Original Article
Diphenyl difluoroketone inhibits energy metabolism and biological behavior of lung cancer cells by down-regulating glucose transporter 1 expression

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Abstract: The present study is to evaluate the effect of diphenyl difluoroketone (EF24), an analogue of curcumin, on energy metabolism and biological behavior of lung cancer A549 cells. Glut1 protein and mRNA expression levels were examined by Western blotting and quantitative real-time polymerase chain reaction, respectively. After EF24 treatment, glucose uptake and the rate of glycolysis were measured. In addition, transwell assay was used to determine the invasive ability of A549 cells. The proliferation ability of A549 cells was detected using MTT assay. Glut1 protein and mRNA expression in tumor tissues was significantly higher than that in adjacent tissues or normal lung tissues. EF24 treatment significantly down-regulated the expression of Glut1 in a dose-dependent manner in A549 cells. In addition, glucose uptake and the rate of glycolysis of A549 cells were also decreased by treatment with EF24. Invasion and proliferation of A549 cells were inhibited by treatment with EF24. The presents study demonstrates that EF24 exerts inhibitory effects on energy metabolism and biological behavior in A549 cells probably through down-regulation of Glut1 expression. EF24 can be a possible drug candidate for the treatment of lung cancer.

Keywords: Diphenyl difluoroketone, glucose transporter 1, lung cancer, A549 cells, glucose uptake, glycolysis, invasion, proliferation

Introduction

Lung cancer is one of the most common tumors. Despite the advances in standardized treatment regimens, 5-year survival rates for lung cancer are still relatively poor [1]. The mortality rate is even higher due to the ineffectiveness of conventional surgery and chemotherapy [2]. New chemotherapy drugs can extend the survival time of patients, but toxicity and side effects limit their clinical application [3]. Cancer cell is characterized by enhanced proliferation and changes of expression of genes and proteins, and these characteristics are found to correlate with glucose uptake and glycolysis [4]. Glucose uptake and glycolysis result in enhanced cell malignant transformation, invasion, metastasis and resistance to chemotherapy and radiotherapy. As a result, current treatments are difficult to achieve expected results. Diphenyl difluoroketone (EF24), a molecule that

has structural similarity to curcumin, is reported to inhibit the proliferation of various cancer cells significantly. Sun et al. show that the cytotoxic effect of EF24 against MDA-MB-231 human breast cancer cells, RPMI-7951 human melanoma cells and DU-145 human prostate cancer cells arises, at least in part, from the induction of cell cycle arrest and subsequent apoptosis by means of redox-dependent mechanism, and EF24-tripeptide chloromethyl ketone drug delivery system can increase the effect of EF24 [5]. Dharmalingam et al. report that EF24 treatment on HCT-116 and HT-29 colon and AGS gastric adenocarcinoma cells results in growth inhibition without affecting the proliferation of normal human fibroblasts [6]. Thomas et al. find that EF24-induced decease of lung cancer cell viability is accompanied by up-regulated mitogen-activated protein kinases as evidenced by increased phosphorylation of ERK1/2, JNK, and p38 [7]. These

results suggest that the novel curcumin-related compound EF24 is a potent antitumor agent [5, 8, 9]. A recent study shows that overexpression of glucose transporter 1 (Glut1), one of the key factors involved in the regulation of aerobic glycolysis, is closely related to aggressive and vigorous growth of non-small cell lung cancer cells accompanied by increasing glucose metabolism [10]. It has been known that facilitative glucose transporter (Glut) is the main carrier that intervenes with the glucose uptake of cells. Glut1 appears to be the most ubiquitously distributed transporter isoform. It is expressed in many fetal and adult mammalian tissues and cell types, although frequently at low levels and in conjunction with more tissue-restricted glucose transporter isoforms. Glut1 mRNA is detected at every stage of early mouse embryo, from oocyte to blastocyst [11]. Ito et al. find that Glut1 is expressed in 45 out of 61 lung carcinomas (74%), including all of 19 squamous cell carcinomas (100%). No Glut1 staining is seen in normal lung epithelium surrounding the tumors. In squamous cell carcinomas and small cell carcinomas, Glut1 immunostaining is stronger in the central area of tumor cell nests corresponding to the hypoperfused region. Focal staining is seen in 14 out of 24 adenocarcinomas (58%), and positive staining is correlated to lesser differentiation, larger tumor size, and positive lymph node metastasis [12]. Ogawa et al. find that Glut1 remains a statistically significant prognostic factor in a multivariate analysis of survival. Their results suggest that Glut1 amplification may participate in sLe(x) synthesis as well as in proliferation, and may be of prognostic value in lung cancer [13]. Thomas et al. find that treatment of MDA-MB231 breast cancer cells and PC3 prostate cancer cells with EF24 leads to the inhibition of HIF-1α transcriptional activity [14]. Previous reports show that the oncogenic factor HIF-1α is implicated in regulating aerobic glycolysis and the expression of Glut1 [15]. It is suggested that downregulation of Glut1 expression inhibits the development of lung cancer. In the present study, we investigate the effect of EF24 on the invasive and proliferation ability of lung cancer cell A549 mediated by Glut1.

Materials and methods

Patients

A total of 65 lung cancer patients who had dissection surgery at the Second Hospital of Shandong University between January 2003 and December 2008 were included in the present study. None of the patients received preoperative chemotherapy or radiotherapy. Tumor tissues were taken from primary tumor area, avoiding necrosis and inflammatory areas. Adjacent tissues were obtained from sites two centimeters away from the tumor tissues, and normal lung tissues were collected from sites five centimeters away from the tumor edge. All procedures were approved by the Ethics Committee of Shandong University. Written informed consents were obtained from all patients or their families.

Cells

Human lung cancer cell line A549 cells were purchased from Shanghai Cell Institute of Chinese Academy of Sciences. A549 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA).

Western blotting

Cytoplasmic protein was extracted from 500 mg tumor tissue, and protein concentration was determined using the Bradford method. The protein (100 μg) was added to each well for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride (PVDF) membrane for Ponceau red staining. PVDF membrane was incubated with 5% bovine serum albumin at 37°C for 1 h, followed by incubation with rabbit anti-human monoclonal antibody (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) at 37°C for 1 h and then donkey anti-rabbit IgG labeled by horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA) at 37°C for 1 h. β-actin was used as control. The signals were detected using electrochemiluminescence (ECL) by Clinical Laboratory of The Second Hospital of Shandong University, Jinan, China. The experiment was repeated for three times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 50-100 mg tissue using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' manual. RNA purity and concentration were measured using a spectrophotometer (Bio-Rad, Hercules, CA, USA). RNA
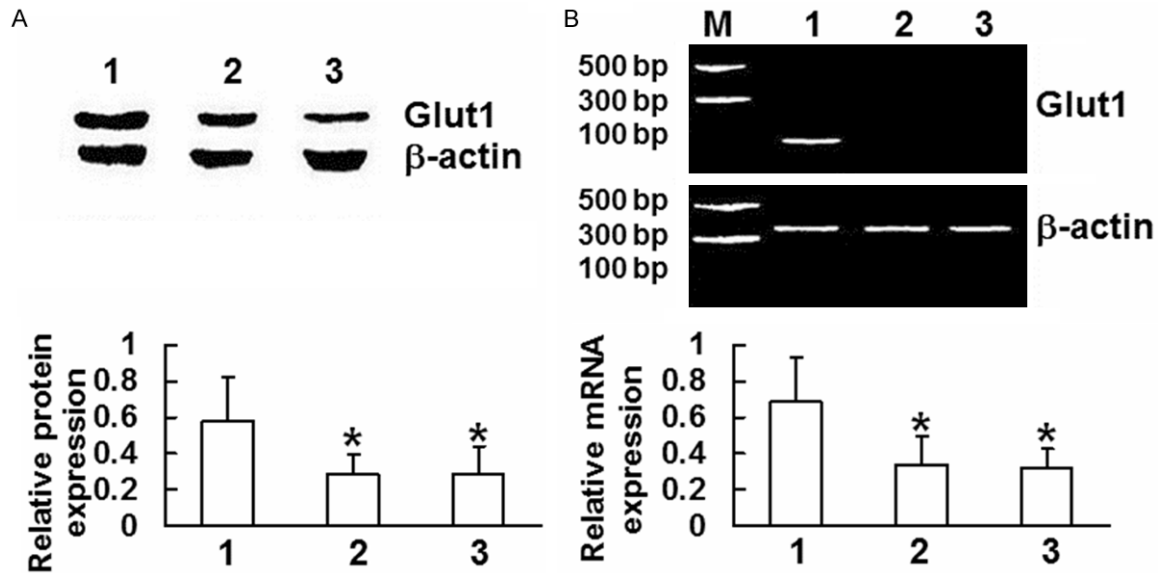
Figure 1. Expression of Glut1 in lung cancer tissues, adjacent tissues and normal lung tissues. A. Glut1 protein expression determined using Western blotting. B. Glut1 mRNA expression measured using quantitative real-time polymerase chain reaction. The data were expressed as means ± standard deviation. 1, tumor tissues; 2, adjacent tissues; 3, normal lung tissues. All experiments were performed in triplicate. *P < 0.05 compared with lung cancer tissues.
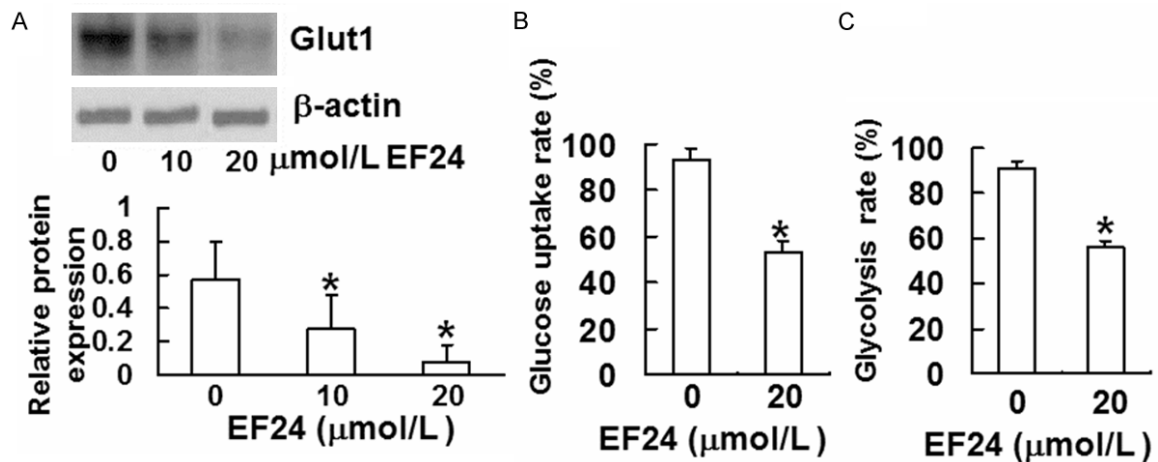

Figure 2. Effect of EF24 treatment on (A) Glut1 protein expression, (B) glucose uptake and (C) glycolysis in A549 cells. After treatment with EF24 (0, 10, and 20 μmol/L), Glut1 protein expression was measured using Western blotting. After treatment with EF24 (0 and 20 μmol/L), glucose uptake and glycolysis was monitored as described previously [5-8]. The data were expressed as means ± standard deviation. All experiments were performed in triplicate. *P < 0.05 compared with lung cancer tissues.

integrity was verified using 1% formaldehyde denaturing gel electrophoresis. Glut1 mRNA expression was detected by semi-qRT-PCR kit (Takara, Tokyo, Japan). β-actin was used as internal reference. Primers were designed according to the literature [13]. Glut1 primers were 5'-CAACTGTGTGGTCCCTACGTCTTC-3' (upstream) and 5'-TCACACTTGG GAATCAGCCCC-3' (downstream). β-actin primers were 5'-CGCTG-CGCTGGTCGT CGACA-3' (upstream) and 5'-GT-CACGCACGATTTCCCGCT-3' (downstream). The amplification reaction involved 28 cycles of reverse transcription at 50°C for 30 min, denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 5 min. PCR products were scanned and photographed

Table 1. The number of invasive cells after treatment with different concentrations of EF24 for different time periods (means ± standard deviation)

EF24 concentrations (µmol/L)	24 h	48 h	72 h
0	48.5 ± 1.22	53.6 ± 1.32	54.9 ± 0.87
10	27.5 ± 0.82*	23.5 ± 0.54*	20.6 ± 0.49*
20	16.3 ± 0.75*	12.3 ± 0.36*	10.1 ± 0.39*
30	10.2 ± 0.66*	8.2 ± 0.35*	7.2 ± 0.65*
40	5.32 ± 0.57*	4.10 ± 0.44*	3.11 ± 0.83*

Note: *P < 0.05 compared with 0 µmol/L EF24.

with the Fluor-S multi-functional imaging system (Bio-Rad, Hercules, CA, USA) after 1.5% agarose gel electrophoresis.

Measurement of glucose uptake and the rate of glycolysis

Glucose uptake and the rate of glycolysis were measured by determining the uptake of 2-[3H] deoxyglucose by cells and the conversion of 5-[3H] glucose to 3 H_2O, respectively, as described previously [16-19].

Transwell assay

The 24-well matrix gel invasion chambers (BD Biosciences, Frankin Lakes, NJ, USA) were used. Extracellular matrix gel was covered onto the upper chamber membrane surface. Different concentrations of EF24 (0, 10, 20, 30, and 40 µmol/L) was added onto A549 cells, and mixed with serum-free RPMI-1640 to reach a density of 2×10⁵ cells/ml after 24 h culture. Cell suspension (200 µl) was added to the upper chamber, and 500 µl RPMI-1640 supplemented with 10% fetal calf serum was added to the lower chamber. After 24, 48, and 72 h culture, cells that moved into the lower chamber were fixed with formaldehyde, and observed under a light microscope (400×; Olympus, Tokyo, Japan). The numbers of cells in six fields were randomly counted for the calculation of mean value, which was used as the number of invasive cells.

MTT assay

Cells were added into 96-well plate after its density was adjusted to 1×10⁴ cells per well using serum-free RPMI-1640 medium. After being cultured overnight, different concentra-

tions of EF24 (10, 20, 30, and 40 µmol/L) was added. RPMI-1640 was used in control group. MTT (20 µl per well) and DMSO (15 µl per well) were mixed after being incubated for 24, 48, 72 h in a humidified atmosphere of 5% CO_2 at 37°C. Absorbance of each well was measured at 490 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell proliferation inhibition rate = (1-absorbance values in experimental groups/absorbance value in control group) × 100.

Statistical analysis

All results were expressed as means ± standard deviation. SPSS 11.5 statistical software (IBM, Armonk, NY, USA) was used for statistical analysis. Student's *t*-test was used for comparison between groups. *P* < 0.05 was considered statistically significant.

Results

Expression of Glut1 is elevated in lung cancer tissues

To measure Glut1 expression, Western blotting and qRT-PCR were employed. The data showed that Glut1 expression in tumor tissues, adjacent tissues and normal lung tissues was 0.581 ± 0.246, 0.290 ± 0.101 and 0.287 ± 0.152, respectively, suggesting that the expression of Glut1 protein in tumor tissues was significantly higher than that in adjacent tissues or normal lung tissues (P < 0.05) (**Figure 1A**). The qRT-PCR data showed that Glut1 mRNA expression in tumor cells (0.688 ± 0.246) was significantly higher than that in adjacent tissues (0.337 ± 0.156) and normal lung tissues (0.322 ± 0.102) (P < 0.05) (**Figure 1B**). These results indicate that Glut1 expression is elevated in lung cancer tissues.

EF24 decreases Glut1 expression in A549 cells

To study the effect of EF24 on the expression of Glut1 in A549 cells, 0, 10, and 20 µmol/L EF24 was added onto A549 cells. The data showed that EF24 treatment significantly decreased Glut1 expression in A549 cells (0.573 ± 0.223, 0.271 ± 0.211 and 0.077 ± 0.102 for 0, 10, and 20 µmol/L EF24, respectively) (P < 0.05)
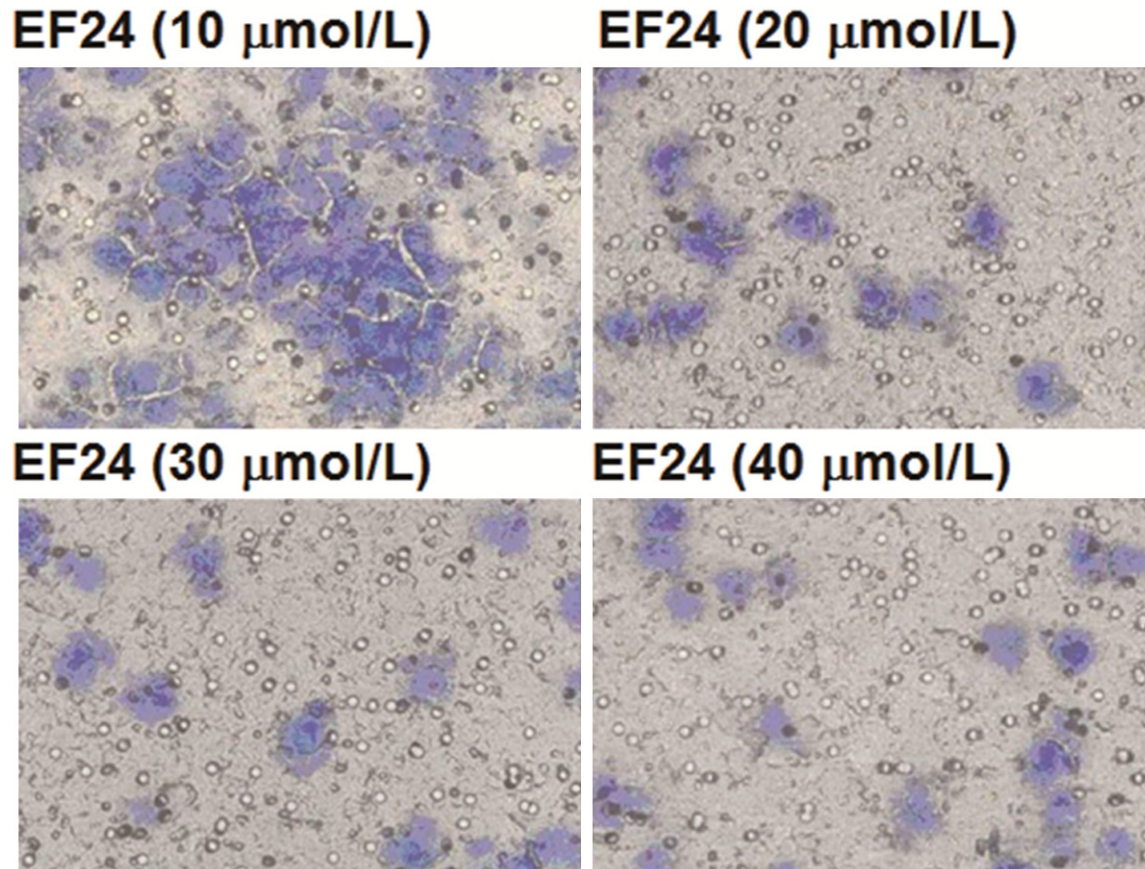
Figure 3. Effect of EF24 on the invasion ability of A549 cells. Transwell assay was performed to measure the invasion ability of A549 cells. Different concentrations of EF24 (10, 20, 30, and 40 μmol/L) was added onto A549 cells, and mixed with serum-free RPMI-1640 to reach a density of 2×10^5 cells/ml after 24 h culture. After culture for 72 h, cells that moved into the lower chamber were fixed with formaldehyde, and observed under a light microscope (400×; Olympus, Tokyo, Japan).

(**Figure 2A**). The results suggest that EF24 decreases Glut1 expression in A549 cells.

EF24 blocks glucose metabolism of A549 cells

To test whether EF24 affects glucose metabolism of A549 cells, glucose uptake and the rate of glycolysis were measured. After treatment with 20 μmol/L EF24, glucose uptake and the rate of glycolysis were both decreased significantly compare to control group (0 μmol/L EF24) ($P < 0.05$) (**Figure 2B** and **2C**). These results indicate that EF24 is an effective inhibitor against aerobic glycolysis.

EF24 inhibits the invasion and proliferation of A549 cells

To test how EF24 affects the invasion and proliferation of A549 cells, Transwell assay and MTT assay were performed. Transwell assay showed that the number of transmebrane cells

was significantly reduced in EF24 groups than in control group in a dose- and time-dependent manner ($P < 0.05$) (**Table 1**). After treatments, the cell structure was not clear under a light microscope, and the invasion on the reconstituted basement membrane was weakened (**Figure 3**). MTT assay showed that the proliferation of A549 cells was significantly inhibited at different degrees in a dose- and time-dependent manner after treatment with different concentrations of EF24 from 24 h to 72 h ($P < 0.05$). The inhibition rate was the highest at 40 μmol/L and 72 h (**Table 2**). These results suggest that EF24 inhibits the invasion and proliferation of A549 cells.

Discussion

Lung cancer is the leading cause of cancer deaths in males and the second in females all over the world. Despite advances in early detection and standardized treatment regimens,

Table 2. Inhibitory effect of EF24 on A549 cells (means ± standard deviation)

EF24 concentrations (µmol/L)	Inhibitory rates		
	24 h	48 h	72 h
0	0.342 ± 0.016	0.486 ± 0.015	0.614 ± 0.016
10	0.287 ± 0.017*	0.367 ± 0.012*	0.445 ± 0.017*
20	0.283 ± 0.021*	0.337 ± 0.023*	0.396 ± 0.022*
30	0.254 ± 0.022*	0.297 ± 0.014*	0.363 ± 0.014*
40	0.227 ± 0.010*	0.273 ± 0.014*	0.331 ± 0.017*

Note: *$P < 0.05$ compared with 0 µmol/L EF24.

5-year survival rates for lung cancer are still poor. Typically, lung cancer is at its advanced stage at the time of diagnosis, and treatments are usually not very effective [20]. Approximately 80% of lung cancer patients are inoperable at diagnosis, and normal chemotherapy is unable to effectively prevent the growth of the tumor. Therefore, it is of great significance to look for a new target for therapy. Warburg et al. discover that tumor cells prefer glycolysis to glucose metabolism even with adequate oxygen supply (Warburg effect) [21]. Aerobic glycolysis, the most basic metabolic changes during tumor malignant transformation process, can promote tumor cell proliferation and invasion, and mediate drug resistance [22, 23]. Many studies [24] have demonstrated that produce of Warburg effect and tumor cells' dependence on Warburg effect are determined by internal and external environments, as well as multiple genes and signal-regulated kinases. Spontaneous changes of many oncogenes and tumor suppressor genes are involved in the regulation of energy metabolism [25].

Glucose transporter (492-524 bp) is a kind of protein embedded in the cell membrane as a carrier for transporting glucose. Glut1 has a higher affinity to glucose and can efficiently transport glucose under normal physiological conditions. Glut1 is the most widely distributed in the body and presents overexpression in almost all tumor tissues [26]. Glut1 is a rate-limiting transporter for glucose uptake, and its expression correlates with anaerobic glycolysis [27]. Therefore, derivatives of Glut1 inhibitors might generate therapeutic effect in lung cancer. Curcumin is an active ingredient extracted from roots or stems of turmeric genus plants, and is characterized by antioxidant, anti-inflammatory, an-ti-viral, safe, and side effect-free properties. Curcumin is regarded as the third generation anti-cancer chemopreventive agent [28]. Adams et al. [29] has studied more than 100 curcumin analogues based on its molecular structure and demonstrated anti-tumor effect, in which EF24 is the most prominent.

In our study, Glut1 expression is high in tumor tissues not only at protein level but also at mRNA level, being consistent with literatures. Considering the important role of Glut1 in regulating glucose metabolism, it is useful to examine whether EF24 targets Glut1 for its anti-metabolism effect. Our results demonstrate that EF24 treatment significantly down-regulates Glut1 expression in a dose-dependent manner in A549 cell, as well as glucose metabolism. Furthermore, biological behaviors of A549 cells including invasion and proliferation are also inhibited after treatment with EF24. In summary, the present study demonstrates the inhibitory effect of EF24 on energy metabolism in lung cancer cells A549 through the down-regulation of Glut1 expression. This effect further leads to the inhibition of cell invasion and proliferation. Therefore, EF24 is a potential drug candidate for the treatment of lung cancer in the future.

Acknowledgements

Disclosure of conflict of interest

None.

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