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Leptomycin B reduces primary and acquired resistance of gefitinib in lung cancer cells

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ABSTRACT

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) gefitinib has demonstrated dramatic clinical efficacy in non-small cell lung cancer (NSCLC) patients. However, its therapeutic efficacy is ultimately limited by the development of acquired drug resistance. The aim of this study was to explore the potential utility of chromosome region maintenance 1 (CRM1) inhibitor leptomycin B (LMB) in combination with gefitinib to overcome primary and acquired gefitinib resistance in NSCLC cells. The combinative effects of gefitinib and LMB were evaluated by MTT and its underlining mechanism was assessed by flow cytometry and Western blot. LMB displayed a synergistic effect on gefitinib-induced cytotoxicity in A549 (IC50: 25.0 ± 2.1 μM of gefitinib + LMB *vs.* 32.0 ± 2.5 μM of gefitinib alone, $p < 0.05$). Gefitinib + LMB caused a significantly different cell cycle distribution and signaling pathways involved in EGFR/survivin/p21 compared with gefitinib. A549 cells then were treated with progressively increased concentrations of gefitinib (A549GR) or in combination with LMB (A549GLR) over 10 months to generate gefitinib resistance. IC50 of gefitinib in A549GLR (37.0 ± 2.8 μM) was significantly lower than that in A549GR (53.0 ± 3.0 μM, $p < 0.05$), which indicates that LMB could reverse gefitinib-induced resistance in A549. Further mechanism investigation revealed that the expression patterns of EGFR pathway and epithelial-mesenchymal transition (EMT) markers in A549, A549GR, and A549GLR were significantly different. In conclusion, LMB at a very low concentration (0.5 nM) combined with gefitinib showed synergistic therapeutic effects and ameliorated the development of gefitinib-induced resistance in lung cancer cells.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths around the world with about 1.3 million deaths per year (Huang, 2014). Histologically, lung cancer can be classified into small-cell lung cancer and non-small cell lung cancer (NSCLC) with 80–85% being NSCLC (Dragnev et al., 2013), most of which are diagnosed at an advanced stage of the disease and have a poor long-term survival from curative surgery or radiation therapy (Marquez-Medina and Popat, 2016). In particular, mutated and overactive epidermal growth factor receptor (EGFR) in NSCLC has emerged as a unique subset of lung adenocarcinoma (Koehler and Schuler, 2013), and targeting the dysregulated EGFR with tyrosine kinase inhibitors (TKIs) have been developed to treat locally advanced or metastatic NSCLC (Lee et al., 2013).

One of such EGFR TKIs is gefitinib (commercial name Iressa; AstraZeneca UK limited) which has been recently approved by FDA as a first-line treatment for metastatic EGFR mutation-positive NSCLC patients (Kazandjian et al., 2016), though recent studies revealed that some NSCLC patients without EGFR mutation(s) also respond to TKIs including gefitinib (the first generation) and afatinib (the second generation) (Home, 2016; Gridelli et al., 2011; Chao et al., 2015). Although EGFR TKI therapies have significantly improved the survival of NSCLC patients, acquired drug resistance eventually emerges and significantly limits the therapeutic potency of EGFR TKI treatments (Koehler and Schuler, 2013). Secondary somatic T790 M mutation in EGFR exon 20 and amplification of MET were frequently identified as the underling mechanisms for EGFR TKI acquired resistance, which have been reported in up to 70% of cases among patients (Pao et al., 2005a; Engelman et al., 2007; Sos et al., 2010). Other resistance mechanisms may exist and need to be further explored. For instance, it has been

Abbreviations: CRM1, chromosome region maintenance 1; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; LMB, leptomycin B; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; TKIs, tyrosine kinase inhibitors; T790M, a substitution mutation of threonine with methionine at position 790 of EGFR exon 20

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suggested that epithelial-mesenchymal transition (EMT) may contribute to the acquired resistance to gefitinib (Rho et al., 2009), by which cancer cells demonstrate morphological changes from the epithelial polarized phenotype to the mesenchymal fibroblastoid phenotype, and thereby enhance their mobility and invasiveness (Nakaya and Sheng, 2013).

Chromosome region maintenance 1 (CRM1), also known as exportin 1 (XPO1), is a nuclear export receptor responsible for transporting a variety of cancer related proteins from nucleus to cytoplasm including p53, p21, p27, pRB, FOXO, and EGFR (Lo et al., 2006; Lu et al., 2012, 2015). Our previous studies have demonstrated that CRM1 is over-expressed in lung cancer and CRM1 inhibitor, leptomycin B (LMB) could serve as an effective adjuvant regimen for lung cancer treatment (Gao et al., 2015; Lu et al., 2015). A recent study further revealed that KRAS-mutant NSCLC is vulnerable to chemical inhibition of CRM1 (XPO1)-dependent nuclear export (Kim et al., 2016). LMB, as the first generation of pharmaceutical CRM1 inhibitor isolated from *Streptomyces* spp., has shown highly inhibitory efficiency in various cancer cell lines including NSCLC cells (IC50 ranging from 0.1 to 10 nM) (Mutka et al., 2009) and more importantly, LMB demonstrated a great potential to reduce drug resistance in various cancer cells induced by different anticancer therapeutic agents (Lu et al., 2015). In addition, short-term LMB treatment (24–72 h) could reverse EMT in snail-transduced primary human mammary epithelial cells (HEMCs) by targeting CRM1 (Azmi et al., 2015). Although the phase I trial of LMB as a single therapeutic agent was unsuccessful due to its gastrointestinal toxicities like malaise and anorexia (Newlands et al., 1996), these side effects may significantly diminish at lower doses when used as a combinative agent (Gao et al., 2015; Lu et al., 2015). Therefore, the clinical application of LMB as an adjuvant therapy also deserves a thorough re-evaluation, and LMB is still used as the paradigm for a novel class of anticancer drugs based on CRM1 inhibition (Gao et al., 2015). Moreover, a series of semi-synthetic LMB derivatives (not commercial available) have been developed, which maintain high potency of LMB and show much better tolerability *in vivo* than LMB (Mutka et al., 2009).

Combination chemotherapy utilizing EGFR TKIs and drugs with different anticancer mechanisms has demonstrated to be one of effective strategies to overcome EGFR TKI resistance or EMT (Huang et al., 2013; Tartarone et al., 2013; Zhu et al., 2015; Zhao et al., 2016a). There are still no reports on the effective combined treatments using CRM1 inhibitors and EGFR TKIs for NSCLC therapy. More importantly, there are no studies investigating the long-term effect of combinative agents on reducing the development of acquired resistance of gefitinib in NSCLC. Also, the effects of inhibiting EMT on the development of EGFR TKI acquired resistance in NSCLC remain unclear.

In the present study, the combined treatment of gefitinib and LMB showed a synergistic cytotoxic effect on NSCLC cell lines A549 and H460. The mechanism of synergism of gefitinib and LMB in A549 was further investigated by flow cytometry and Western blot analyses. A549 co-treated with gefitinib and LMB exhibited significantly different profiles of survival signaling and cell cycle arrest from A549 treated by gefitinib alone. More importantly, the effects of LMB on reducing acquired gefitinib resistance in A549 were testified in A549 generated by co-treatment of gefitinib and 0.5 nM LMB for 10 months, which remained a much higher gefitinib-sensitivity compared to gefitinib-resistant A549. Finally, Western blot, quantitative real time PCR (qRT-PCR), and series of phenotype assays revealed that the two resistant A549 cells had significantly different expressions of EGFR pathways and EMT biomarkers as well as malignant transformation activities compared to parental A549 cells.

2. Materials and methods

2.1. Cell lines and reagents

The NSCLC cell lines A549 and H460 were purchased from American Type Culture Collection (ATCC). Gefitinib-resistant A549 (A549GR) was generated by an intermittent selection method through exposing A549 to a stepwise increased concentration of gefitinib (from 24 μM to 50 μM) for 10 months, which simulates the median time (6–12 months) for the development of acquired resistance of gefinitib in clinical applications (Nguyen et al., 2009; Rho et al., 2009). After each treatment of gefitinib for 48 h, the surviving cells were sub-cultured and grew to 70–80% confluence in drug-free medium for the next treatment. The dosages of gefitinib would increase continuously based on the tolerance of A549 cells. Concurrently, gefitinib + 0.5 nM LMB-resistant A549 (A549GLR) was generated by treating A549 with 0.5 nM LMB as well as the same concentration and exposure time of gefitinib as A549GR. A549 (within five passages), A549GR, and A549GLR were cultured in RPMI 1640 medium (Thermo Scientific, Logan, UT) containing 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 incubator at 37 °C. For all the *in vitro* studies, the established A549GR and A549GLR were cultured in drug-free medium for at least 1 week to eliminate the effects of gefitinib and/or LMB.

Gefitinib (≥ 98%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA,) and LMB (1 mM) was purchased from LC labs (Woburn, MA). Afatinib (> 99%) was obtained from Selleckchem (Houston, TX). The stocks of gefitinib (10 mM), afatinib (10 mM), and LMB (10 μM) were diluted to the required concentrations immediately before use in the growth media. Primary antibodies including EGFR, phospho-EGFR (Tyr1068), p44/42 MAPK (Erk1/2), phospho-p44/22 MAPK (Erk1/2) (Thr202/Tyr204), Akt, phospho-Akt (Ser473), phospho-STAT3 (Ser727), MET (D1C2), HER2/ErbB2 (D8F12), p21, survivin, E-cadherin, vimentin, and α-tubulin were purchased from Cell Signaling Technology (Danvers, MA). Twist1 antibody was purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG, anti-mouse IgG, and chemiluminescence kit were purchased from Cell Signaling Technology. Radioimmunoprecipitation assay (RIPA) lysis buffer was obtained from Santa Cruz Biotechnology.

2.2. Cell viability assay

Cell viability was evaluated by the MTT assay as described previously (Shao et al., 2011; Lu et al., 2012; Gao et al., 2015). Briefly, based on the cytotoxicity of LMB observed in this study and our previous reports (Shao et al., 2011; Lu et al., 2012; Gao et al., 2015), 0.5 nM LMB was selected for co-treatment. The synergistic effect of gefitinib and LMB was evaluated by comparing to vehicle controls for gefitinib treatments or LMB (0.5 nM) for gefitinib + LMB treatments as described in our previous studies (Gao et al., 2015; Lu et al., 2012). Experiments were repeated independently three times.

2.3. Analysis of cell cycle by flow cytometry

Based on the cell viability assay, a total of six groups of A549 with different treatments for 48 h were analyzed, including control, 0.5 nM LMB (LMB), 12 μM gefitinib (Gefitinib12), 24 μM gefitinib (Gefitinib24), 12 μM gefitinib + 0.5 nM LMB (Gefitinib12 + LMB), and 24 μM gefitinib + 0.5 nM LMB (Gefitinib24 + LMB). Cells were stained with Guava Cell Cycle Reagent (Millipore, Billerica, MA) and run on a Guava EasyCyte™ Flow Cytometer (Millipore) as previously described (Lu et al., 2012). Each sample was run in triplicate and each experiment was repeated three times.