Research Article

Diosmetin Affects Gene Expression on Human Lung Adenocarcinoma Cells

Changshan Song,1 Shunfu Deng,1 Hui Hu,1 Zheng Zheng,2 Bairu Shen,3 Xuhui Wu,3 Minqian Huang,3 Jiaqing Wang,3 and Zhenyu Wang2

1Department of Thoracic Surgery, Foshan Clinical Medical School of Guangzhou University of Chinese Medicine, Guangdong 528000, China
2Department of Thoracic Surgery, Affiliated Foshan Foxing Chancheng Hospital of Guangdong Medical University, Guangdong 528000, China
3Department of Thoracic Surgery, Foshan Foxing Chancheng Hospital, Guangdong 528000, China

Correspondence should be addressed to Changshan Song; songcs2000@163.com

Received 16 February 2022; Revised 25 April 2022; Accepted 26 April 2022; Published 20 May 2022

Academic Editor: Dong-Hua Yang

Copyright © 2022 Changshan Song et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Objective. This study was aimed at investigating the effects of diosmetin (a natural flavonoid) on the gene expression of human lung adenocarcinoma (LUAD) cells.

Methods. HCC827 and A549 cells were used. MTT and colony formation assay were used to investigate the effects of diosmetin on cell proliferation and colony forming activity. The expression of mRNA, microRNA, and lncRNA in HCC827 and A549 cell lines after diosmetin treatment was measured using DNA microarray, microRNA chromatin immunoprecipitation assay (ChIP), and long noncoding RNA (lncRNA) ChIP. Part of the results were cross-validated by quantitative reverse transcription PCR (RT-qPCR), while some others were analyzed using bioinformatic tools.

Results. Diosmetin inhibited proliferation and colony formation of HCC827 and A549 cells. Investigation on gene expression profiles of A549 and HCC827 cells revealed that compared with the control group, diosmetin can up- or downregulated the expression of mRNAs, microRNAs, and lncRNAs. The top three candidates in each RNA category were cross-validated by RT-qPCR, from which single peaks were observed in the melt curves, showing a great specificity. After a comprehensive selection of the results from the mRNA ChIP, we performed GO and KEGG functional clustering analyses on the differentially expressed genes.

Conclusion. Diosmetin treatment induced gene expression of A549 and HCC827 cells. Our results will provide guidance for development of new diagnostic and therapeutic targets.

1. Introduction

Lung cancer is one of the most common types of malignant tumors. It is the third most common cancer after breast cancer and prostate cancer, which seriously threatens human health and quality of life. Lung cancer has been the focus of attention of cancer researchers around the world [1]. Due to the lack of specific clinical manifestations, early-stage lung cancer can be easily confused with general respiratory tract inflammatory diseases, which leads to low rate of diagnosis. Most of the lung cancer cases are found in the middle and late stages, which has lost the best opportunity for surgery. For patients with surgical indications, surgery includes tumor resection and lymph node dissection. Surgery is currently the preferred treatment for lung cancer. However, according to statistics, after the surgical treatment, the 5-year survival rate of lung cancer is only 15%-20%. In the past two decades, anticancer drugs have been developed. Natural ingredients in Chinese herbal medicine, including some flavonoids and semisynthetic taxane derivatives, play a very important role in improving antitumor efficacy and developing specific chemotherapeutic drugs. Current chemotherapeutic drugs for lung adenocarcinoma (LUAD) have no
selectivity, low efficacy, severe toxicity, and side effects. Therefore, it is very important to find a safe and effective chemotherapeutic drug with more targets and less side effects.

Diosmetin is a monomeric flavonoid polyphenol compound. It is derived from various plants such as *Galium verum*, chrysanthemum, lemon, and citrus. In recent years, increasing numbers of studies on diosmetin have shown its effects on anti-inflammation, antitumor, anti-thrombosis, immune regulation, free radical scavenging, and other pharmacological effects [2, 3]. Ma and Zhang [4] found that diosmetin inhibits the proliferation of liver cancer cells by targeting checkpoint kinase 2 (Chk2) and promotes cancer cell apoptosis and cell cycle arrest. Yan et al. [5] found that diosmetin may activate E-cadherin expression and inhibit cell apoptosis and cell cycle arrest.

2. Materials and Methods

2.1. Cell Culture. The LUAD cell line HCC827 and A549 were provided by the Department of Thoracic Surgery, Foshan Clinical Medical College, Guangzhou University of Chinese Medicine. The cells were cultured in RPMI-1640 complete medium containing 10% fetal bovine serum, penicillin, and streptomycin and placed in a 5% CO2, saturated humidity, and 37°C constant temperature incubator. The cells in the logarithmic phase were taken for the experiment.

2.2. MTT Assay. HCC827 and A549 cells (2 × 10^3 per well) were seeded into 96-well plates and cultured in complete culture medium. After 12-hour incubation, the cells were incubated in the presence or absence of different concentrations of diosmetin for 24 hours. Then, 25 mL of MTT (5 mg/mL) was added and incubated for an additional 3 hours. After that, the medium was replaced with 100 mL DMSO to dissolve the crystals by shaking the plate for 10 minutes. Absorbance at 450 nm was read with a microplate reader (ELx800, BioTek Instruments Inc., USA).

2.3. Colony Formation Assay. HCC827 and A549 cells were seeded into 6-well plates (500/well) and placed in a 5% CO2, saturated humidity, and 37°C constant temperature incubator. After 12 hours, the cells were incubated in the presence or absence of different concentrations of diosmetin for 14 days. Then, the cells were fixed by 4% paraformaldehyde for 10 min and stained by 0.1% crystal violet for 5 min. The number of colonies (at least 50 colonies) was counted.

2.4. RNA Extraction and Quality Control and Microarray. HCC827 and A549 cells were seeded into 6-well plates (1 × 10^4/well). After 12 hours, the cells were incubated in the presence or absence of different concentrations of diosmetin for 24 hours. Then, total RNA was extracted from cells using TRIzol reagent according to the manufacturer’s instructions. NanoDrop was used to detect the purity and concentration of the extracted RNA. The microarray experiments were performed using the Affymetrix GeneChip Human Transcriptome Array (Affymetrix, MA, USA). The microarray comprises more than 40,000 noncoding transcripts (including IncRNAs, miRNAs, and small nucleolar RNAs). The cDNA labeling, microarray analysis, and bioinformatics analysis were performed by Gennminix Informatics (Gennminix, Shanghai, China). The aberrantly expressed transcripts (IncRNAs, miRNAs, and mRNAs) were identified using P values <0.05.

2.5. Purification of Total RNA. RNA was purified by using the Ambion miRVana miRNA Isolation Kit. Briefly, the RNA was dissolved in 50μl RNase-free water, and 200μl lysis/binding buffer and 24μl miRNA homogenate additive were added and mixed. The mixture was centrifuged and solution collected. It was put on ice bath for 10 min, added 330μl of ethanol, mixed and transferred to the RNA purification column, centrifuged, and the eluent discarded. The purification column was washed with 700μl miRNA Wash Solution 1, then 50μl of preheated miRNA Elution Solution.
2.6. **RT-qPCR Reaction.** The RT-qPCR reaction mixture (Table 1) was added to the AB TaqMan Human microRNA array plate and centrifuged at room temperature and 1200 rpm for 1 min, repeat for one time. The AB TaqMan Human MicroRNA Array plate was sealed.

2.7. **ChIP Result Clustered Analysis.** The results of miRNA expression were normalized. Cluster 3.0 was used to determine the cluster, and cluster analysis chart was drawn. The diosmetin group was compared with the control group with a 2-fold difference after normalization as the screening criterion for expression difference.

2.8. **RT-qPCR Verified Target Gene Expression in Cell Lines.** The RT-qPCR reaction condition was 95°C for 5 min, 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s, 40 cycles. After the reaction, the results were analyzed by the comparative Ct value method, and the formula is as follows: $\triangle \Delta Ct = [\text{Ct} \text{target} - \text{CtU6}] / [\text{Ct} \text{control} - \text{CtU6}]$. The expression level of target gene was calculated using the $2^{-\Delta \Delta Ct}$ method in the diosmetin-treated cells relative to the control cells.

2.9. **Statistical Analysis.** ChIP data was analyzed via Cluster 3.0 and Significance Analysis of Microarrays (SAM, version 2.1). The difference of 2 times in expression was used as the criterion for identifying differentially expressed genes.
3. Result

3.1. Diosmetin Inhibited the Viability and Colony Formation of HCC827 and A549 Cells. Previous studies have shown that diosmetin (Figure 1(a)), as a natural flavonoid present in legumes, olive leaves, and citrus plants, has anticancer activity. In the present study, the cell viability was assessed using MTT assays. The results showed that diosmetin treatment for 24 hours significantly inhibited cell growth in a dose-dependent manner (Figure 1(b)). Furthermore, colony formation assay showed that the proliferation of both HCC827 and A549 cells was inhibited by diosmetin with increasing concentration. Quantification analysis of the colony numbers showed that diosmetin inhibited cell viability and colony formation of HCC827 and A549 cells.

Table 2: Top 10 differentially expressed mRNAs in A549 cells treated with diosmetin.

| Upregulated mRNAs                          | Times of difference | Downregulated mRNAs                               | Times of difference |
|--------------------------------------------|---------------------|---------------------------------------------------|---------------------|
| Glucoside xylosyltransferase 2             | 25.52               | Phospholipase A2, group VI                        | 6.90                |
| Interleukin 7 receptor                     | 20.37               | Mucin 6, oligomeric mucus/gel forming             | 6.86                |
| Collagen, type V, alpha 1                  | 18.84               | 3-hydroxy-3-methylglutaryl-CoA reductase          | 6.84                |
| Bone morphogenetic protein 5               | 13.83               | Cytochrome P450, family 51, subfamily A, polypeptide 1 | 6.19                |
| Carboxypeptidase A4                        | 12.71               | Squalene epoxidase                                | 5.55                |
| Fibroblast growth factor 21                | 12.50               | Low density lipoprotein receptor                 | 4.24                |
| Insulin-like growth factor 1 (somatomedin C) | 9.64               | G protein-coupled receptor 180                    | 4.04                |
| Interleukin 6 (interferon, beta 2)         | 6.52                | Neuropilin 1                                      | 3.88                |
| p53-responsive gene 1                      | 5.86                | Myelin basic protein                              | 3.85                |
| GTP binding protein 2                      | 2.80                | Glutamate receptor interacting protein 2          | 3.75                |
proliferation in a dose-dependent manner (Figure 1(c)). These results indicated that diosmetin has the inhibitory effects on the proliferation and cell viability of lung adenocarcinoma cells.

3.2. ChIP Analysis. In order to investigate the effects of diosmetin on the gene expression of HCC827 and A549 cells, total RNA was isolated from cells, and the results showed that the RNA concentration was in the range of 100 ng/
μl~ 500 ng/μl, and the values at A260/A280 were between 1.8 and 2.0. RNA samples were subjected to formaldehyde denaturation agarose gel electrophoresis. 5s rRNA, 18s rRNA, and 28s rRNA bands are complete and clear. 28s:18s rRNA band brightness is greater than or close to 2 : 1, indicating that the RNA was not degraded (Figure 2).

The microarray results of mRNAs, microRNAs, and lncRNAs after applying diosmetin on A549 and HCC827 cells were clustered with cluster3.0, and part of the cluster analysis results is shown in Figure 3.

3.3. Diosmetin Affected the Expression of mRNAs, miRNAs, and lncRNAs in A549 and HCC827 Cells. The expression of mRNAs, miRNAs, and IncRNAs was different in A549 and HCC827 cells treated with diosmetin. Compared with the control group, the top 10 up- and downregulated mRNAs, microRNAs, and IncRNAs in A549 and HCC827 cells are shown in Tables 2–7.

3.4. RT-qPCR Verification. Three mRNAs, miRNAs, and IncRNAs with the highest expression in each group were
selected, and the ChIP results were verified by the RT-qPCR method. The RT-qPCR results are consistent with the ChIP (Figures 4–6).

3.5. Clustered Analysis of ChIP Results. After the A549 and HCC827 cells were treated with 4 μM diosmetin, the expression of mRNAs ChIP results was screened and integrated. The results obtained were subjected to GO and KEGG functional cluster analyses. It can be seen from Figures 7 and 8 that some differentially expressed gene clusters were enriched, which provides inspiration for further studies.

4. Discussion

LUAD is one of the most common cancers threatening human health. The main clinical treatment for LUAD is surgery removal combined with radiotherapy and/or chemotherapy. Due to the low sensitivity and side effect of radiotherapy and/or chemotherapy, the current clinical approaches are often not satisfactory. Thus, development of new antitumor drugs for LUAD has attracted a lot of attention. Diosmetin has an antitumor effect that closely related to the activity of cytochrome P450. Diosmetin is an inhibitor of the cytochrome P450 1A1 and 1B1 by acting as an antagonist of aryl hydrocarbon receptor and affects the activity of cytochrome P450 1A1. Thus, diosmetin can mediate the drug metabolism by inhibiting cytochrome P450 2C9 and 2C8 [11]. The antitumor effects of diosmetin through cytochrome P450 family 1 (CYP1) could be seen in three aspects: (1) inhibiting CYP1 enzyme activity; (2) acts as the substrate of CYP1; and (3) the combined effect of 1 and 2. A recent study revealed that in breast cancer, diosmetin can inhibit cell proliferation by inducing cell cycle arrest. Additionally, diosmetin has the antiproliferation and proapoptosis effects in MDA-MB-231 cells [12]. In ovarian cancer, it was found that ovarian cancer apoptosis is induced by activating reactive oxygen species and inhibiting NRF2 gene [13]. In lung cancer, some studies indicate that diosmetin can selectively induce cell apoptosis through ROS accumulation by disrupting the PI3K/Akt/GSK-3β/Nrf2 pathway and enhance the efficacy of paclitaxel in NSCLC cells [14]. However, a comprehensive understanding of diosmetin effect in LUAD is still lacking.

Noncoded RNA (ncRNA) is a class of noncoding transcripts involved in regulation of a variety of biological processes such as cell growth, proliferation, apoptosis, and differentiation. It affects target gene after reverse transcription, thereby regulating the translational level and
expression of that gene. In 180,000 transcripts of human cells, approximately 20,000 are protein coding, and the remaining 160,000 were noncoded transcripts. miRNA and lncRNA have the strongest association with lung cancer. miRNA is a set of noncoded RNAs between 20 NT and 25 NT that inhibits mRNA translation or enhances mRNA regulatory associated gene expression, thereby participating in cell proliferation, differentiation, and apoptosis [15]. Studies have shown that miR-33a-5p can activate Wnt/β-catenin signals through the JPX/Twist1 axis to participate in the EMT process, making lung cancer cells easy to metastasize [16, 17]. lncRNA is a class of noncoded RNA larger than 200 nT. It can be used as sponges of miRNA, or combined with enhancers to help their activity. For instance, it promotes the formation of chromatin loops and the recruitment of remodeling complexes. Zhou et al. demonstrated that lncRNA DLEU2 is upregulated in NSCLC tissues and cells, and by targeting miR-30c-5p, it can enhance the proliferation, invasion, and migration and reduce apoptosis of A549 and LLC cells [18]. L. Xu et al. revealed that compared to A549 cells, lncRNA SNHG14 and HOXB13 were upregulated, while miR-133a was downregulated in A549/DDP cells [19]. Knockdown of SNHG14 or overexpression of miR-133a has been shown to increase the DDP sensitivity of A549/DDP cells. Thus, this study uses RNA ChIP to observe the cell epigenetics changes after A549 and HCC827 cells were treated with diosmetin. Furthermore, bioinformatics was used to explore gene expression pathway regulated by diosmetin. In this study, the differentially expressed mRNAs, microRNAs, and lncRNAs caused by the effects of diosmetin on A549 and HCC827 cells were screened using RNA ChIP. The results were verified by RT-qPCR and both results are consistent. These results lay the foundation for future clinical uses of diosmetin.

In addition to diosmetin, other natural or synthetic compounds also have inhibitory effects on LUAD. In terms of inducing apoptosis, for example, curcumin and dimethylolcoumarin can partially inhibit the ERK/MAPK signaling pathway through the ROS-independent mitochondrial pathway to induce cell apoptosis. Digitalis flavonoids induce mitochondrial apoptosis in LUAD cells. Myricetin extracted from bayberry bark can inhibit the proliferation and induce apoptosis of A549 cells. Leucocephalus can regulate c-Myc/S phase protein, WD repeat sequence protein, 7/histidine deacetylase, and other signaling pathway to induce apoptosis of LUAD cells. Pine cones induce apoptosis of LUAD cells by activating caspase-3. In A549 cells, the epidermal growth factor inhibitor gefitinib can upregulate apoptotic molecules and downregulate antiapoptotic molecules through a p53-dependent pathway. Other molecules, oxidized carotene, can inhibit proliferation of A549 cells. Green tea can upregulate the expression of annexin 1 to promote actin rearrangement in A549 cells. The proanthocyanidins in grape seeds can inhibit the metastasis of non-small-cell lung cancer by inhibiting the activities of nitric oxide, guanylate cyclase, and ERK1/2. Schizandrin inhibits the proliferation of A549 cells by controlling cell cycle and inducing cell apoptosis.

Taken together, diosmetin affects the gene expression and proliferation of human lung adenocarcinoma A549 and HCC827 cells. This study provides a rationale for using diosmetin for lung cancer treatment.

![Figure 8: The GO function clustering analysis on A549 and HCC827 cells treated with 4 μM diosmetin.](image-url)
**Data Availability**

Emails could be sent to the address songcs2000@163.com to obtain the shared data.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**References**

[1] G. S. Hoes and D. R. Baldwin, “Recent advances in the management of lung cancer,” *Clinical Medicine*, vol. 18, Supplement 2, pp. s41–s46, 2018.

[2] T. Ahmad, A. J. Shah, T. Khan, and R. Roberts, “Mechanism underlying the vasodilation induced by Diosmetin in porcine coronary artery,” *European Journal of Pharmacology*, vol. 884, article 173400, 2020.

[3] S. A. Park, S. K. Bong, J. W. Lee et al., “Diosmetin and its glycoside, diosmin, improve atopic dermatitis-like lesions in 2,4-dinitrochlorobenzene-induced murine models,” *Biomolecules & Therapeutics*, vol. 28, no. 6, pp. 542–548, 2020.

[4] A. Ma and R. Zhang, “Diosmetin inhibits cell proliferation, induces cell apoptosis and cell cycle arrest in liver cancer,” *Cancer Management and Research*, vol. 12, pp. 3537–3546, 2020.

[5] Y. Yan, X. Liu, J. Gao, Y. Wu, and Y. Li, “Inhibition of TGF-β signaling in gliomas by the flavonoid diosmetin isolated from Draccephalum peregrinum L,” *Molecules*, vol. 25, no. 1, p. 192, 2020.

[6] A. Friedlaender, A. Drilon, G. J. Weiss, G. L. Banna, and A. Addeo, “KRAS as a druggable target in NSCLC: rising like a phoenix after decades of development failures,” *Cancer Treatment Reviews*, vol. 85, article 101978, 2020.

[7] V. Aran and J. Omerovic, “Current approaches in NSCLC targeting K-RAS and EGFR,” *International Journal of Molecular Sciences*, vol. 20, no. 22, p. 5701, 2019.

[8] Y. Lu, Y. Liu, S. Oeck, G. J. Zhang, A. Schramm, and P. M. Glazer, “Hypoxia induces resistance to EGFR inhibitors in lung cancer cells via upregulation of FGFR1 and the MAPK pathway,” *Cancer Research*, vol. 80, no. 21, pp. 4655–4667, 2020.

[9] S. Hokari, Y. Tamura, A. Kaneda et al., “Comparative analysis of TTF-1 binding DNA regions in small-cell lung cancer and non-small-cell lung cancer,” *Molecular Oncology*, vol. 14, no. 2, pp. 277–293, 2020.

[10] D. Jiao, J. Chen, Y. Li et al., “miR-1-3p and miR-206 sensitizes HGF-induced gefitinib-resistant human lung cancer cells through inhibition of c-met signalling and EMT,” *Journal of Cellular and Molecular Medicine*, vol. 22, no. 7, pp. 3526–3536, 2018.

[11] M. Poór, G. Boda, V. Mohos et al., “Pharmacokinetic interaction of Diosmetin and silibinin with other drugs: inhibition of CYP2C9-mediated biotransformation and displacement from serum albumin,” *Biomedicine & Pharmacotherapy*, vol. 102, pp. 912–921, 2018.

[12] C. Wang, S. Li, H. Ren et al., “Anti-proliferation and pro-apoptotic effects of diosmetin via modulating cell cycle arrest and mitochondria-mediated intrinsic apoptotic pathway in MDA-MB-231 Cells,” *Medical Science Monitor*, vol. 25, pp. 4639–4647, 2019.

[13] F. Zhao, X. Hong, D. Li, Z. Wei, X. Ci, and S. Zhang, “Correction to: Diosmetin induces apoptosis in ovarian cancer cells by activating reactive oxygen species and inhibiting the Nrf2 pathway,” *Medical Oncology*, vol. 38, no. 7, 2021.

[14] X. Chen, Q. Wu, Y. Chen et al., “Diosmetin induces apoptosis and enhances the chemotherapeutic efficacy of paclitaxel in non-small cell lung cancer cells via Nrf2 inhibition,” *British Journal of Pharmacology*, vol. 176, no. 12, pp. 2079–2094, 2019.

[15] M. Piwecka, P. Głąż, L. R. Hernandez-Miranda et al., “Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function,” *Science*, vol. 357, pp. 6357–6357, 2017.

[16] J. Pan, S. Fang, H. Tian et al., “IncRNA JPX/miR-33a-5p/Twist1 axis regulates tumorigenesis and metastasis of lung cancer by activating Wnt/β-catenin signaling,” *Molecular Cancer*, vol. 19, no. 1, 2020.

[17] Z. Huang, S. Li, Y. Ma, N. Wu, and Y. Yang, “Expression of MiR-148b-3p in lung adenocarcinoma and its correlation with prognosis,” *Zhongguo Fei Ai Za Zhi*, vol. 22, no. 5, pp. 306–311, 2019.

[18] Y. Zhou, H. Shi, Y. Du et al., “IncRNA DLEU2 modulates cell proliferation and invasion of non-small cell lung cancer by regulating miR-30c-5p/SOX9 axis,” *Aging*, vol. 11, no. 18, pp. 7386–7401, 2019.

[19] L. Xu, Y. Xu, M. Yang, J. Li, F. Xu, and B. L. Chen, “IncRNA SNHG14 regulates the DDP-resistance of non-small cell lung cancer cell through miR-133a/HOXB13 pathway,” *BMC Pulmonary Medicine*, vol. 20, no. 1, p. 266, 2020.