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Research Paper

Losmapimod Overcomes Gefitinib Resistance in Non-small Cell Lung Cancer by Preventing Tetraploidization

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ABSTRACT

The epidermal growth factor receptor (EGFR) is known to play a critical role in non-small cell lung cancer (NSCLC). Constitutively active EGFR mutations, including in-frame deletion in exon 19 and L858R point mutation in exon 21, contribute about 90% of all EGFR-activating mutations in NSCLC. Although oral EGFR-tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, show dramatic clinical efficacy with significantly prolonged progression-free survival in patients harboring these EGFR-activating mutations, most of these patients will eventually develop acquired resistance. Researchers have recently named genomic instability as one of the hallmarks of cancer. Genomic instability usually involves a transient phase of polyploidization, in particular tetraploidization. Tetraploid cells can undergo asymmetric cell division or chromosome loss, leading to tumor heterogeneity and multi-drug resistance. Therefore, identification of signaling pathways involved in tetraploidization is crucial in overcoming drug resistance. In our present study, we found that gefitinib could activate YAP-MKK3/6-p38 MAPK-STAT3 signaling and induce tetraploidization in gefitinib-resistance cells. Using p38 MAPK inhibitors, SB203580 and losmapimod, we could eliminate gefitinib-induced tetraploidization and overcome gefitinib-resistance. In addition, shRNA approach to knockdown p38α MAPK could prevent tetraploidy formation and showed significant inhibition of cancer cell growth. Finally, in an *in vivo* study, losmapimod could successfully overcome gefitinib resistance using an in-house established patient-derived xenograft (PDX) mouse model. Overall, these findings suggest that losmapimod could be a potential clinical agent to overcome gefitinib resistance in NSCLC.

1. Introduction

Lung cancer is one of the most lethal cancers worldwide (Siegel et al., 2016). Non-small cell lung cancer (NSCLC) is the major type of lung cancer and the overall 5-year survival rate is <10% (Miller et al., 2016). In NSCLC, mutations are frequently observed in the epidermal growth factor receptor (EGFR), including an in-frame deletion in exon 19 and the L858R point mutation in exon 21 (Lynch et al., 2004). These mutations comprise about 90% of all EGFR-activating mutations in NSCLC. Oral EGFR-tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, show dramatic clinical efficacy initially with significantly prolonged progression-free survival in patients harboring these EGFR-activating mutations (Bria et al., 2011). However, most of these patients will eventually develop acquired resistance. Different mechanisms have been identified to contribute to the acquired resistance (Sequist et al., 2011). Among them, the development of a secondary T790 M mutation in exon 20 of the *Egfr* gene and mesenchymal-epithelial transition factor (*Met*) amplification are the two main mechanisms, which account for 50% and 20% of acquired resistance, respectively (Engelman et al., 2007; Kobayashi et al., 2005a). Based on this heterogeneity of mechanisms, single-agent treatment strategies to successfully overcome acquired resistance have severe limitations.

In recent years, researchers have identified genomic instability as one of the hallmarks of cancers (Hanahan and Weinberg, 2011). Genomic instability usually involves a transient phase of polyploidization, in particular, tetraploidization (Vitale et al., 2007). In response to DNA damage or cellular stress, cells can undergo cell cycle checkpoints to delay cell cycle progression and allow time for cell repair. G1/S and

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G2/M are two main cell cycle checkpoints that have been identified. Prolonged G2-arrest can induce G1-state tetraploidization by allowing cells to enter S-phase by *endo*-reduplication (Lilly and Duronio, 2005). This p53-induced tetraploidy provides a survival mechanism by preventing 4 N cells to undergo mitosis prematurely (Shen et al., 2013). Most importantly, these tetraploid cells can undergo asymmetric cell division or chromosome loss, leading to tumor heterogeneity (Vitale et al., 2011; Zhang et al., 2014). Tetraploid cells have been identified in 37% of early stage cancers and have been demonstrated to promote uncontrolled cancer cell growth (Zack et al., 2013). Moreover, accumulating evidence shows that tetraploid cells are associated with multidrug resistance and lead to poor prognosis (Bakhoum and Compton, 2012; Lee et al., 2011). Therefore, identification of the signaling pathways that are involved in tetraploidization is crucial in overcoming drug resistance.

The p38 MAPK belongs to the mitogen activated protein kinase (MAPK) superfamily and is a major cellular signal transducer of extracellular stress (Han et al., 1994; Schaeffer and Weber, 1999). The p38 MAPK is activated by phosphorylation of the Thr-Gly-Tyr dual phosphorylation motif at residues Thr180 and Tyr182 (Doza et al., 1995). The p38 MAPK family consists of four members: p38α, p38β, p38γ and p38δ (Jiang et al., 1997). Among the four isoforms, the p38α MAPK isoform is the best characterized. The role of the p38 MAPK pathway in regulating cell cycle checkpoints, cell differentiation and cell survival is well-established (Thornton and Rincon, 2009). Both cell cycle checkpoints, G1/S and G2/M, have been reported to be associated with p38 MAPK (Bulavin et al., 2001; Lafarga et al., 2009). However, the role of p38 MAPK in tetraploidization remains largely unknown.

Several p38 MAPK inhibitors are under development and are being evaluated in various phases of clinical trials (Kumar et al., 2003; Triantaphyllopoulos et al., 2010). However, most of these inhibitors have failed because of unacceptable side effects. Losmapimod is the only p38 MAPK inhibitor that has progressed through Phase III clinical trials and has been shown to be well-tolerated in human clinical studies (O'Donoghue et al., 2016). In the current study, we determined whether inhibition of p38 MAPK could overcome gefitinib resistance by using the prototypical p38 MAPK inhibitor, SB203580, or losmapimod. We found that gefitinib could induce tetraploidization by activating YAP-MKK3/6-p38 MAPK-STAT3 signaling in gefitinib-resistant cells. We further used losmapimod to examine whether the ablation of p38 MAPK activation could inhibit tetraploidization and overcome gefitinib-resistance. Our data indicate that ablation of p38 MAPK signaling could eliminate tetraploidy formation induced by gefitinib and significantly inhibit cell proliferation and anchorage-independent cell growth. Knockdown of p38 MAPK also showed substantial inhibition of cancer cell growth. Finally, we found that losmapimod could successfully overcome gefitinib resistance *in vivo* in an in-house established PDX mouse model. Overall, these findings demonstrate that losmapimod could be a potential clinical agent to overcome gefitinib resistance in NSCLC.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. SB203580 was purchased from Selleck Chemicals (Houston, TX) and losmapimod was from Medchemexpress (Princeton, NJ). Gefitinib was obtained from LC Laboratory (Woburn, MA). All the above reagents were dissolved in dimethyl sulfoxide (DMSO), stored at -80 °C, and diluted in culture medium for experiments. Rosewell Park Memorial Institute Medium (RPMI)-1640, DMEM, gentamicin, antibacterial-antimycotic solution, trypsin-EDTA and Opti-MEM were all from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Biological Industries (Beit-Haemek, Israel). The primary antibody against Ki-67 (Thermo Fisher Scientific Cat# PA5-19462, RRID:AB_10981523) was purchased from ThermoScientific (Fremont, CA) and the secondary antibody against rabbit (Santa Cruz Biotechnology Cat# sc-2004, RRID: AB_631746) and mouse (Santa Cruz Biotechnology Cat# sc-2005, RRID:AB_631736) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies, including phospho-p38 MAPK (Cell Signaling Technology Cat# 9211, RRID:AB_331641), p38 MAPK (Cell Signaling Technology Cat# 9212, RRID:AB_330713), p38α MAPK (Cell Signaling Technology Cat# 9218S, RRID:AB_10694846), p21 (Cell Signaling Technology Cat# 2947S, RRID:AB_823586), cyclin D1 (Cell Signaling Technology Cat# 2922, RRID:AB_2228523), p-MKK3 (Ser189)/MKK6 (Ser207) (Cell Signaling Technology Cat# 9236S, RRID:AB_491009), MKK3 (Cell Signaling Technology Cat# 8535S, RRID:AB_1122023), MKK6 (Cell Signaling Technology Cat# 8550S, RRID:AB_1122022), p-Stat3 (Tyr705) (Cell Signaling Technology Cat# 9145, RRID:AB_2491009), Stat3 (Cell Signaling Technology Cat# 9139, RRID:AB_331757), p-YAP (Ser109) (Cell Signaling Technology Cat# 46931), p-YAP (Ser127) (Cell Signaling Technology Cat# 13008, RRID: AB_2650553), YAP (Cell Signaling Technology Cat# 14074, RRID:AB_2650491) and GAPDH (Cell Signaling Technology Cat# 2118, RRID:AB_561053) were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Tissue Specimens

A total of 25 primary lung adenocarcinoma tissues and matched non-tumorous adjacent specimens were collected from 25 patients who underwent surgical resection at the Henan Cancer Hospital (Henan, China). The histomorphology and molecular characteristics of all the samples were analyzed and tested by the Department of Pathology at Henan Cancer Hospital. Written informed consent from each patient and institutional review board approval were obtained for the current study.

2.3. Immunohistochemistry (IHC) Staining

Tissue specimens were fixed in 10% (v/v) formaldehyde in phosphate-buffered saline, embedded in paraffin and cut into 5 μm sections. The sections were deparaffinized in xylene solution and rehydrated using gradient ethanol concentrations. Antigen retrieval was performed using sodium citrate and the slides were then incubated with H_2O_2 to block endogenous peroxidases. Thereafter, primary antibodies: Ki-67 (1:100), phosphorylated (p)-p38 (1:75), and cyclin D1 (1:75) were incubated at 4 °C overnight and the signals were visualized by the indirect avidin biotin-enhanced horseradish peroxidase method according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). After developing, all sections were observed by microscope (400×) and quantitative analysis was performed using the Image-Pro Premier software (v.9.0) program.

2.4. Cell Culture

HCC827 (ATCC Cat# CRL-2868, RRID:CVCL_2063) and H1975 (ATCC Cat# CRL-5908, RRID:CVCL_5908) human lung adenocarcinoma cell lines and the HEK293T (ATCC Cat# CRL-3216, RRID:CVCL_0063) human embryonic kidney cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA). HCC827GR (RRID: CVCL_V620) cells were kindly provided by Professor Pasi A. Jane from Dana-Farber Cancer Institute (Boston, MA). All cells were cytogenetically tested and authenticated before freezing. All cell culture conditions were performed following ATCC's instructions. All lung adenocarcinoma cells were cultured in RPMI-1640, whereas HEK293T cells were cultured in DMEM, supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Each vial of frozen cells was thawed and maintained in culture for 10 to 20 passages.

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