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Introduction

Lung cancer has long been the most common cancer and cause of cancer-related deaths worldwide, with 1.61 million new cases and 1.38 million deaths in 2008 alone, representing 12.7% of new cancers and 18.2% of cancer mortality (Jemal et al., 2011). Non-small-cell lung cancer (NSCLC), which accounts for ~85% of all cases of lung cancer, is a leading cause of cancer deaths worldwide (Janku et al., 2010). In China, men’s high lung cancer rates reflect high smoking rates but high rates among non-smoking women appear to be related to other factors (Lam et al., 2004). For example, women living within 200 m of factories in Shenyang, China, had a significantly higher risk of lung cancer (Xu et al., 1989). Current treatments for NSCLC may extend survival but are rarely curative (Greenlee et al., 2000; Subramanian and Govindan, 2008). During the past years, the discovery of crizotinib, targeted the kinase domain of the epidermal growth factor receptor (EGFR) gene, became a revolutionized mark of the treatment of NSCLC (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). It has been approved by the U.S. Food and Drug Administration for the treatment of patients with advanced NSCLC (Hallberg and Palmer, 2010). Crizotinib was originally developed to target the c-MET pathway in advanced solid tumors, although it was also known to inhibit ALK (Kwak et al., 2010). However, other mechanisms of crizotinib remain unclear.

In our studies, we confirmed crizotinib-induced apoptosis in lung cancer cell A549. Based on the evidence of apoptotic A549 cells, we detected the changes of signaling pathway. Small ligand docking has become an increasingly important tool for the computational analysis of binding interactions between proteins and ligands since 1980s (Lengauer et al., 1996; Taylor et al., 2002). We firstly estimated the possible proteins that could interact with crizotinib. Then we demonstrated our hypothesis by using western blot.

Materials and Methods

Cell culture and reagents

Lung cancer cell A549 were obtained from the American Type Culture Collection (Bethesda, MD) and grown in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 ug/ml streptomycin). Cells were maintained in a humidified cell incubator with 5% CO2 at 37 ºC. Crizotinib was purchased from Sigma (Carlsbad, CA).

Cell apoptosis assay

Aptoptosis was determined using an apoptosis detection kit (Keygene, Nanjing, China). Briefly, cells were collected, washed twice in ice-cold PBS, and then resuspended in binding buffer at a density of 1 × 10⁶ cells/ml. Cells treated with crizotinib (500 nM) were incubated simultaneously with fluorescein-labeled Annexin V and propidium iodide (PI) for 20 min. Then the mixture was analyzed by a FACScanLibur machine. Data were analyzed using Cell Quest software from BD. Untreated cells were used as a negative control.
Preparation of the proteins and ligands structures for docking

We applied our approach to four target proteins: Smad3, TGF-β, TNF, and ERK. These structures with crystallographic resolutions of less than 3.0 Å, were retrieved from the Protein Data Bank (http://www.rcsb.org). The PDB codes of the selected proteins are: 1MK2 (Qin et al., 2002), 2TGI (Daopin et al., 1992), 1ICH (Sukits et al., 2001) and 3ERK (Wan et al., 1998). The molecular structure of crizotinib was downloaded from Pubchem Compound (http://www.ncbi.nlm.nih.gov/pccompound) and the format was changed into PDB using the software Mol2mol. Data were imported into the modeling software iGEMDOCK (version 2.1) (Drug Design and Systems Biology Laboratory of National Chiao Tung University, Taiwan). All non-protein components such as water molecules, metal ions, and lipids were deleted and hydrogen atoms were added to the protein structures. The interaction of crizotinib and proteins was analyzed by iGEMDOCK.

Antibodies and western blotting

Cells treated with crizotinib were lysed in RIPA buffer (150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na₃VO₄, 1 mM Na₂MoO₄, 10 μM aprotinin, 10 μM leupeptin) on ice. Untreated cells were used as a negative control. Crude cell lysates were then centrifuged at 14,000 × g for 10 min, and cleared lysates were collected and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk-TBST then incubated with primary antibodies. Anti-phospho-Smad3 (pSer425) and anti-Smad3 (Abcam, Cambridge, UK) were used to identify the changes of signaling pathway. β-actin (Santa Cruz, CA) was used as an internal control. The reaction was followed by probing with peroxidase-coupled secondary antibodies at dilutions ranging from 1:1000 to 1:2000 (Santa Cruz, CA), and binding results were visualized by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Differences between groups were analyzed using Student’s t-test for continuous variables. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS, Inc.) and significance was established at p < 0.05.

Results

The antitumor activities of crizotinib on A549

In order to further study the function of crizotinib, we first utilized FCM to detect the apoptotic cells, including A549 and A549 treated with crizotinib. The results showed crizotinib could suppress viability of cells. The untreated ones were used as a negative control (p < 0.05, Figure 1).

Model of crizotinib bound to Smad3 and binding site identification

The identification of candidate binding sites of crizotinib is the first step to detect the signaling pathway. Crizotinib was docked into Smad3 using iGEMDOCK (version 2.1) with postdocking minimization and the top-scoring pose chosen for further analysis (Yang, 2004; Yang and Chen, 2004). We performed the docking run three times. Figure 2 also showed the performance of the binding sites between crizotinib and Smad3. Other proteins showed no interaction with crizotinib (Data were not shown).

The role of crizotinib in phosphorylation of Smad3

In order to further detect biological effects of crizotinib on Smad3, we performed western blot analysis using antibodies that recognize phosphorylated, active Smad family members. We found that the level of P-Smad3 was increased in A549 after treatment with crizotinib. While Smad3 levels were no essentially changes (Figure 3). The results indicated that Smad signaling pathway was activated by crizotinib.
Discussion

Preclinical studies demonstrate that ALK inhibition induces apoptosis and tumor regression in models of ALK-expressing tumors, identifying ALK as a driver mutation and underscoring its potential as a therapeutic target (McDermott et al., 2008). Crizotinib, a dual MET/ALK inhibitor, has produced a high response rate and prolonged median progression-free survival in patients with ALK-positive NSCLC (Kwak et al., 2010). In our studies, in order to detect other possible mechanisms of crizotinib, we carried out a novel computer simulation method (small molecular docking technology) to prognosis the proteins that could interaction with crizotinib. In our screening proteins, we found that Smad3 could dock with crizotinib. However, the biological effect of crizotinib on Smad3 remains unclear. In further studies, we found that the level of P-Smad3 was increased by using western blot. In other words, crizotinib could activate Smad3. The transforming growth factor-β (TGF-β) signaling pathway plays an important role in cell differentiation, growth, and apoptosis (Zhou et al., 1998). Smad3, a key mediator of the Smad family of proteins, is the “receptor-regulated SMADs” (Schmierer and Hill, 2007). Consistent with previous studies, we also found that A549 cells after treatment with crizotinib showed more apoptotic ratio than untreated ones. Crizotinib induced apoptosis in A549 cells through Smad signaling pathway.

In summary, we used a novel computer simulation to prognosis the possible proteins that could interaction with crizotinib. The hypothesis was demonstrated by using western blot. Our results confirmed that small molecular docking technology was a reliable, economical and feasible method. It can be used as a preliminary screening of signaling pathway.

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