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Antibiotic drug rifabutin is effective against lung cancer cells by targeting the eIF4E-β-catenin axis

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ABSTRACT

The essential roles of overexpression of eukaryotic translation initiation factor 4E (eIF4E) and aberrant activation of β-catenin in lung cancer development have been recently identified. However, whether there is a direct connection between eIF4E overexpression and β-catenin activation in lung cancer cells is unknown. In this study, we show that antibiotic drug rifabutin targets human lung cancer cells via inhibition of eIF4E-β-catenin axis. Rifabutin is effectively against lung cancer cells in *in vitro* cultured cells and *in vivo* xenograft mouse model through inhibiting proliferation and inducing apoptosis. Mechanistically, eIF4E regulates β-catenin activity in lung cancer cells as shown by the increased β-catenin phosphorylation and activity in cells overexpressing eIF4E, and furthermore that the regulation is dependent on phosphorylation at S209. Rifabutin suppresses eIF4E phosphorylation, leads to decreased β-catenin phosphorylation and its subsequent transcriptional activities. Depletion of eIF4E abolishes the inhibitory effects of rifabutin on β-catenin activities and overexpression of β-catenin reverses the inhibitory effects of rifabutin on cell growth and survival, further confirming that rifabutin acts on lung cancer cells via targeting eIF4E- β-catenin axis. Our findings identify the eIF4E- β-catenin axis as a critical regulator of lung cancer cell growth and survival, and suggest that its pharmacological inhibition may be therapeutically useful in lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide with poor cure rate [1]. Although the identification of activating epidermal growth factor receptor (EGFR) mutations and development of EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib, have revolutionized treatment for lung cancer, resistance to EGFR inhibitors develops due to secondary EGFR-T790M mutation [2]. β-catenin has been recently identified to contribute to lung tumor development induced by EGFR mutations. Mutant EGFR preferentially bound to tyrosine phosphorylated β-catenin, leading to an increase in β-catenin-mediated transactivation, particularly in cells harboring EGFR-T790M mutation [3].

Translation regulator eukaryotic translation initiation factor 4E (eIF4E) is essential for cap-dependent mRNA translation and protein expression [4]. eIF4E overexpression contributes to cellular transformation due to its ability to promote translation of genes involved in proliferation and survival [5], and correlates with poorer prognosis in lung cancer [6,7]. Recent work has highlighted the importance of eIF4E phosphorylation at serine 209 (S209) in transformation and suggested that eIF4E phosphorylation regulates self-renewal of leukemia stem cell via activating Wnt/β-catenin signaling [8].

Rifabutin is a semisynthetic antibiotic derived from the rifamycins, and primarily used to treat tuberculosis [9]. It kills bacterial by blocking the DNA-dependent RNA synthesis [10]. Our encounter with a non-small cell lung cancer patient combined with pulmonary tuberculosis, who showed blunted tumor progression while receiving rifabutin, prompted us to study the possible effects of rifabutin in lung cancer cells. We are the first to reveal that rifabutin inhibits proliferation and induces apoptosis of EGFR-mutated lung cancer cells *in vitro* and *in vivo*. Importantly, we show that the inhibitory effects of rifabutin in lung cancer cells are attributed to its inhibition of eIF4E-β-catenin signaling pathway. Finally, we

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show that eIF4E overexpression and phosphorylation (S209) coordinately regulate β-catenin signaling in lung cancer cells.

2. Materials and methods

2.1. Cells and drugs

Human lung cancer cell lines H3255, H1650, H1975 were purchased from American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle Medium (DMEM) (life technologies, US) containing 10% fetal bovine serum (FBS, Hyclone, UK). Rifabutin (Sigma, US) was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C.

2.2. MTS and apoptosis assays

Cells were incubated in DMSO or drug for 3 days. Cell proliferation activity was evaluated by the CellTiter 96 AQueous One Solution Cell Proliferation assay kit (Promega, US). For apoptosis assay, treated-cells were stained with Annexin V-FITC and propidium iodide (BD Pharmingen, US) according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry using Beckman Coulter FC500.

2.3. Western blot analyses

Cells were lysed by RIPA buffer (Life Technologies Inc, US). Proteins from whole-cell extracts were resolved using denaturing SDS–PAGE and analyzed by Western blot using anti-eIF4E, p-eIF4E (S209), p-β-catenin (S552), β-catenin, Ki67 (Cell Signaling Technologies, US), cleaved PARP, Mcl-1 and GAPDH (Santa Cruz Inc, US).

2.4. Small interfering RNA

Small interfering RNA (siRNA) to human eIF4E was custom-ordered from Ambion. The two sequences tested were siRNA eIF4E a (GGAUGGUAUUGAGCCUAUG) and siRNA eIF4E b (GGUGGGCACUCUGGUUUUU) [11]. Control siRNA was purchased from Ambion. Transfection was done suing DharmaFECT1 reagent according to the manufacturer's protocol.

2.5. Plasmids transfection assays

To assess β-catenin activity, cells were firstly transfected with a M50 Super 8× TOPFlash plasmid (a kind gift from Dr. Randall Moon) [12]. At 24 h post-transfection, cells were treated with drugs for 24 h and then were harvested by using the Luciferase Reporter Assay System (Promega, US). For β-catenin overexpression, cells were transfected with 1.5 μg pcDNA, or pcDNA-β-cat (human β-catenin pcDNA3 plasmid, a kind gift from Dr. Eric Fearon) using Lipofectamine® Transfection Reagent (Thermo Scientific, US). Western blot and rescue experiments were performed at 24 h post-transfection. H3255 cell line overexpressing eIF4E and its mutant forms were generated by retroviral transduction by using MSCV-internal ribosome entry site (IRES) constructs as previously described [13].

2.6. Lung cancer xenograft in SCID mouse

SCID mice at 6 weeks old were purchased from the Jackson Laboratory. All procedures were conducted according to the guidelines approved by the Institutional Animal Care and Use Committee. Briefly, one million H3255 cells were subcutaneously injected into the flank of each mouse. When tumor volume reached approximately 200 mm^3, the mice were treated with vehicle control and intraperitoneal rifabutin at 20 mg/kg once daily for three weeks. For ki67 and active caspase-3 immunostaining, tumor frozen section slides were fixed with 4% paraformaldehyde (Sigma, US). The slides were incubated with ki67 or active caspase-3 antibody (Santa Cruz, US) at 4 °C for overnight and secondary antibodies at room temperature for 2 h. The cytoplasm and nuclei were stained with hematoxylin and eosin.

2.7. Real time PCR

The total RNA was isolated from H3255 cells with TRIzol Reagent (Ambion, US) and used to produce the first-strand cDNA using iScript cDNA Synthesis Kit (Bio-rad, CA). PCR were performed to amplify the cDNA using a SsoFast EvaGreen Supermix (Bio-rad, CA). The sequences of the primers are as follows: MYC (5′-AAT GAA AAG GCC CCC AAG GTA GTT ATC C-3′and 5′-GTC GTT TCC GCA ACA AGT CCT CTT C-3′), Cyclin D (5′-CCG TCC ATG CGG AAG ATC-3′ and 5′-ATG GCC AGC GGG AAG AC-3′), LEF1 (5′-CGA CGC CAA AGG AAC ACT GAC ATC-3′, and 5′- GCA CGC AGA TAT GGG GGG AGA AA-3′) and GAPDH (5′-AAC GGG AAG CTT GTC ATC AAT GGA AA-3′ and 5′-GCA TCA GCA GAG GGG GCA GAG-3′).

2.8. Statistical analyses

All experiments in this study were repeated at least three times with similar results. The data are expressed as mean and standard deviation (SD). Statistical analyses were performed by unpaired Student's t test, with p-value < 0.05 considered statistically significant.

3. Results

3.1. Rifabutin effectively inhibits proliferation and induces apoptosis of multiple EGFR-mutated lung cancer cell lines

We firstly investigated the effects of rifabutin on the proliferation and apoptosis in several non-small cell lung cancer cell lines, including H3255, H1650 and H460. These cell lines harbor EGFR tyrosine kinase domain mutation L858R [14]. H3255 is gefitinib-sensitive whereas H1650 and H460 are gefitinib-resistant due to additional mutations, such as phosphatase and tensin homologue (*PTEN*) loss (H1650) or *KRAS* mutations (H640). We found that rifabutin dose-dependently inhibited proliferation of these cell lines, with IC_{50} of 5–7 μM (Fig. 1A). Rifabutin also induced apoptosis in lung cancer cell lines as assessed by flow cytometry for Annexin V staining (Fig. 1B). Decreased level of proliferating marker protein Ki67 and anti-apoptotic protein Mcl-1, and increased level of pro-apoptotic protein active caspase-3 were observed in these cell lines treated with rifabutin (Fig. 1C), further demonstrating the anti-proliferative and pro-apoptotic effects of rifabutin in lung cancer cells.

3.2. Rifabutin inhibits β-catenin signaling via suppressing eIF4E phosphorylation in lung cancer H3255 cells

It has been reported that β-catenin plays an essential role in lung cancer development induced by EGFR mutations [3]. We therefore tested whether rifabutin affects β-catenin activities in lung cancer cells. Phosphorylation of β-catenin on Ser552 induces its translocation into the nucleus and increase its transcriptional function [15]. Rifabutin dose-dependently inhibited phosphorylation of β-catenin on Ser552 in H3255 cells (Fig. 2A). By using a β-catenin reporter assay, we found that rifabutin decreased β-catenin activities (Fig. 2B). Consistent with the decreased β-catenin activities, expression of β-catenin target transcriptional genes Myc, Cyclin D