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G	2.90
hsa00512	Mucin type O-Glycan biosynthesis	0.003111	9	GALNTL4 GCNT4 GALNT2 GCNT3 GALNT1 GALNT3 GALNT14 GALNT9 GALNT5	2.88
hsa05310	Asthma	0.003935	9	FCER1A HLA-DMB RNASE3 HLA-DOA HLA-DPA1 HLA-DRA HLA-DPB1 HLA-DOB CD40LG	2.79
hsa04672	Intestinal immune network for IgA production	0.000614	14	IL15RA CD28 HLA-DMB CCR9 HLA-DOA HLA-DPA1 AICDA HLA-DRA CD80 HLA-DPB1 CD86 ITGA4 HLA-DOB CD40LG	2.74
hsa00770	Pantothenate and CoA biosynthesis	0.055820	4	VNN1 VNN2 BCAT1 DPYD	2.40
hsa04512	ECM-receptor interaction	0.000327	21	SDC2 COL6A1 RELN LAMB4 CHAD COL6A3 COL5A3 COL4A6 THBS2 COL4A1 IBSP TNXB ITGA11 COL5A2 COL5A1 LAMB1 THBS4 COL4A2 COL11A2 ITGA4 LAMA5	2.37
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.000946	18	CACNG2 CACNA2D3 TCF7L1 CACNA2D1 CACNG8 CACNA1D TCF7L2 CACNG6 DMD CACNB4 DES SGCG ITGA11 CTNNA2 LEF1 ITGA4 CACNB2 SGCB	2.33
hsa05216	Thyroid cancer	0.025390	7	TCF7L1 RXRG TCF7L2 PAX8 NTRK1 PPARG LEF1	2.32
hsa05320	Autoimmune thyroid disease	0.004412	13	FASLG CD28 HLA-DMB TG HLA-DOA HLA-DPA1 HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G CD40LG	2.31
hsa04960	Aldosterone-regulated sodium reabsorption	0.011420	10	SGK1 PRKCA KCNJ1 ATP1A4 PIK3CG PIK3R1 PRKCG HSD11B1 IGF1 IRS4	2.29
hsa05416	Viral myocarditis	0.001751	17	FYN CD28 HLA-DMB DMD HLA-DOA SGCG HLA-DPA1 HLA-DRA CD80 MYH15 HLA-DPB1 CD86 MYH10 HLA-DOB HLA-G CD40LG SGCB	2.27
hsa00140	Steroid hormone biosynthesis	0.007065	13	CYP3A4 HSD3B2 UGT2B4 AKR1C4 UGT1A1 CYP7B1 UGT1A10 AKR1D1 HSD11B1 AKR1C3 UGT1A7 SRD5A2 UGT1A5	2.19
hsa04610	Complement and coagulation cascades	0.006678	15	KNG1 CFB MASP1 CFI C8A PROS1 THBD C1S F13B BDKRB1 FGG F9 SERPINA1 SERPINA5 C8B	2.09

hsa05150	Staphylococcus aureus infection	0.014960	12	CFB HLA-DMB MASP1 CFI FPR3 C1S FGG HLA-DOA HLA-DPA1 HLA-DRA HLA-DPB1 HLA-DOB	2.06
hsa04514	Cell adhesion molecules (CAMs)	0.000413	29	SDC2 NRXN3 CADM1 NRCAM PDCD1LG2 JAM2 CLDN10 CD28 CNTNAP2 CADM3 HLA-DMB NLGN3 CLDN14 SELL HLA-DOA PTPRM HLA-DPA1 HLA-DRA PVRL1 CD80 NCAM1 HLA-DPB1 CD86 ITGA4 CDH4 HLA-DOB HLA-G CLDN18 CD40LG	2.05

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

Pathway ID	Description	GeneRatio	BgRatio	pvalue	qvalue	genes	enrich_factor
hsa04512	ECM-receptor interaction	22/617	85/5894	0.000135	0.013760	RELN ITGA7 COL6A2 LAMC1 COL4A6 COL2A1 ITGA2 CD44 TNXB COL4A5 LAMA3 COL1A1 SDC3 SDC1 COL4A4 ITGA5 VWF THBS4 COL4A2 COL11A2 SV2C THBS1	2.47
hsa04010	MAPK signaling pathway	51/617	268/5894	0.000108	0.021990	CACNA1F CACNA1A MAP3K5 DUSP9 PDGFRA FLNC HSPA1L CACNA1C PRKCA TRAF6 NGF DUSP4 MAPT CACNA2D1 PDGFRB CACNA1H CACNG8 HSPA2 NFATC4 GNG12 NTF3 CACNA1S IL1A HSPA1A FGF9 MAP2K6 GADD45A FGFR1 DUSP7 PRKCB DUSP3 RAC2 NF1 DAXX MAP4K4 CACNA2D2 MEF2C TGFBR2 MAPK8IP3 CACNB4 NFATC2 PRKCG TGFB1 STMN1 FGFR2 RAPGEF2 RASGRP2 CACNG4 FGFR3 CACNB2 FGF2	1.82
hsa04510	Focal adhesion	38/617	200/5894	0.000704	0.047880	ACTN3 RELN ITGA7 PDGFRA FLNC PRKCA COL6A2 CAPN2 PTK2 LAMC1 PDGFRB COL4A6 SHC3 COL2A1 ITGA2 PRKCB PPP1CC RAC2 PDGFD TNXB FLT4 COL4A5 LAMA3 COL1A1 PRKCG COL4A4 VAV3 ITGA5 PDPK1 VWF ZYX THBS4 COL4A2 BCL2 COL11A2 RAPGEF1 THBS1 DOCK1	1.82

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

Pathway ID	Description	GeneRatio	BgRatio	pvalue	qvalue	genes	enrich_factor
hsa04080	Neuroactive ligand-receptor interaction	54/614	272/5894	0.000021	0.004404	GALR1 HTR2A GABRG3 GRM1 MTNR1B GPR35 GABRB3 GRIN2D LPAR3 GRIK1 HTR5A MC2R TACR3 MCHR2 GRID2 TAAR9 GRM5 NPY2R DRD2 LEP ADORA3 MC5R GRM8 GPR50 PRL PTGDR FPR3 GABRA2 P2RY10 NMBR BDKRB1 HTR2C OPRK1 F2RL1 TRHR HTR7 GABRA5 DRD5 PTGFR GRM2 BRS3 EDNRA GRID1 GHSR TBXA2R GRIN2A GABRA3 TACR1 AGTR2 HTR1F ADRA2C TACR2 HTR1E GRM3	1.91
hsa04510	Focal adhesion	41/614	200/5894	0.000087	0.006060	FYN COL6A1 AKT3 RELN LAMB4 TLN2 FLNC PRKCA CHAD COL6A3 SHC4 KDR COL5A3 PIK3CG COL4A6 PIK3R1 SHC3 THBS2 RASGRF1 COL4A1 IBSP PDGFD TNXB FLT4 EGF ITGA11 CCND2 VAV1 PRKCG MYLK3 COL5A2 COL5A1 LAMB1 IGF1 PAK7 THBS4 COL4A2 COL11A2 ITGA4 DOCK1 LAMA5	1.97
hsa04940	Type I diabetes mellitus	15/614	45/5894	0.000074	0.007695	FASLG GAD1 CD28 HLA-DMB LTA PTPRN2 HLA-DOA HLA-DPA1 IL12B HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G	3.20
hsa05330	Allograft rejection	13/614	39/5894	0.000196	0.010250	FASLG CD28 HLA-DMB HLA-DOA HLA-DPA1 IL12B HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G CD40LG	3.20
hsa04512	ECM-receptor interaction	21/614	85/5894	0.000327	0.013670	SDC2 COL6A1 RELN LAMB4 CHAD COL6A3 COL5A3 COL4A6 THBS2 COL4A1 IBSP TNXB ITGA11 COL5A2 COL5A1 LAMB1 THBS4 COL4A2 COL11A2 ITGA4 LAMA5	2.37
hsa04514	Cell adhesion molecules (CAMs)	29/614	136/5894	0.000413	0.014390	SDC2 NRXN3 CADM1 NRCAM PDCD1LG2 JAM2 CLDN10 CD28 CNTNAP2 CADM3 HLA-DMB	2.05

						NLGN3 CLDN14 SELL HLA-DOA PTPRM HLA-DPA1 HLA-DRA PVRL1 CD80 NCAM1 HLA-DPB1 CD86 ITGA4 CDH4 HLA-DOB HLA-G CLDN18 CD40LG	
hsa05332	Graft-versus-host disease	13/614	43/5894	0.000523	0.015620	FASLG CD28 HLA-DMB KLRD1 KIR2DL3 HLA-DOA HLA-DPA1 HLA-DRA CD80 HLA-DPB1 CD86 HLA-DOB HLA-G	2.90
hsa04672	Intestinal immune network for IgA production	14/614	49/5894	0.000614	0.016040	IL15RA CD28 HLA-DMB CCR9 HLA-DOA HLA-DPA1 AICDA HLA-DRA CD80 HLA-DPB1 CD86 ITGA4 HLA-DOB CD40LG	2.74
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	18/614	74/5894	0.000946	0.021970	CACNG2 CACNA2D3 TCF7L1 CACNA2D1 CACNG8 CACNA1D TCF7L2 CACNG6 DMD CACNB4 DES SGCG ITGA11 CTNNA2 LEF1 ITGA4 CACNB2 SGCB	2.33
hsa05416	Viral myocarditis	17/614	72/5894	0.001751	0.036600	FYN CD28 HLA-DMB DMD HLA-DOA SGCG HLA-DPA1 HLA-DRA CD80 MYH15 HLA-DPB1 CD86 MYH10 HLA-DOB HLA-G CD40LG SGCB	2.27

Table 7 Distribution of the methylated sites on the abnormally expressed genes

UCSC_REFGENE_NAME	Foldchanges	beta_dfference	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; 1stExon; 5'UTR
		-0.36606718	1stExon; 5'UTR; 1stExon; 5'UTR; 5'UTR; 1stExon; 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 cells relative to H446 cells

Expression	Methylation status	Description	Genes
Upregulated	Hypomethylated	Pathways in cancer	BMP4 FGFR2 KIT RARB TOX3 DAPK1 FGFR1 RUNX1 ITGA2 EPAS1 LAMA3
		MAPK signaling pathway	FGFR2 FGFR1 FLNC CACNG4 HSPA1A NFATC2 NFATC4
		Cytokine-cytokine receptor interaction	KIT IL7 TNFRSF19 TNFSF10 OSMR TNFRSF11B
		Cell adhesion molecules (CAMs)	CNTN1 ALCAM CLDN1 HLA-DMB
		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 IL1R1 MEF2C MRAS FGF13 HSPA1L
		Pathways in cancer	EGF PIK3R1 TGFA ETS1 MMP2 FGF13 IGF1 PPARG BCL2
		Melanoma	EGF PDGFD PIK3R1 FGF13 IGF1
		Osteoclast differentiation	IL1R1 PIK3R1 TNSRSF11A FYN PPARG
		Prostate cancer	EGF PIK3R1 IGF1 TGFAPDGFD BCL2
		Glioma	EGF PIK3R1 IGF1 TGFA
		Cell adhesion molecules (CAMs)	NLGN3 NRCAM SDC2
		Focal adhesion	EGF PDFGD PIL3R1 BCL2
		Hypertrophic cardiomyopathy (HCM)	CACNA2D1 CACNG6 IGF1
		Dilated cardiomyopathy	CACNA2D1 CACNG6 IGF1
		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