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PPARγ agonist efatutazone and gefitinib synergistically inhibit the proliferation of EGFR-TKI-resistant lung adenocarcinoma cells via the PPARγ/PTEN/Akt pathway

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ABSTRACT

Development of acquired resistance to EGFR-TKI therapy continues to be a serious clinical problem in Lung adenocarcinoma management. Peroxisome proliferator-activated receptor gamma (PPARγ) agonists demonstrate anti-tumor activity likely via transactivating genes that regulate cell proliferation, differentiation and apoptosis. Efatutazone, a novel later generation PPARγ agonist, selectively activates PPARγ target genes and has anti-proliferative effects in a range of malignancies. However, the exact function and molecular mechanism of PPARγ agonists efatutazone in EGFR-TKI gefitinib-resistance of Lung adenocarcinoma has not been determined. In this study, we studied the development of acquired resistance to an EGFR-TKI gefitinib in lung adenocarcinoma cells and investigated the antiproliferative effects of efatutazone in the acquired resistant cells. The treatment of gefitinib-resistant cells with efatutazone reduced the growth of gefitinib-resistant cells in a dose- and time-dependent manner, and facilitated the anti-proliferative effects of gefitinib. Mechanistic investigations suggested that efatutazone acted by upregulating protein expression of PPARγ, phosphatase and tensin homolog (PTEN), inactivating the Akt pathway, followed by dephosphorylation of p21Cip1 at Thr145 without affecting the transcriptional levels. Our results suggested that efatutazone, alone or in combination with gefitinib, might offer therapeutic effects in lung adenocarcinoma.

1. Introduction

Lung cancer, the most common malignancy worldwide, is the number one killer in cancers [1]. Lung cancer consists of two histologic types, small cell lung cancer and non-small cell lung cancer (abbreviated as NSCLC). In recent years, lung adenocarcinoma (abbreviated as LAC) has been the most common subtype of NSCLC, which accounts for 85% of all lung cancers [2]. The patients who harbor activating epidermal growth factor receptor (EGFR) mutation response to tyrosine kinase inhibitors (TKIs) specifically, which opens a new chapter in molecular targeted therapies for NSCLC patients [3]. Gefitinib has been recommended for the treatment of NSCLC patients who are identified as mutations of EGFR [4]. The clinical application of EGFR-TKIs benefits approximately 80% of the advanced NSCLC patients, resulting in the treatment revolution of the disease [4]. However, acquired resistance to TKI remains a major obstacle for therapeutic success. After treatment for 10–16 months, most of the patients fail to respond to the reversible first-generation of TKI [5]. In previous papers, potential mechanisms associated with acquired resistance to gefitinib include the loss of PTEN, the T790M mutation in exon 20 of EGFR, MET amplification and the consequent hyperactivation of Akt [6–9].

PPARγ (also known as NR1C3) belongs to the nuclear hormone receptor superfamily which are ligand-activated transcription factors [10]. PPARγ plays a role in a variety of biological processes, including modulation of metabolism, inflammatory response and adipose cell differentiation [10,11]. PPARγ ligands consist of endogenous ligands

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and exogenous ligands. Endogenous ligands include $\triangle^{12,15}$ prostaglandin J2 and fatty acids. Exogenous ligands include troglitazone and efatutazone. Troglitazone, the first-generation thiazolidinedione (PPARγ ligands), has been demonstrated by numerous studies that ligands for PPARγ show prominent anti-proliferative effects in colorectal cancer [12], breast carcinoma [13], and hepatocellular cancer [14]. PPARγ protein regulates or interacts with a great deal of signal channels, such as PTEN pathway [15] which represses the activation of downstream targets and cell cycle associated proteins p21Cip1 [16] and p27 [14]. Thiazolidinedione could activate PTEN expression. PTEN is a tumor suppressor protein thereby inhibiting the activation of Akt and inducing G1 phase cell cycle arrest and apoptosis [17]. Induction of the activity of PTEN and its functional protein may be an effective treatment for multiple malignancies. Efatutazone, a novel third-generation PPARγ agonist of thiazolidinedione, is 500-fold more potent over troglitazone at least in PPAR response–element activation and cancer cell growth inhibition respect [16]. The antitumor effects of efatutazone were demonstrated in patients with advanced malignancies [18]. Therefore, this agent shows tremendous potential for practical cancer therapy.

Based on the close relationship between PPARγ and PTEN signaling pathway downstream, furtherly as well as our preliminary results, we proposed a hypothesis that the PPARγ agonist efatutazone can reverse the acquired resistance of gefitinib via PPARγ-PTEN-Akt signaling pathway. The results of this study might provide a new therapeutic strategy for clinical treatment of acquired gefitinib-resistant lung cancer.

2. Materials and methods

2.1. Reagents

Efatutazone and gefitinib (Iressa) were purchased from MedChemExpress(USA). Before added to cell cultures, efatutazone and gefitinib were prepared in dimethyl sulfoxide (DMSO) in the vitro analyses. Cell counting kit-8(CCK-8) was purchased from Dojindo (Kumamoto, Japan). RPMI 1640 (Gibco) were supplemented with 10% fetal bovine serum (FBS) (Wisent, Canada). Penicillin-Streptomycin Solution was purchased from Gibco.

2.2. Cell culture

HCC827 cell line is a lung adenocarcinoma cell line harboring the EGFR exon 19 deletion (Del E746-A750), and was purchased from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The lung adenocarcinoma cell line PC-9 was derived from an untreated Japanese patient that carried an in-frame deletion in EGFR exon 19 (delE746-A750) [19], which was kindly provided by Dr. Z Chen (Nanjing Medical University, Nanjing, China). These cells were maintained in an environment of 5% CO_2 at 37 °C in RPMI-1640 medium supplemented with 10% FBS.

2.3. Establishment of the gefitinib-resistant HCC827-GR and PC-9-GR subline cells from HCC827 and PC-9 cells

To establish gefitinib-resistant subline cells, HCC827 and PC-9 cells were exposed to increasing the concentration of gefitinib according to previously described method [20,21]. Finally, the HCC827 and PC-9 cells generated stable gefitinib-resistance. HCC827-GR and PC-9-GR cell line was isolated and it was confirmed resistance to gefitinib independently. These gefitinib-resistant cell lines were passed more than 25 times in the presence of gefitinib and the resistance was confirmed by CCK-8 assays. HCC827 and PC-9 cell lines were maintained concomitantly without gefitinib. So parental cells HCC827 and PC-9 were still sensitive to gefitinib and we examined it every 5 passages. HCC827, HCC827-GR, PC-9 and PC-9-GR cells were cultured in RPMI1640 supplemented with 10% heating-inactivated FBS and 1% Penicillin-Streptomycin Solution.

2.4. Statistical analysis of PPARG expression in lung adenocarcinoma

To determine the expression pattern of PPARG in lung adenocarcinoma, we used the datasets in the Oncomine (https://www.oncomine.org) database [22,23], which is an online database consisting of formerly published and publically available microarray data; the significance of the gene expression across the available studies was also taken into account. PPARG gene was queried in the database and the results were filtered by selecting lung adenocarcinoma and Normal vs. Cancer Analysis. Three publically available Gene Expression Omnibus (GEO) datasets (http://www.ncbi.nlm.nih.gov/geo/) GSE74575, GSE72197 and GSE83666 were used to analyze PPARG expression with respect to lung adenocarcinoma EGFR-TKI-resistance. An online database named Kaplan–Meier Plotter (http://kmplot.com/analysis/), which is capable to assess the effect of 54,675 genes on survival using 2437 lung cancer samples on the HGU133 Plus 2.0 array. The correlation between PPARG expression and overall survival (OS) in lung adenocarcinoma was analyzed by Kaplan-Meier plotter [24,25]. The log rank *p*-value and hazard ratio with 95% confidence intervals were also computed.

2.5. Measurement of cell viability

The cell viability was determined by the CCK-8 assay. After 24 h incubation, the cells were plated in 96-well plates in various gefitinib concentrations with or without different concentrations of efatutazone, and the plates were incubated for additional 48 h. According to the manufacturer's protocol, CCK-8 reagents were mixed to each wells, the absorbance was measured at 450 nm with an ELISA plate reader. The IC_{50} values [26] were calculated according to the percentages. Each experiment was performed in triplicate.

2.6. Sequencing of the EGFR gene

To determine the EGFR sequence of HCC827-GR and PC-9-GR cell, DNA was extracted from each cell line using a QIA-amp DNA Mini Kit (Qiagen, Japan), and the exons encoding the intracellular domain (exons 18–22) were amplified by PCR. Primer sequences: EGFR18 forward, 5′-AGCATGGTGAGGGCTGAGGTGAC-3′ and reverse, 5′-ATATA CAGCTTGCAAGGACTCTGG-3′. EGFR19 forward, 5′-CCAGATCACTG GGCAGCATGTGGCACC-3′ and reverse, 5′-AGCAGGGTCTAGAGCAG AGCAGCTGCC-3′. EGFR20 forward, 5′-GATCGCATTCATGCGTCTTC ACC-3′ and reverse, 5′-TTGCTATCCCAGGAGCGCAGACC-3′. EGFR21 forward, 5′-TCAGAGCCTGGCATGAACATGACCCTG-3′ and reverse, 5′-GGTCCCTGGTGTCAGGAAAATGCTGG-3′. The sequencing was conducted using an ABI 3500 sequencer (ABI). Each experiment was performed in triplicate.

2.7. Colony formation assay

HCC827-GR and PC-9-GR cell were seeded at a density of 400 cells per well in flat-bottomed 6-well plates. After 24 h of incubation, cells were treated with gefitinib (5 μM) alone, efatutazone (40 μM) alone or both drugs diluted with the medium to appropriate concentrations and replaced medium every 3 or 4 days. After two weeks, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet [27]. Visible colonies were counted, and each experiment was repeated three times.

2.8. Small-interference RNA transfection

Small-interference RNA (siRNA) duplexes for PPARG were designed and synthesized by RiboBio Co., Ltd. (Guangzhou, China). HCC827-GR