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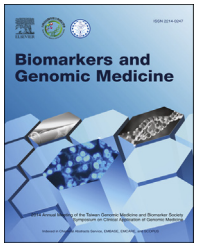

SHORT COMMUNICATION

Secretomic analysis uncovers the mechanisms of gefitinib resistance in non-small-cell lung carcinoma

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Abstract For 2 decades, lung cancer has been the most deadly of all malignant neoplasms in Taiwan. Novel strategies for the discovery and treatment of lung cancers are becoming a particularly important research topic in the field of biomedicine. Gefitinib (trade name Iressa; AstraZeneca, Wilmington, DE, USA) is a biologic agent used to treat lung cancer. The clinical antitumor action of gefitinib is primarily the inhibition of the epidermal growth factor receptor. Gefitinib is indicated as a first-line therapy for lung cancer, although the occurrence of chemoresistance limits the longterm results of this drug. This study was divided into two research directions. The first part is to generate the drug-resistant cancer cell line as a platform to understand the mechanism of drug inactivation of gefitinib for lung cancer. The second part is to collect cell secretomes to find potential biomarkers for the diagnosis and treatment of lung cancer by using minimally invasive assays. Therefore, the non-small-cell lung carcinoma line PC9 and gefitinib-resistant cancer cell line PC9/gef were used in this study. With the analysis of secretomics, differentially expressed extracellular secreted proteins were identified to study the desired biomarkers for molecular diagnostics and gefitinib-resistance. These cell lines provide a useful tool for the further study of the biologic properties in lung cancer *in vitro*.

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Introduction

Lung cancer is one of the most difficult cancers to treat. According to the international statistics on lung cancer survival rates, the overall 5-year survival rate for stage I and stage II lung cancers is 30—50% and the overall 5-year survival rate for stage III and stage IV lung cancers is <10%. Lung cancer patients in the early stage of this disease have a high survival rate; however, most patients are diagnosed with lung cancer at an advanced stage.[1] That is why the mortality rate of lung cancer is always high. Gefitinib is a first-line cancer drug for treating lung cancer. By reversibly competing with adenosine triphosphate (ATP), gefitinib is able to bind into a critical ATP binding site to inhibit intracellular tyrosine kinase activity of the epidermal growth factor receptor (EGFR), which inhibits cancer cell growth.[2] Acquired resistance to gefitinib nevertheless has been reported in patients after a median of approximately 10 months from the initiation of treatment.[3] By contrast, a secretome represents all proteins secreted by cells. Many secreted proteins such as cytokines, growth factors, angiogenesis factors, and proteases are important in the progression of tumors and their signal transduction. By collecting the secretomes and performing two-dimensional differential gel electrophoresis (2D-DIGE) analysis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis to find the proteins that are expressed differentially between the cancer cell line and the drug-resistant cell line, we hope to establish a system with a noninvasive mode to investigate the biomarkers for gefitinib-resistant lung cancer, and thereby improve overall survival rates in patients.

Materials and methods

Cell line and culture

Cell lines used in this study were human lung cell line PC9 (American Type Culture Collection, Manassas, VA USA) and its gefitinib-selected drug-resistant cell line, PC9/gef. The PC9 and PC9/gef cells were cultured at 37°C and 5% carbon dioxide (CO_2) in Roswell Park Memorial Institute (RPMI) medium with 1.5 g/L sodium bicarbonate containing 10% (v/v) fetal bovine serum, and 1 IU/mL penicillin/streptomycin (all items were purchased from Gibco-Invitrogen Corp., Paisley, UK).

MTT cell viability assay

Cells were seeded in 96-well plates (8000 cells/well) and incubated for 24 hours, prior to treating them with the indicated gefitinib concentrations for 48 hours. After removing gefitinib, 100 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (1 mg/mL) were added to each well and incubated at 37°C at 5% CO_2 for 4 hours. The supernatant was removed and 100 mL dimethyl sulfoxide (DMSO) was added to stop the reaction. The absorbance of the samples was measured at a wavelength of 545nM.

Time course of the starve

Cell lines were incubated in serum-free RPMI medium. The cell culture medium was harvested at the indicated hours to collect the secretome. The Western blot test was used to ensure that the cell was intact. (The secretome needs to be collected before the cell is destroyed during starvation.)

Collecting the secretome

The secretome was collected from three 15-cm Petri dishes and then centrifuged at 2683.2 g for 90 minutes until the supernatant was concentrated to 1 mL.

Western blot

Protein samples were diluted with sample buffer and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the separated proteins to Immobilon-P membranes (with a pore size of 0.45 mm), the membranes were blocked for 1 hour by 5% bovine serum albumin (BSA). The membranes were then incubated with primary antibody against asparagine synthetase (ASNS) for 24 hours and washed with tris-buffered saline and Tween 20 (TBS-T; 4× 10 minutes). They were thereafter incubated with secondary antibody for 1 hour and washed again with TBS-T (6 × 15 minutes). Immunoreactive proteins were detected by a chemiluminescence method.

Two dimensional-differential gel electrophoresis analysis

Two dimensional-differential gel electrophoresis analysis was performed with cyanine dyes containing Cy2, Cy3, and

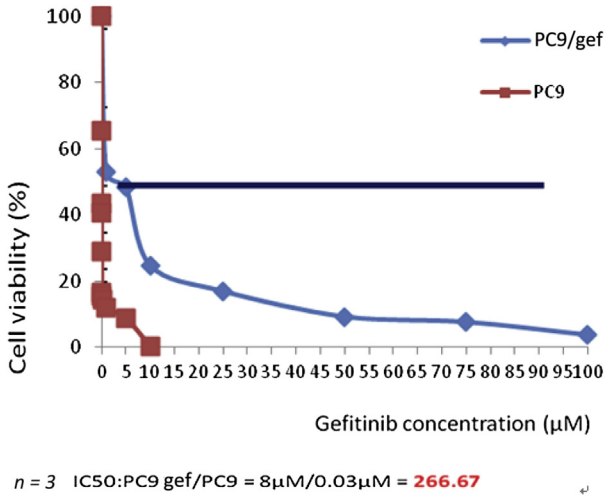

Figure 1 The MTT assay results. The PC9 and PC9/gef cells were grown overnight and treated with a range of doses of gefitinib. An MTT assay is used to determine cell viability. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.