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Better survival with EGFR exon 19 than exon 21 mutations in gefitinib-treated non-small cell lung cancer patients is due to differential inhibition of downstream signals ☆

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Abstract

Somatic mutations in the epidermal growth factor receptor (EGFR) kinase domain are associated with sensitivity to tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung cancer (NSCLC). Our clinical data showed NSCLC patients with exon 19 deletions survived longer following gefitinib treatment than those with exon 21 point mutations. We aimed to investigate whether these two mutations produced differences in phosphorylation of EGFR and downstream signals. Two stable cell lines expressing these mutations were obtained by transfection. Inhibition of phosphorylation of EGFR, Akt, and Erk by gefitinib was detected using Western blotting, and cell inhibition tests were conducted to evaluate the bio-behavior. Gefitinib inhibited the phosphorylation of EGFR, Akt, and Erk to a greater degree in exon 19 deletion cells than in L858R cells. Gefitinib produced G1 arrest in more of the cells with exon 19 deletion than with L858R. This might be attributable to patient selection in TKIs therapy.

Keywords: Epidermal growth factor receptor; Mutation; Non-small cell lung cancer; Tyrosine kinase inhibitor; Autophosphorylation

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC), 70–80% of which is diagnosed at an advanced stage, accounts for about 85% of the total cases. At present, chemotherapy is the first-line treatment for advanced cases, with a median overall objective response rate of

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17% [1]. NSCLC shows a promising response to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, which target the molecular pathways of tumor cell growth and survival. The objective response rate to EGFR-TKIs was higher in patients harboring somatic EGFR mutations [2,3].

Mutations of EGFR are found in fewer than 10% of non-Asian patients but occur in 30% of East Asians [4–6]. The two most important mutations are L858R in exon 21, which involves a thymine-to-guanine transversion that replaces leucine with arginine, and exon 19 deletion, which eliminates a leucine-arginine-glutamate-alanine motif. These two account for more than 80% of EGFR mutations [4,7–9]. The altered structure of the EGFR kinase domain allows TKIs binding more tightly to the receptor. Both *in vivo* and *in vitro* studies have shown that these mutations increase the sensitivity of NSCLC patients to EGFR-TKIs [10–12].

An individual patient data (IPD) meta-analysis, conducted by Wu et al., showed that the clinical selected population for gefitinib are non-smokers with adenocarcinoma and that a more favorable response occurred in patients with somatic EGFR mutations [5]. Moreover, patients with exon 19 deletions survived longer than those with exon 21 point mutations, consistent with previous studies [13–15]. Few *in vitro* studies were found to have specifically investigated the mechanism of the distinction. We hypothesized that these two mutations might affect the autophosphorylation of residues in EGFR, probably via changes in the kinase domain structure. It would lead to a different activation model of downstream signaling, causing a biological alteration [8,16]. Therefore, to further understand different types of mutations in EGFR and downstream signals, we mimicked the biological behavior of the two mutations by generating EGFR mutation cell line, which originally did not express EGFR. Thus the transfected mutant EGFR, instead of other genes, should be the cause of the difference.

2. Materials and methods

2.1. Patients

Our retrospective analysis examined 57 patients with advanced NSCLC who had received gefitinib therapy and the specimen were available at Guangdong Provincial People's Hospital and Peking Union Medical College Hospital from July 2002 to January 2005. In the NSCLC patients, tumor tissues obtained either from paraffin-embedded or frozen and mutations in EGFR exons 18–21 were analyzed by direct sequencing as previously described [17,18]. In this study, patients of refractory NSCLC with performance status (PS) 0–2 were included. All patients had been treated with gefitinib 250 mg daily until disease progression was confirmed. The last follow-up date was 20 June 2007. All specimens were collected with informed consent.

2.2. Statistical analysis

The relationship between response to gefitinib and EGFR mutation status was analyzed using the Kruskal–Wallis H test. Univariate survival estimates were derived from a Kaplan–Meier analysis, and log-rank tests were used to assess differences in overall survival between the two major mutation types. The P values were two-sided, and $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS 13.0 and Sigmastata 3.5 integrated with Sigmaplot 10.0.

2.3. Transfection of mutant EGFR using a retrovirus system

The pcDNA3.1(−) expression vectors with inserts containing the entire length of the EGFR gene with either a point mutation (L858R) or a deletion (del746–750) were generously provided by Dr. William Pao of Memorial Sloan-Kettering Cancer Center. Each of the mutated EGFR was cloned into pLNCX2, a retrovirus package plasmid, and the cloned sequences were confirmed by sequencing. The pLNCX2 vectors containing the EGFRs were transfected into the PA317 cell line by using Lipofectamine 2000 (Invitrogen) and retrovirus were collected for transfection of 293 human embryonic kidney cells.

2.4. Transfection of 293 cell line

The 293 cell line was maintained in RMPI-1640 (Gibco) with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin–streptomycin. Each PA317 supernate containing Polybrene (Sigma) at 2 μmol/L was transferred to a 293 cell culture, and positive clones were selected with G418. Immunoblotting was used to select cells expressing each type of EGFR in about an equal amount.

2.5. Cell proliferation and inhibition tests

A total of 5000 of the 293-EGFR (del746–750) or 293-EGFR (L858R) cells were seeded in each well of a 24-well plate. After culture for 16 h, the cells were exposed to medium containing 10 μM gefitinib (AstraZeneca), or medium alone as a negative control. The concentration was selected since it could completely inhibited the phosphorylation of EGFR (our immunoblotting showed) and lower than the IC50 of 293 cells (data not shown). Each subsequent day, one well was digested and counted.