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Research article

Synergistic effect of pacritinib with erlotinib on JAK2-mediated resistance in epidermal gowth factor receptor mutation-positive non-small cell lung Cancer

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ABSTRACT

The combination effect of pacritinib, a novel JAK2/FLT3 inhibitor, with erlotinib, the epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), on non-small cell lung cancer cells with EGFR activating mutations was investigated. The combination showed synergistic effects on JAK2-mediated EGFR TKI-resistant PC-9/ER3 cells in some cases. The combination markedly suppressed pAKT and pERK although pSTAT3 expression was similar regardless of treatment with the pacritinib, pacritinib + erlotinib, or control in PC-9/ER3 cells. Receptor tyrosine kinase array profiling demonstrated that pacritinib suppressed MET in the PC-9/ER3 cells. The combined treatment of pacritinib and erlotinib in PC-9/ER3 xenografts showed more tumor shrinkage compared with each drug as monotherapy. Western blotting revealed that pMET in tumor samples was inhibited. These results suggest MET suppression by pacritinib may play a role in overcoming the EGFR-TKI resistance mediated by JAK2 in the PC-9/ER3 cells. In conclusion, pacritinib combined with EGFR-TKI might be a potent strategy against JAK2-mediated EGFR-TKI resistance.

1. Introduction

Lung cancer accounts for a leading cause of cancer mortality worldwide. Patients with non-small cell lung cancer (NSCLC) harboring activating mutations in the epidermal growth factor receptor (EGFR) benefit from treatment with EGFR tyrosine kinase inhibitors (TKIs). However, almost all of the patients eventually develop resistance to EGFR-TKIs within approximately one year. An acquired resistance through the secondary mutation T790M can be overcome by third generation EGFR-TKIs [1,2]. However, there are a wide variety of mechanisms by which EGFR-TKI resistance may arise and the approach to overcome the vast majority of resistant cases remains unclear.

In addition to the RAS/MAPK and PI3K/AKT pathways, the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is an essential signal cascade for cell proliferation, cell differentiation, migration and apoptosis. The JAK family in mammals consists of four members: JAK1, JAK2, JAK3 and TYK2 [3]. These proteins interact with a variety of cytoplasmic signaling molecules including EGFR. JAK activation leads to downstream activation of transcription through their major substrates, STATs. Most notably, STAT3 has been shown to be a key molecule mediated by JAK family signaling [4]. STAT3 has been suggested to play a role in the carcinogenesis of early stage EGFR mutation-positive lung adenocarcinoma [5]. Additionally, EGFR inhibition was shown to activate STAT3 signaling in EGFR-mutant NSCLC cells [6]. Previously, we established a novel EGFR-TKI resistant cell line derived from PC-9 (a cell line highly sensitive to treatment with EGFR-TKIs). We showed that activation of the JAK2/STAT3 pathway mediated one of the key mechanisms in EGFR-TKI resistance [7]. Ruxolitinib is a JAK inhibitor approved by the US Food and Drug Administration in 2011 for the treatment of primary and

Abbreviations: JAK, Janus kinase; STAT, signal transducers and activators of transcription; EGFR, epidermal growth factor receptor; FLT3, FMS-like tyrosine kinase 3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor

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secondary myelofibrosis, and polycythemia vera on the basis of the result of phase III trials [8–10]. Ruxolitinib is a pan-JAK inhibitor that strongly inhibits both JAK1 and JAK2 [11]. Pacritinib is a novel macrocyclic pyrimidine-based JAK2 inhibitor with clinical activity in patients with myelofibrosis and lymphoma [12,13]. Specifically, in a multi-center phase II study, pacritinib demonstrated favorable efficacy with limited hematologic toxicity in patients with myelofibrosis compared with the historical safety profile of ruxolitinib [8,9,13]. Pacritinib is also expected to have activity in acute myeloid leukemia (AML) because it is known to target FMS-like tyrosine kinase 3 (FLT3) [14].

Our hypothesis is that JAK2 inhibition would overcome some resistance to EGFR-TKI. Since multiple mechanisms for resistance can occur, a multikinase inhibitor such as pacritinib blocks a number of pathways that may also be of importance in overcoming resistance in addition to JAK2. In the present study, we demonstrate that the combination of pacritinib and erlotinib showed synergistic effects on JAK2-related EGFR-TKI-resistant lung cancer *in vitro* and *in vivo* under some circumstances, and suppression of MET by pacritinib may contribute its mechanism.

2. Materials and methods

2.1. Cell lines

The PC-9 cell line was derived from a patient with pulmonary adenocarcinoma, carrying an in-frame deletion in EGFR exon 19 (del_E746-A750), and is highly sensitive to EGFR-TKIs (e.g., gefitinib, erlotinib and afatinib) [15,16]. The PC-9/ER3 cell line was derived from the PC-9 cell line following chronic exposure to erlotinib, after which resistance to erlotinib was demonstrated and the cell line was found to exhibit the JAK2-mediated EGFR-TKI-resistant mechanism as described previously [7].

2.2. Reagents and antibodies

Pacritinib was kindly given by CTI BioPharma, Inc. (Seattle, WA). Gefitinib and erlotinib were purchased from Selleck Chemicals. Rabbit monoclonal antibodies against phosphorylated JAK2 (pJAK2: Tyr1007/1008), pJAK2 (Tyr221), pFLT3 (Tyr591), FLT3, EGFR, pEGFR (Tyr1068), pMET (Tyr1234/1235), p44/42 mitogen-activated protein kinase (MAPK) (ERK1/2), pMAPK (pERK1/2) (Thr202/Tyr204), AKT, pAKT (Ser473), STAT3, pSTAT3, and GAPDH were purchased from Cell Signaling Technology. Because there are multiple phosphorylation sites on JAK2 with different functions and Tyr1007/1008 and Tyr221 were inhibited by pacritinib as previously shown [12–14], we used two antibodies for pJAK. JAK2, IRAK-1, pIRAK1 (Ser376), and polyclonal MET antibodies were purchased from Santa Cruz Biotechnology, a CLK4 antibody was obtained from Abcam, and a pIRAK1 (Thr209) antibody was purchased from Assay Bio Technology. Peroxidase-labeled anti-rabbit or anti-mouse antibodies (GE Healthcare Biosciences) were used as secondary antibodies.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Dose-response curves were determined using an MTT assay. Briefly, cells were seeded in 96-well plates at 1500/well and incubated with each drug for 96 hat 37 °C in 5% CO_2 incubator, and then quantified using a microplate reader (Bio-Rad, Hercules, CA). The half maximal inhibitory concentration (IC_{50}) was used to evaluate the effect of the drug. Each assay was performed in triplicate. All IC_{50} values are presented as means ± standard error (SE).

2.4. The calculation of combination index

The isobologram and combination index (CI) were calculated according to the methods based on the median-effect analysis by Chou and Talaly using CompuSyn software (ComboSyn, Inc. Cambridge, MA) [17,18]. The combination effect was evaluated by the following combination index (CI): <0.9 indicates synergism, 0.9–1.1 indicates additive, >1.1 indicates antagonism as previously described [18].

2.5. Immunoblotting analysis

Cells were lysed in lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerolphosphate, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor tablets [Roche Applied Sciences, Indianapolis, IN]). The proteins were then separated by electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes, and probed with specific antibodies. Peroxidase-labeled anti-rabbit or anti-mouse antibodies (GE Healthcare Biosciences, Piscataway, NJ) were used as the secondary antibody followed by detection with an ECL Prime Western blotting detection reagent (GE Healthcare Biosciences).

2.6. HotSpot kinase assay, human phospho-RTK array and human phospho-kinase antibody array

In order to determine the selectivity of pacritinib, the HotSpot kinase assay, a radioactivity based kinase assay, was performed at Reaction Biology Corp (Malvern, PA). Human phospho-RTK and phospho-kinase antibody arrays were also performed to reveal the mechanism of action of pacritinib (Table S1 and Table S2, respectively). Protein expression was assessed by densitometry using ImageJ software (developed at the National Institutes of Health).

2.7. Xenograft model

Seven-week-old female athymic immunodeficient mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). All mice were provided with sterilized food and water and were housed with a 12-h/12-h light/dark cycle. PC-9 and PC-9/ER3 cells (2×10^6) were injected subcutaneously into the backs of the mice. One week after injection, the mice were randomly assigned to the following four groups: vehicle, 10 mg/kg/day of erlotinib, 75 mg/kg/day of pacritinib, or 10 mg/kg/day of erlotinib plus 75 mg/kg/day of pacritinib. Drugs were administered by oral gavage once per day, five times per week. Tumor volume (width × width × length/2) was measured twice per week. All tumor volumes are expressed as means ± standard deviation (SD). Differences in tumor volume were evaluated using Student's *t*-test. Mice were sacrificed and tumors were harvested after two weeks. Protein were extracted from those tumors and analyzed as described above. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University (Okayama, Japan).

2.8. Statistical analyses

For the experimental data, all *P*-values correspond to two-sided tests, with the significance set at $P<0.05$. Statistical analyses were conducted using Stata software (v. 12; StataCorp, College Station, TX).