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Effect evaluation of cisplatin-gemcitabine combination chemotherapy for advanced non-small cell lung cancer patients using microarray data

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Abstract. – OBJECTIVE: This study was performed to evaluate the therapeutic effect of cisplatin-gemcitabine combination chemotherapy for advanced non-small cell lung cancer (NSCLC).

MATERIALS AND METHODS: Dataset GSE39345 from patients who underwent cisplatin-gemcitabine combination chemotherapy and normal controls was downloaded from Gene Expression Omnibus. Differentially expressed genes (DEGs) were identified using Limma package and divided into 3 datasets: unique DEGs in NSCLC before chemotherapy vs. control samples (dataset A), common DEGs (dataset B), unique DEGs in NSCLC after chemotherapy vs. control samples (dataset C). Enrichment analysis was to identify functions Protein-protein interaction (PPI) analysis was to identify hub nodes and interacting pairs in dataset C and PPI network was constructed using Cytoscape software, followed by screening of small molecules using Connectivity Map.

RESULTS: Herein, 320 unique DEGs in dataset A, 584 common DEGs in dataset B and 1562 unique DEGs in dataset C were obtained. The 320 DEGs were significantly enriched in methylation and positive regulation of cell differentiation; the 584 DEGs were significantly enriched in positive regulation of cell differentiation and cytokine-cytokine receptor interaction pathway; the 1562 DEGs were enriched in functions associated with defense response. RELA and PLCB3 correlated with PLCE1 and INADL were hub nodes in the PPI network. Cefoperazone was the small molecule negatively correlated with DEGs.

CONCLUSIONS: Chemotherapy could prevent genes from aberrant methylation, partially restore cell differentiation process, fail to regulate cytokine-cytokine receptor interaction and induce weakened defense response. Cefoperazone could be used as a supplementary drug.

Key Words:
Cisplatin-gemcitabine combination chemotherapy, Defense response, Cefoperazone.

Introduction

Non-small cell lung cancer (NSCLC) accounting for 80-90% of lung cancer could contribute to paraneoplastic syndromes through metastasis and lead to cancer-related death[1,2]. The patients with NSCLC are often diagnosed in advanced stage and even those diagnosed in early stage often experience recurrence and metastatic period[3]. Chemotherapy with cisplatin-gemcitabine has been developed as the first-line treatment method for advanced or metastatic NSCLC based on its tolerability profile and favorable efficacy[4-6], in which gemcitabine acts as inhibitor of exonuclease and DNA repair by incorporating its triphosphate into DNA[7] whilst cisplatin functions through forming platinum-DNA adducts[8]. Previous study has evaluated the tumor-response and survival of patients with advanced NSCLC treated with four chemotherapy regimens including cisplatin-gemcitabine[9]. Nevertheless, the overall survival of advanced NSCLC patients has not been improved after first-line chemotherapy.

Some attempts have been made to deeply characterize the molecular mechanisms underlying the first-line chemotherapy with cisplatin-gemcitabine in order to improve the efficacy and reduce side-effects. Gene expression profiles of peripheral blood mononuclear cells reveal the *IL4* pathway are partially or totally reversed by chemotherapy in NSCLC patients, suggesting its

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immune surveillance[10]. The cytokine-cytokine receptor interaction pathway could not be recovered by chemotherapy because the associated genes including *CCL21*, *CCL3*, *IL9*, *PRLR* remain dysregulated in NSCLC patients after chemotherapy[11]. Small molecules being used for the treatment of NSCLC patients in combinations with other drugs have attracted the interest of researchers. For instance, ATN-224 is reported to be potentially clinical agent on the basis of its antioxidant inhibition selectively in cancer cells[12]. Tyrosine kinase inhibitors erlotinib is also proposed as supplementation to chemotherapy or radiotherapy[13]. However, small molecular specifically targeting the side-effects of first-line chemotherapy with cisplatin-gemcitabine remains rarely researched.

This study aimed to evaluate the therapeutic effect of first-line chemotherapy with cisplatin-gemcitabine using microarray expression data deposited in the public database by Chen et al[10]. The differentially expressed genes (DEGs) in NSCLC patients before and after chemotherapy vs. control subjects were separately screened and divided into three datasets, followed by enrichment analysis and protein-protein interaction (PPI) network construction in an attempt to explicit the molecular mechanisms underlying the treatment of chemotherapy and identify small molecule as drugs for complementary therapy.

Materials and Methods

Gene Expression Profiling

The gene expression profiling dataset GSE39345[10] of peripheral blood mononuclear cells was downloaded from Gene Expression Omnibus (GEO)[14], including 32 advanced NSCLC samples before chemotherapy, 17 NSCLC samples treated with cisplatin-gemcitabine combined chemotherapy and 20 matched healthy controls. The gene expression profiling was previously investigated by the GPL6104 Illumina humanRef-8 v2.0 expression bead chip.

Data Processing and DEGs Screening

Raw data were converted into recognizable format using package Affy in R[15], followed by median normalization and $\log_2$ transformation[16]. The expression level of each gene was calculated by taking the average of expression levels of probes corresponding to the same gene. Limma package[17] in R was used to identify DEGs in NSCLC samples before chemotherapy vs. health samples (group 1) and NSCLC samples after chemotherapy vs. health samples (group 2) with the cutoffs of $|\log \text{fold change (FC)}| > 1$ and false discovery rate (FDR) < 0.05.

Comparative Analysis of DEGs in the Two Groups

To understand whether DEGs were affected by chemotherapy in NSCLC samples, unique DEGs in each group and common DEGs in both two groups were screened out and constructed into interactive Venn diagram[18] in which all the DEGs were divided into three datasets of set A, set B and set C. Unique DEGs in group 1 (dataset A) represented the genes recovered by chemotherapy while the unique DEGs in group 2 (dataset C) represented the side-effects induced by chemotherapy. The common DEGs in two groups (dataset B) represented the genes that could not be regulated by chemotherapy.

Enrichment Analysis of DEGs in Three Datasets

To further identify the functions involved by DEGs in the three datasets, GO (Gene Ontology)[19] enrichment analysis was performed with the threshold of $p < 0.05$ using DAVID (Database for Annotation, Visualization and Integrated Discovery)[20]. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis of DEGs in dataset B was also performed to identify the pathways that could not be changed in NSCLC patients after chemotherapy with the criterion of $p < 0.05$ using KOBAS (KEGG Orthology Based Annotation System)[20].

PPI Analysis of DEGs in Dataset C

To further understand the interactions between DEGs in dataset C in a global perspective, PPI analysis was performed using STRING (Search Tool for the Retrieval of Interacting Genes)[21] database and visualized by constructing PPI network using Cytoscape software[22].

Screening of Small-Molecules

The DEGs from PPI network were divided into up-regulated genes and down-regulated genes and then respectively mapped into Connectivity Map (CMAP) database[23,24] to identify the small-molecules closely associated with side-effects of chemotherapy with criterion of the $|\text{connective score}| > 0.8$.

Results

Screened DEGs in Two Groups and Interactive Venn Diagram Analysis

With the cutoffs of |log FC| > 1 and FDR < 0.05, a total of 904 DEGs were identified in NSCLC samples before chemotherapy vs. health samples while 2146 DEGs were identified in NSCLC samples after chemotherapy vs. health samples. Furthermore, these DEGs were divided into three datasets as shown in the Venn diagram (Figure 1): (1) 320 unique DEGs in dataset A which were normalized by chemotherapy treatment; (2) 584 common DEGs in dataset B which may be not regulated by chemotherapy treatment and need other intervention methods; (3) 1562 unique DEGs in dataset C which represented side-effects induced by chemotherapy and needed some adjuvant therapy.

GO and KEGG Enrichment Analysis of DEGs

From the enrichment analysis using DAVID and KOBAS, the DEGs in datasets A were significantly enriched in different GO terms such as methylation (p = 3.91E-04, such as *CARM1* and *DNMT3B*) and positive regulation of cell differentiation (p = 8.06E-04, such as *PPARG*, *IGF2* and *CCL5*); The DEGs in dataset B were significantly enriched in different GO terms including positive regulation of cell differentiation (p = 1.45E-03, such as *FOXA1* and *CTNNB1*) and KEGG pathways of cytokine-cytokine receptor interaction (p = 9.28E-03, such as *CCL3*, *IL9*, *IL24* and *CXCL6*) and adherens junction (p = 3.61E-02); The DEGs in dataset C were enriched in various GO terms including defense response (p = 1.26E-03, such as *RELA*), defense response to virus (p = 2.01E-02, such as *RELA*), response to bacterium (p = 4.43E-02, such as *RELA*) and intracellular signaling cascade (p = 4.76E-02, such as *PLCB3*, *PLCE1* and *INADL*) (the top 10 significantly enriched GO terms of DEGs in three datasets are shown in Figure 2).

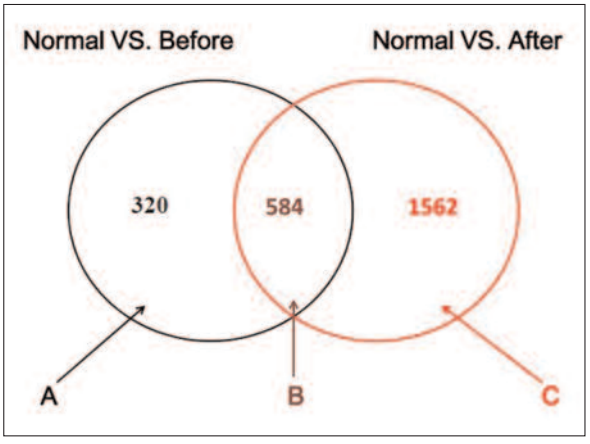

Figure 1. Venn diagram for differentially expressed genes in advanced non-small cell lung cancer (NSCLC) samples before chemotherapy vs. health samples, as well as NSCLC samples after chemotherapy vs. health samples.

PPI Network Analysis

Using Cytoscape software, we obtained the PPI network consisting of 72 nodes representing DEGs and 69 edges representing interactions. According to the PPI network, *RELA* (degree = 10) and *PLCB3* (degree = 6) were the hub nodes in the network (Figure 3). *PLCB3* was correlated with *PLCE1* and *INADL*.

Screened Small-Molecules

A total of 14 small-molecules that may be resistant to the side-effects induced by chemotherapy were identified using CMAP database with the criterion of the |connective score| > 0.8 (Table I). Cefoperazone (correlation coefficient = -0.917, p = 0.001) was identified as small-molecular drug with the highest negative correlation with DEGs.

Discussion

By performing the comparative analysis on microarray data, this study identified 320 unique DEGs in NSCLC samples before chemotherapy vs. control samples, 584 common DEGs and 1562 unique DEGs in NSCLC samples after chemotherapy vs. control samples. The 320 unique DEGs were enriched in functions of methylation and positive regulation of cell differentiation. The 584 common DEGs were enriched in cytokine-cytokine receptor interaction pathway and positive regulation of cell differentiation function. The 1562 unique DEGs were enriched in functions associated with defense response and intracellular signaling cascade. Moreover, *RELA* and *PLCB3* correlated with *PLCE1* and *INADL* were the hub nodes in the PPI network and cefoperazone was identified with the highest negative correlation with the 1562 unique DEGs.

Chemotherapy probably played a role in preventing genes from aberrant methylation in NSCLC patients. Gene methylation is demon-

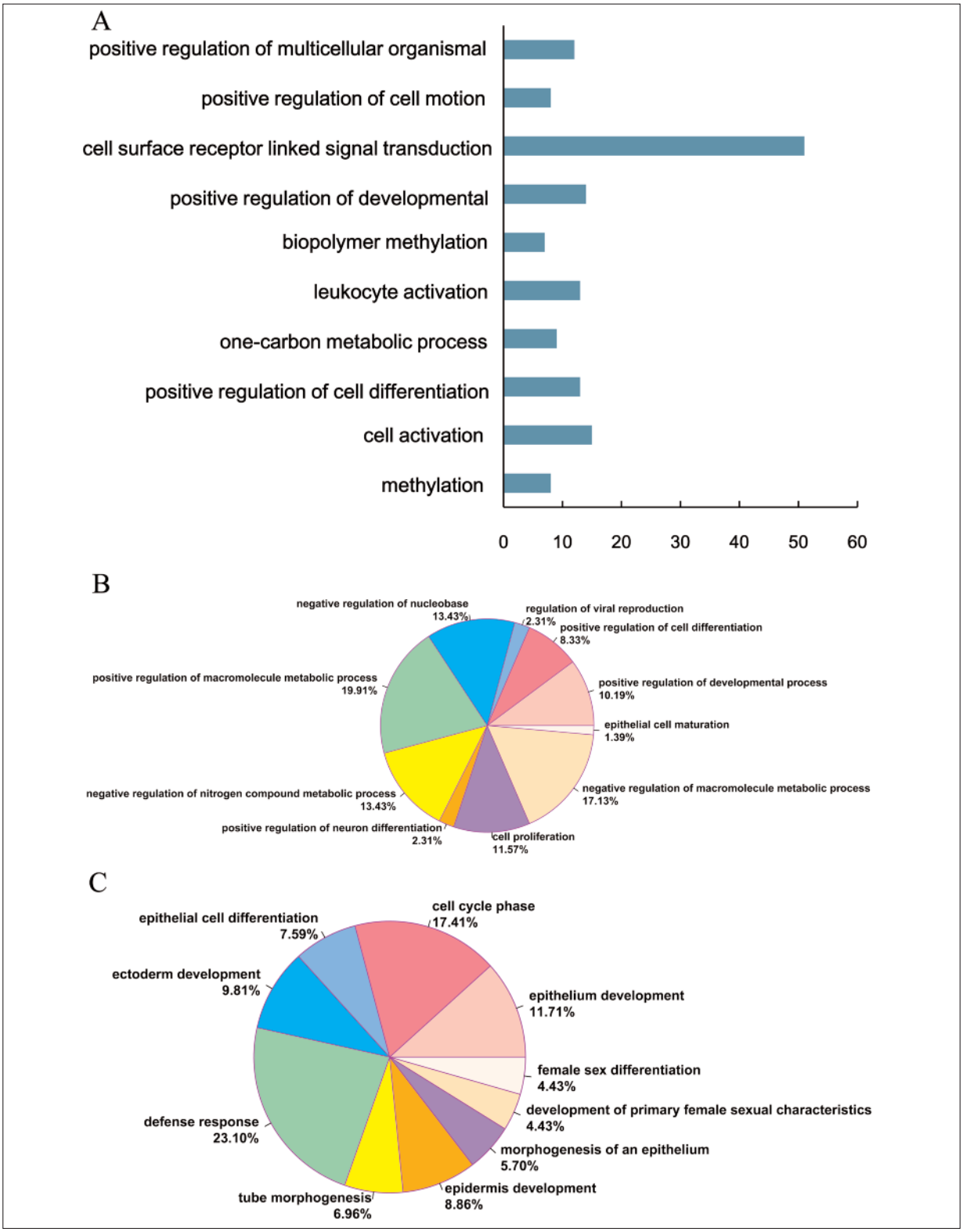

Figure 2. Significantly Gene Ontology terms of DEGs in three datasets. ***A,*** Histogram of functional terms of unique DEGs in NSCLC samples before chemotherapy vs. health samples. ***B,*** Pie chart of common DEGs. ***C,*** Pie chart of unique DEGs in NSCLC samples after chemotherapy vs. health samples. The height of columns represents the number of DEGs. Different colors represent different functional terms. Percentage of each functional term represents the percent of DEGs enriched in this term in all DEGs. DEGs, differentially expressed genes; NSCLC, non-small cell lung cancer.

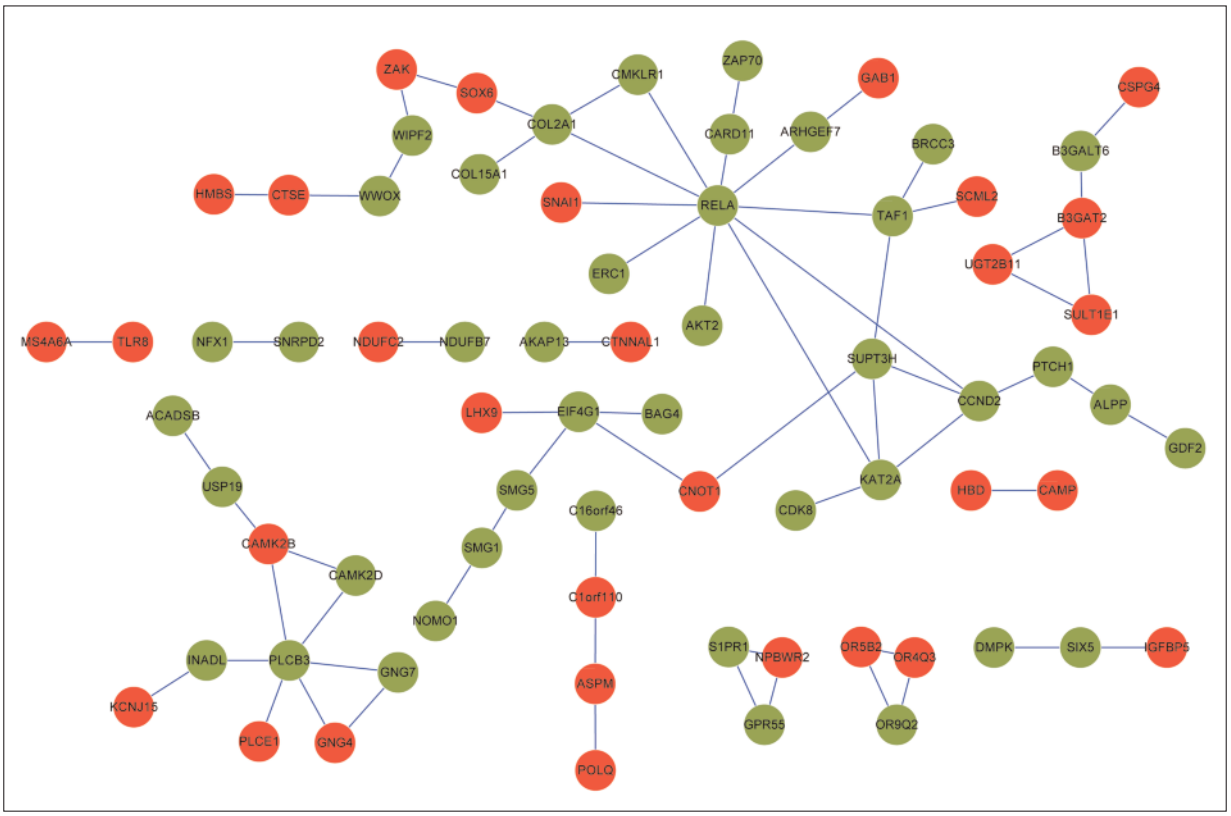

Figure 3. The protein-protein interaction network of differentially expressed genes in non-small cell lung cancer samples after chemotherapy vs. control samples. Red represents up-regulation; green represents down-regulation.

strated to increase with lung cancer risk[25]. *CARM1* (coactivator-associated arginine methyltransferase 1) associated with DNA methyltransferase activity may experience copy number changes and *DNMT3B* (DNA-methyltransferase 3 beta) involved in aberrant methylation is reported with polymorphism in lung cancer[26,27]. Herein, we found that the expressions of these two genes were normalized after chemotherapy, probably suggesting the role of chemotherapy in inhibiting gene aberrant methylation. Besides, chemotherapy could partially restored cancer cell differentiation process which is a useful therapeutic target for NSCLC patients[28]. As exemplified, *CCL5* (chemokine ligand 5) associated with the migration and metastasis of human cancer[29], *IGF2* (insulin-like growth factor 2) related to cell proliferation and tumor growth[30], *PPARG* (peroxisome proliferator-activated receptor gamma) involved in inhibiting invasive metastasis and inducing differentiation[28,31], were normalized by chemotherapy, suggesting the regulatory effect on cell differentiation of chemotherapy. However, some other genes involved in cell differentiation were not recovered, such as *CTNNB1* (catenin, beta 1) and *FOXA2* (forkhead box A2) which are associated with cancer cell survival and cancer metastasis[32,33]. Also, in accordance with a previous study, chemotherapy could not play a role in the cytokine-cytokine receptor interaction pathway due to the failure to regulate the expression of *CCL3*, *IL9*, *IL24* and *CXCL6*[11].

Notably, this study identified a large number of newly emerging DEGs induced by chemotherapy, implying the molecular mechanisms of its side-effects. Among the DEGs, *RELA* (v-rel avian reticuloendotheliosis viral oncogene homolog A) and *PLCB3* (phospholipase C, beta 3) with high degree obtained the major interest. *RELA*, a component of *NFkB* may contribute to increased expression of inflammatory genes such as *IL-6* and *TNF-* [34]. This gene was enriched in defense response, defense response to virus and response to bacterium, implying a weakened defense ability of cells induced by chemotherapy in NSCLC patients. The other hub node *PLCB3* correlated with *PLCE1* (phospholipase C, epsilon 1) and

Table I. Small molecules significantly correlated with differentially expressed genes in advanced non-small cell lung cancer after chemotherapy vs. health samples.

CMAP name	Enrichment	*p*-value
Cefoperazone	-0.917	0.001
PHA-00767505E	-0.865	0.0006
SC-560	-0.835	0.0089
Emetine	-0.818	0.002
Solanine	0.803	0.0028
CAY-10397	0.822	0.0113
Levcycloserine	0.833	0.0012
Corynanthine	0.842	0.0077
Trapidil	0.843	0.0076
MS-275	0.864	0.0373
Dienestrol	0.87	0.0041
Triflusal	0.876	0.0036
Ioxaglic acid	0.879	0.0035
Carbarsone	0.937	0.00002

INADL (InaD-like) were enriched in intracellular signaling cascade through which cells respond to the extracellular signals so as to perform normal function and survive[35]. *PLCB3* and *PLCE1* both functioning as tumor suppressors are key enzymes in signal transduction[36,37]. *INADL* encoding a protein with multiple *PDZ* domains mediates protein-protein interactions[38]. The dysregulation of these three genes induced by chemotherapy in NSCLC patients may suggest the aberration of intracellular signaling cascade to respond to extracellular stimuli and thus leading to a weakened defense response system.

Cefoperazone was identified to be the drug mostly negatively related to the DEGs in NSCLC patients after chemotherapy, suggesting its potential use of being supplementary drug for NSCLC patients. This small molecule has been used in clinical trial successfully. For instance, cefoperazone is reported to be as effective as clindamycin/gentamicin and cefazolin/gentamicin being least expensive antibiotic regimen for the treatment of hospital-acquired pneumonia[39]. Intramuscular cefoperazone is also demonstrated to be safely used in the treatment of home-acquired pneumonia[40]. In terms of cancer treatment, cefoperazone could effectively play a role with low toxicity in the treatment of infections in patients[41]. With these successful clinical applications, cefoperazone is speculated to be a supplementary drug used in the treatment of NSCLC patients jointly with first-line chemotherapy.

Conclusions

On the basis of the comparative analysis in a global sense, this study revealed cisplatin-gemcitabine combination chemotherapy could prevent genes from aberrant methylation probably via regulating *CARM1* and *DNMT3B*, partially restored cell differentiation process by normalizing the expression of *CCL5*, *PPARG* and *IGF2* and failing to regulate *FOXA2* and *CTNNB1* expression, and fail to play a role in cytokine-cytokine receptor interaction pathway. Notably, this chemotherapy may induce a weakened defense response to bacteria and virus which may be remedied by cefoperazone as a supplementary drug.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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