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Original article

P21 (waf1/cip1) is required for non-small cell lung cancer sensitive to Gefitinib treatment☆

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ABSTRACT

Lung cancer is the leading cause of death from cancer in the world. Gefitinib is known to its inhibition of EGFR tyrosine kinase and worldwide used for antitumor in non-small cell lung cancer (NSCLC). Here, we show that Gefitinib reduces p-Akt levels, concomitant with elevation of p21 levels and suppression of cdk2/4 and cyclinE/D1 activities which result in impaired cell cycle progression through G1 arrest only in NSCLC cells in which it inhibits growth. We find that Gefitinib-induced p21 protein stability, rather than increased RNA accumulation, was responsible for the elevated p21 levels. More, treatment of beta-elemene, a natural plant drug extracted from Curcuma wenyujin, restored sensitivity to Gefitinib via the mechanism modulated the elevation of p21 levels in the cells which are acquired resistance to Gefitinib. These data suggest that administration of Gefitinib in combination with beta-elemene may offer great opportunities for NSCLC which are acquired resistance to Gefitinib. The p21 effect on the cells to response to Gefitinib was further confirmed by p21 over-expression and knockdown studies pointing to a requirement of p21 for the cells sensitive to Gefitinib. Thus, we propose that p21 is required for Gefitinib-sensitive NSCLC cells.

1. Introduction

Gefitinib is a small-molecule quinazoline derivative that was developed as a tyrosine kinase inhibitor (TKI) of the EGF receptor (EGFR). EGFR is known to promote growth of cells and functions as an oncogene and expressed in up to 80–90% of non-small cell lung cancer (NSCLC) [1]. However, it is now clear that there are limited subgroups of (12–18%) NSCLC patients who derive particular benefit from this treatment [2]. Gefitinib has been recently registered as first-line treatment of NSCLC patients with EGFR activating mutations. This approval is based on the data of the Phase III IPASS study, which demonstrated superior progression-free survival, greater objective response rate, improved tolerability and significant quality of life benefits for gefitinib compared to carboplatin/paclitaxel doublet chemotherapy in clinically selected first-line patients in Asia [3–5]. Mutations and amplification of the EGFR gene, and other molecules such as phosphorylated Akt and ErbB-3 expression have been well described as markers of a better outcome in patients treated with Gefitinib [6–8]. However, molecular approaches linked to Gefitinib sensitivity in NSCLC are still unknown. Furthermore, correlation between some of the predictors and clinical benefit is urgently required.

In the other hand, even in cases sensitive to Gefitinib, resistance is acquired through continuous drug administration. Additional treatments for cases of NSCLC relapsing with treatment Gefitinib are urgently needed. Beta-elemene, a natural plant drug extracted from Curcuma wenyujin, has been used as an antitumor drug for different tumors, including NSCLC via mechanism that inhibits Ras/Mapk signaling and cell cycle progression [9,10]. Beta-elemene acts synergistically with cytotoxic drugs against a variety of tumor cells, and the observation that beta-elemene is able to do so in drug-resistant patients and, thereby, overcome drug resistance is especially provocative.

Alterations in cell cycle control are a universal feature of lung cancers. P21, the product of the WAF1/CIP1/SDI1 gene, is an inhibitor of cyclin-dependent kinases and is activated through p53-dependent or p53-independent pathways [11]. Studies have clearly indicated that p21 plays an important role in regulation of the cell cycle, especially in G1 arrest [12,13]. Recent studies have showed that p21 plays important role in antiproliferative effort of Gefitinib [14–17]. However, the clinical significance of p21 expression in NSCLC response to Gefitinib remains unclear.

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In this study, the correlations between p21 expression and the incidence of Gefitinib-induced cytostasis, proliferative activity, and p53 status in the cell lines were assessed. Our data implicated that p21 plays an important role in NSCLC response to Gefitinib.

2. Materials and methods

2.1. Cell cycle analysis by FACS

Cells were washed twice with PBS, trypsinized and resuspended in PBS containing 0.1% Triton X-100 and RNase (1 mg/ml) (Sigma). The cell suspension was incubated at 37 °C for 30 minutes. Propidium iodide (molecular probes) was added at a final concentration of 50 μg/ml and the cell suspension was kept at 4 °C for 1 hour. The cells were filtered and the cell cycle was analysed by flow cytometry with the FACScan system (Becton Dickinson).

2.2. Cells and transfection

Human lung cancer cell lines pc-9 and H1299 cells and the Gefitinib-resistant cell line pc-9-ZD cells were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone). MYC tagged p21 expression vector was generated as previously described [18]. ShRNA sequence against p21 (#1, CTT CGA CTT TGT CAC CGA G; #2, GAC CAT GTG GAC CTG TCA C) were clone into pSUPER-EGFP1 constructs (OligoEngine). The pSUPER-Scramble plasmid (gat ccc cTT CTC CGA ACG TGT CAC GTt tca aga gaA CGT GAC ACG TTC GGA GAA ttt ttg gaa a) was used as the nonsense control [19], which were synthesized by Sangon Ltd. (Shanghai). Cells were transiently transfected with expression constructs using GeneJammer transfection reagents (Stratagene). For half-life experiments, cells were treated with cycloheximide (CHX, Sigma) at a final concentration of 10 μg/ml.

2.3. Measurement of cell death

Cells were seeded into 96-well microtiter plate and treated with Gefitinib (10,100,1000 nM) or beta-elemene (40 μg/ml). A 10 μl of the CCK-8 solution was added and then incubated 2 hours according to the procedure of Cell Counting Kit-8 (Dojindo Laboratories). The absorbance was measured at 450 nm using a microplate reader (BioTeK).

2.4. Western Blotting

The cells were prepared in lysis buffer of MC-CelLytics Kit (Shenergy Biocolor). The protein content was determined using the Bradford calorimetric assay method (Shenergy Biocolor). The lysate was resolved by 10% polyacrylamide-sodium lauryl sulfate gel electrophoresis and transferred to a Hybond-C Super membrane (Amersham). Antibodies used for detection as follows, p21 (2946, Cell Signaling), p53 (2524, Cell Signaling), p-Akt (05-669, Upstate), CDK2 (PC44, CalBiochem), CDK4 (sc260, Santa Cruz), cyclinD1 (sc20044, Santa Cruz), cyclinE (sc25303, Santa Cruz) and β-actin (Beyotime). Then, the blot was incubated with a secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or antirabbit IgG (Rockland Immunochemicals) and detected with Odyssey Infrared Imaging System.

2.5. Real-time PCR

Total RNA was extracted by homogenization in 1 mL TRIzol reagent (Invitrogen), followed by chloroform reextraction and isopropanol precipitation. 1 μg of RNA were reverse transcribed using RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas) and random hexamer primer (Fermentas). Real-time PCR was done in a final volume of 20 uL containing 1.6 μL of each cDNA template, 1 μL of each primer(10 Mm), and 10 μL of a SYBR Green master mix (Takara). Primers used were 5′-GGCAGACCAGCATGACA GATT-3′ (sense) and 5′-GCGGATTAGGGCTTCCTCTT-3′ (antisense) for p21(waf/cip1); 5′-CACGATGGAGGGGCCGGACTCATC-3′ (sense) and 5′-TAAAG ACCTCTATGCCAACACAGT-3′ (antisense) for human β-actin. The average of p21 gene was normalized to β-actin as endogenous housekeeping gene.

3. Results

3.1. P21 was elevated by Gefitinib in non-small lung cancer cells that are sensitive to Gefitinib

Recent studies have led to understand some mechanism of Gefitinib induced cytostasis. Akt activity is commonly reduced by Gefitinib in these tumors that are sensitive to Gefitinib. To confirm this point, we assessed Gefitinib induced cytostasis and Akt activities in two NSCLC cell lines: pc-9 and H1299 cells. As shown in Fig. 1a, pc-9 cells have an IC50 < 1 μM, indicated as Gefitinib-sensitive cells, whereas H1299 cells have an IC50 > 1 μM, indicated as Gefitinib-resistant cells. Western blotting analysis was performed using antiphosphor-Akt antibody. As demonstrated in Fig. 1b above, Gefitinib led to a reduction in phosphor-Akt only in the Gefitinib-sensitive pc-9 cells but not in Gefitinib-resistant H1299 cells. These data suggested that reducing of Akt activation levels plays an important role in mediating of Gefitinib induced cytostasis.

Studies have shown that antitumor agent suppresses cell growth likely related to upregulation of cell cycle inhibitors such as p27 and p21 in NSCLC [20]. Thus, we asked if p21 plays a role in response to Gefitinib selectively in NSCLC cell lines whose growth is inhibited by Gefitinib. We examined p21 levels in pc-9 and H1299 cells by Western blotting analysis. As seen again in Fig. 1b, the p21 protein levels were high in pc-9 cells. More, exposure to Gefitinib actually increased p21 expression, effects that were most pronounced in cells which are sensitive to Gefitinib. In contrast, no detectable p21 could be seen in H1299 cells with or without treatment of Gefitinib. We further sought to test some other key cell cycle regulators which showed in Fig. 1b. Thus, we observed a significant decrease in the levels of cdk2, cdk4, cyclinE and cyclinD1 by Gefitinib in pc-9 cells but these did not occurred to H1299 cells. These data was consistent with results of cell cycle analysis showed that Gefitinib induced retention of cells in the G1 phase, a sharp decrease in the S phase population but no significant change in the G2/M fraction in pc-9 cells. In contrast, there was no apparent change of cell cycle pattern by treatment of Gefitinib in Gefitinib-resistant H1299 cells (Fig. 1c). These data here indicated that Gefitinib elevated p21 levels and suppressed cdk2/4 and cyclinE/D1 activities which resulted in impaired cell cycle progression through G1 arrest. Taken together, Gefitinib treatment induced cytostasis through multiple mechanisms such as reducing phosphorylation of Akt activity and suppressing cell cycle progression by induction of p21 protein in Gefitinib-sensitive pc-9 cells.

We suggested that Gefitinib promoted p21 protein elevation was, at least partly contribute to its negative regulation of cell progression by Gefitinib in pc-9 cells. As p21 acts primarily as a transcription target of p53, we next examined the possibility that the induction of p21 caused by p53 transcriptional activity in pc-9 cells treated with or without Gefitinib, the amount of p53 protein was assessed. As shown in Fig. 2a, no apparent correlation between p53 levels and p21 levels was seen in, suggesting that the former molecule is not under the latter's direct control.