Research Paper

Effect of Erlotinib Combined with Radiotherapy on Proliferation and Apoptosis of Human Non-Small Cell Lung Cancer Cells and its Possible Mechanism Exploration

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To explore the effects and possible mechanisms of erlotinib combined with radiotherapy on the proliferation and apoptosis of human non-small cell lung cancer cells. Blank control group, non-small cell lung cancer group and erlotinib group were set cells in the blank control group were human bronchial epithelioid cells without any treatment and cultured routinely. The proliferation ability of the cells in the three groups was detected by cell counting kit-8, Western blot was used to assess protein expression, quantitative polymerase chain reaction was used to measure messenger ribonucleic acid expression, and Transwell was used to determine metastatic potential. The quantity of migrating cells and the rate of cell growth of non-small cell lung cancer group were higher than that of blank control group; the cell metastasis activity number of erlotinib group were reduced than that of non-small cell lung cancer group. The cell invasion number of non-small cell lung cancer group was higher than blank control group; erlotinib group was reduced than that of non-small cell lung cancer group. The overall apoptosis rate was higher than the non-small cell lung cancer group. The apoptotic proteins Fas, B-cell lymphoma 2-associated X protein and Fas ligand were higher in the erlotinib group than in the non-small cell lung cancer group, and the B-cell lymphoma 2 was reduced than the non-small cell lung cancer group. The 5' adenosine monophosphate-activated protein kinase and peroxisome proliferator-activated receptor coactivator-1 alpha in the non-small cell lung cancer group were reduced than the blank control group and erlotinib group were higher than the non-small cell lung cancer group. The protein mitogen-activated protein kinase and peroxisome proliferator-activated receptor coactivator-1 alpha in the non-small cell lung cancer group were reduced than the blank control group and erlotinib group were higher than those in the non-small cell lung cancer group. The mechanism of erlotinib's capacity to lower the viability of non-small cell lung cancer cells, block their proliferation, migration, and invasion ability, and induce apoptosis may be connected to the control of the 5' adenosine monophosphate-activated protein kinase/ peroxisome proliferator-activated receptor coactivator-1 alpha signaling pathway.

Key words: Erlotinib, radiotherapy, non-small cell lung cancer, proliferation, apoptosis

Lung cancer is becoming more common each year due to the world economy's rapid growth and the speed at which industry is developing[1]. Among them, Non-Small Cell Lung Cancer (NSCLC) is the main pathological type in the clinical diagnosis of lung cancer and about 80 % of lung cancer patients are part of its incidence group, which has a high death and morbidity rate[2]. Clinical studies have found that Epidermal Growth Factor Receptor (EGFR) gene exon 19 mutations are the most typical in NSCLC, which can trigger enhanced activity of EGFR tyrosine kinase, promote biological behaviors such as tumor cell implantation, metastasis and invasion and then lead to tumor progression[3]. Because NSCLC patients do not have obvious clinical symptoms in the early

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stage of the disease, most patients have progressed to advanced pathological stage when lung cancer is diagnosed, and distant metastases have occurred, and the best time for surgical treatment is lost[4]. In recent years, with the continuous improvement of medical technology, molecularly targeted therapy drugs have played an important role in the drug treatment of patients with EGFR-mutated NSCLC, among which small molecule tyrosine kinase inhibitors can be multi-targeted anti-angiogenic and have significant clinical effects in preclinical studies[5]. As one of the tyrosine kinase inhibitors, erlotinib can effectively inhibit tumor growth by effectively blocking the receptors for both epidermal and fibroblast growth factors[6]. In addition, the 5' Adenosine Monophosphate-activated Protein Kinase (AMPK)/Peroxisome Proliferator-Activated Receptor Coactivator-1 Alpha (PGC-1α) signaling pathway is closely related to tumorigenesis and development, and is an important proliferative and anti-apoptotic pathway in cells[7]. In order to identify novel therapeutic targets for the treatment of clinical NSCLC, we examined the impact and potential mechanism of erlotinib in combination with radiation on the proliferation and apoptosis of human NSCLC cells in this study.

MATERIALS AND METHODS

Materials and reagents:

Human Bronchial Epithelioid cells (HBE) and human NSCLC cell line (NCI-H524) cells were from the ATCC cell bank. Erlotinib was purchased from Roche. AMPK, PGC-1α, messenger Ribonucleic Acid (mRNA) primers (Sigma, United States of America (USA)); quantitative Polymerase Chain Reaction (qPCR) detection kit, Cell Counting Kit-8 (CCK-8) detection kit, Annexin V- Fluorescein Isothiocyante (FITC)/Propidium Iodide (PI) apoptosis kit (Shanghai Biyuntian Company); Transwell Cab (Corning, USA); Artificial reconstituted basement membrane adhesive (Matrigel) was from BD Company; AMPK, PGC-1α, Fas, B-cell lymphoma 2 (Bcl 2)- Associated X Protein (BAX), Fas Ligand (FasL), Bcl-2 primary antibody (Abcam, United Kingdom).

Methods:

Cell culture: Following the cell's freezing, they were inoculated in T25 flasks with Dulbecco's Modified Eagle Medium (DMEM) media that included 10 % Fetal Bovine Serum (FBS) and kept in an incubator at a constant temperature, and trypsinized, passaged and frozen according to the cell growth. The Blank Control Group (BCG), NSCLC group (NSG) and Erlotinib Group (ERG) were set up. The cells in the BCG were HBE cells and were routinely cultured without any treatment. The cells in the NSG were human NSCLC cell line (NCI-H524), radiotherapy treatment, and routine culture. The cells in the ERG were treated with NCI-H524 after radiotherapy treatment with erlotinib at 50 μmol/l and the culture continued in the incubator for 48 h. Each experiment is repeated 6 times.

Western blot:

NSCLC cell line (NCI-H524) cells to erlotinib 50 μmol/l and continue to culture in the incubator for 48 h, the protein content was measured by Bicinchoninic Acid (BCA) after each set of cells was added to the cell protein lysate and homogenized at 4° to create a 10 % homogenate. The supernatant was then centrifuged for testing, gelatinization was made, electrophoresis was 90 min, glue cutting, film transfer was 90 min and milk was sealed. After washing, they were generated using Bio-Rad imaging laboratory software, treated with primary and secondary antibodies and then examined.

qPCR: NSCLC cell line (NCI-H524) was added to erlotinib 50 μmol/l and continued to be cultured in the incubator for 48 h. The complementary Deoxyribonucleic Acid (cDNA) synthesis kit reverse transcribed microRNA (miRNA) into cDNA and the miRNA fluorescence qPCR detection kit was used for quantitative real-time PCR and the cycle was finished in accordance with the kit's guidelines. Once the reaction was finished, the program estimated PGC-1α and AMPK mRNA.

CCK8: After the human NSCLC cell line (NCI-H524) was added to erlotinib 50 μmol/l and continued to be cultured in the incubator for 48 h, cells was added with 10 μl of CCK8 solution per well, and after 4 h of culture, the proliferation ability of each group of cells was detected by microplate reader (Optical Density (OD) 450 nm). Using flow cytometry, each group's apoptosis was identified following the addition of the appropriate reagents in accordance with the apoptosis kit's instructions.

Transwell method to detect cell migration and invasion: After the human NSCLC cell line (NCI-H524) cells were added to erlotinib 50 µmol/l and continued to be cultured in the incubator for 48 h, the cell density of each group was adjusted to 5×10^5 cells/well and seeded in the upper chamber of Transwell, the whole medium in the lower chamber was supplemented with 15 % FBS. Following a 48 h period of propofol administration for both the high-dose and low-dose groups, the control group received an identical quantity of DMEM culture media, fixed and stained. The Transwell chamber's top chamber is covered with Matrigel in a clean bench to evaluate the cell invasion capacity. The next stages are the same as those for cell migration.

Statistical methods:

The data were processed and analyzed using the statistical software Statistical Package for the Social Sciences (SPSS) 22.0. The continuous data were expressed by ($\bar{x}\pm s$), the multi-group comparison was done using F-variance analysis, the pairwise comparison between multiple groups was done using the Least Significant Difference (LSD)-t test, and the difference was statistically significant at $p<0.05$. Compared with the BCG $^ap<0.05$, compared with NSG $^bp<0.05$

RESULTS AND DISCUSSION

The cell proliferation activity and cell migration number in the NSG were higher than the BCG. The cell proliferative activity and cell migration number of ERG were reduced than those of NSG, as shown in Table 1. The number of cell invasion in the NSG was higher than that in the BCG. Compared to the NSG, there were less cell invasions in the ERG, and the overall apoptosis rate was higher than that in the NSG, as shown in Table 2.

The apoptosis proteins Fas, Bax and FasL in the ERG were higher than the NSG and the Bcl-2 were reduced than the NSG as shown in Table 3. The AMPK and PGC-1α in the NSG were reduced than the BCG. The AMPK and PGC-1α in the ERG were higher than the NSG, as shown in Table 4.

TABLE 1: EFFECT OF ERLOTINIB ON THE PROLIFERATION AND MIGRATION OF NSCLC CELLS

Group	n	Proliferative activity	Number of migrations
BCG	6	0.10±0.01	73.25±15.70
NSG	6	0.22±0.04[a]	142.74±24.34[a]
ERG	6	0.15±0.03[b]	106.48±17.44[b]
F		25.154	19.022
p		0.000	0.000

Note: Compared with the NSG, [a]p<0.05 and compared with ERG, [b]p<0.05

TABLE 2: EFFECT OF ERLOTINIB ON INVASION AND APOPTOSIS OF NSCLC CELLS

Extent	n	Overall apoptosis rate (%)	Number of invasion
BCG	6	9.97±1.23	51.72±10.31
NSG	6	9.51±1.13	121.05±20.26[a]
ERG	6	31.34±4.25[b]	93.12±16.35[b]
F		134.293	26.157
p		0.000	0.000

Note: Compared with the NSG, [a]p<0.05 and compared with ERG, [b]p<0.05

TABLE 3: ERLOTINIB'S IMPACT ON NSCLC CELLS APOPTOTIC PROTEIN EXPRESSION

Group	n	Fas	Bax	FasL	Bcl-2
BCG	6	0.35±0.14	0.37±0.09	0.41±0.14	0.54±0.03
NSG	6	0.33±0.17	0.43±0.14	0.44±0.14	0.49±0.04
ERG	6	0.84±0.18[b]	0.86±0.16[b]	0.78±0.12[b]	0.31±0.03[b]
F		13.446	20.294	18.641	72.177
p		0.001	0.000	0.000	0.000

Note: Compared with the ERG, [b]p<0.05

TABLE 4: EFFECT OF ERLOTINIB ON THE mRNA LEVELS OF AMPK AND PGC-1α IN NSCLC CELLS

Group	n	AMPK mRNA	PGC-1α mRNA
BCG	6	1.65±0.40	1.76±0.43
NSG	6	1.24±0.32[a]	0.83±0.35[a]
ERG	6	2.47±0.54[b]	2.59±0.67[b]
F		12.744	18.451
p		0.000	0.000

Note: Compared with the NSG, [a]p<0.05 and compared with ERG, [b]p<0.05

The MPK and PGC-1α in the NSG were reduced than the BCG. The protein expression levels of MPK and PGC-1α in the ERG were higher than those in the NSG, as shown in Table 5. Being the most prevalent kind of lung cancer based on histopathology, NSCLC has the characteristics of high metastasis rate, high degree of malignancy and strong aggressiveness, most patients are at an advanced stage when they are diagnosed, and surgery is one of the important treatment methods in the clinical treatment of NSCLC, and it is also the first choice and most effective treatment method for patients with NSCLC in the early and middle stages, but EGFR mutant NSCLC, as the most prevalent kind of lung cancer according to histopathology, has the characteristics of strong invasiveness, high malignancy and high metastasis rate, and most patients are at an advanced stage when they are diagnosed, the best opportunity for surgical treatment has been lost, the mortality rate is high, and other effective treatments need to be sought urgently[8]. With the in-depth study of tumor genetics and molecules, drugs that target tumor molecules have been utilized extensively in the treatment of cancer patients[9]. Thus, research into the etiology and pathophysiology of NSCLC, the molecular mechanisms underlying the growth, invasion, and migration of NSCLC cells, and the identification of novel therapeutic targets are crucial for improving patient quality of life, reducing disease progression, and improving clinical symptoms. Patients with EGFR-mutant NSCLC have been found to respond significantly to treatment with small-molecule tyrosine kinase inhibitors[10]. In this work, we examined the impact and potential mechanism of erlotinib in conjunction with radiation treatment on the growth and death of NSCLC cells, in order to provide potential therapeutic targets for the treatment of clinical NSCLC.

Erlotinib is a commonly used molecularly targeted tumor drug in clinical practice, which can effectively inhibit tumor growth[11]. By competing with adenosine triphosphate for the EGFR receptor enzyme binding site, erlotinib can inhibit tyrosine kinase activity. It can also stop the activation of the EGFR downstream signaling pathway, which in turn prevents tumor cell proliferation, invasion and metastasis while encouraging tumor cell apoptosis[12]. In addition, erlotinib can exert a stronger anti-angiogenic effect by inhibiting microvascular density and small vessel proliferation, thereby inhibiting tumor angiogenesis, thereby effectively reducing tumor volume[13]. The study's findings demonstrated that the NSG had higher levels of both cell migration and proliferation activity than the BCG. Compared to the NSG, the ERG had less cell migration and less cell proliferative activity. The number of cell invasion in the NSG was higher than that in the BCG. The cell invasion in the ERG was reduced than that in the NSG, and the overall apoptosis rate was higher than that in the NSG. These results suggest that erlotinib can reduce the viability of NSCLC cancer cells and reduced the growth, metastasis of NSCLC cells.

An important factor in preventing apoptosis is the aberrant expression of the Fas pathway, also known as the exogenous apoptotic pathway. Fas and FasL are members of the tumor necrosis factor family of cell surface receptors, and their interactions result in ligand-mediated cell death as well as the downregulation of both Fas and its ligand[14]. In clinical practice, the ratio of Bcl-2 to Bax is frequently used to assess the degree of apoptosis in tumor cells. Bax is one of the most typical pro-apoptotic molecules, whereas Bcl-2 is an inhibitory protein[15]. The study's findings demonstrated that the apoptosis proteins Fas, bax and FasL in the ERG were higher than the NSG, and the Bcl-2 were lower than the NSG. It has been found that erlotinib can induce apoptosis in NSCLC cancer cells through the mitochondrial pathway[16].

TABLE 5: EFFECT OF ERLOTINIB ON THE EXPRESSION LEVELS OF MPK AND PGC-1α PROTEINS IN NSCLC CELLS

Group	n	AMPK	PGC-1α
BCG	6	0.57±0.08	0.52±0.09
NSG	6	0.28±0.04[a]	0.30±0.03[a]
ERG	6	0.86±0.28[b]	0.76±0.11[b]
F		15.521	45.156
P		0.000	0.000

Note: Compared with the NSG, [a]p<0.05 and compared with ERG, [b]p<0.05

One often utilized signal transduction system in the human body that is crucial for controlling cell division, growth, migration and apoptosis is the AMPK/PGC-1α pathway[17]. A serine/threonine protein kinase, AMPK plays a crucial role in the body's regulation of inflammation and cellular energy homeostasis. It is expressed in a variety of organs and tissues related to metabolism and can be activated by a variety of bodily stimuli by detecting changes in the status of cellular energy metabolism, which affects multiple links of cellular material metabolism to coordinate the body's metabolism and energy balance[18]. Research indicates that when the body's energy and metabolism are out of balance, AMPK activation can control the phosphorylation of genes involved in lipid synthesis and downstream malonyl-Coenzyme A (CoA) expression, which in turn controls the body's fatty acid production[19]. PGC1-α is a mitochondria-related gene transcriptional coactivator, which acts as a nuclear transcription coactivator to increase transcriptional efficiency by binding to other coactivators to different target genes, thereby regulating important physiological processes such as fatty acid oxidation, oxidative phosphorylation, and mitochondrial biogenesis[20]. PGC1-α activates the TFAM of mitochondrial DNA, and Together with Mitochondrial Transcription Factor B (TFBM) and mitochondrial RNA polymerase, it forms a transcriptional initiation complex that regulates mitochondrial biogenesis and function[21]. It has been demonstrated that over activation of the AMPK/PGC-1α signaling pathway is crucial in controlling the proliferation, survival, and aggressiveness of endometrial cancer cells, breast cancer cells, and gastric cancer cells[22]. The results of this study revealed that the AMPK and PGC-1α in the NSG were reduced than the BCG. The AMPK and PGC-1α in the ERG were higher than those in the NSG. The protein of MPK and PGC-1α in the NSG were reduced than the BCG. The MPK and PGC-1α in the ERG were higher than the NSG. These results suggest that erlotinib can regulate the AMPK/PGC-1α signaling pathway, which gives a theoretical foundation for treating clinical tumor invasion and metastasis.

In summary, Erlotinib has the capacity to decrease the viability of NSCLC cells, prevent their growth, metastasis, and trigger their apoptosis. Its mechanism of action may be associated with the modulation of the AMPK/PGC-1α signaling pathway.

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Conflict of interests:

The authors declared no conflict of interests.

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