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Inhibition of mitochondrial translation effectively sensitizes renal cell carcinoma to chemotherapy

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ABSTRACT

The functional importance of mitochondrial protein translation has been recently documented in the context of various cancers but not renal cell carcinoma (RCC). In lines with these efforts, our work demonstrates that mitochondrial translation inhibition by tigecycline or depletion of EF-Tu mitochondrial translation factor effectively targets RCC and significantly sensitizes RCC response to chemotherapy. We show that antibiotic tigecycline inhibits multiple biological functions of RCC, including growth, colony formation and survival. It also significantly enhances *in vitro* and *in vivo* efficacy of paclitaxel in RCC. Tigecycline preferentially inhibits translation of mitochondrial DNA-encoded proteins, activities of mitochondrial respiratory complexes that contain mitochondrially encoded subunits. As a consequence of mitochondrial respiratory chain inhibition, decreased mitochondrial respiration is observed in RCC cells exposed to tigecycline. In contrast, tigecycline is ineffective in RCC ρ0 cells that lack mitochondrial DNA and subsequent mitochondrial respiration, further confirm mitochondrial translation inhibition as the mechanism of tigecycline's action in RCC. Importantly, genetic inhibition of mitochondrial translation by EF-Tu knockdown reproduced the inhibitory effects of tigecycline. Finally, we show the association between mitochondrial translation inhibition and suppression of PI3K/Akt/mTOR signaling pathway. Our work used pharmacological and genetic strategies to demonstrate the important roles of mitochondrial translation in RCC and emphasize the therapeutic value of sensitizing RCC to chemotherapy.

1. Introduction

Renal cell carcinoma (RCC) is an epithelial tumor derived from the proximal tubules of nephrons and the most common malignant tumor in the adult kidney [1]. Clear cell and papillary RCC are two major subtypes with distinct morphological and cytogenetic features [2,3]. The current available therapeutic options including chemotherapy, radiotherapy, targeted therapy (eg, tyrosine kinase inhibitor and antibodies) and immunotherapy are ineffective and the clinical management of advanced RCC is still challenging [4–6]. A primary cause of the resistance and subsequent treatment failure is RCC intra-tumoral heterogeneity [7–9]. Targeting common between different molecular/genetic subclasses of RCC may therefore represent a potential therapeutic strategy.

Mitochondria functions are involved in a diverse collection of intracellular signaling, energy production, cellular homeostasis and survival [10,11]. It is known that critical respiratory chain subunits that are essential for proper mitochondrial functions are encoded by mitochondrial DNA [12]. Inhibition of mitochondrial translation that leads to loss of mitochondrial respiration and subsequent mitochondrial dysfunction has been demonstrated to be effective in eliminating cancer and cancer stem cells [13,14]. Some types of cancer cells have been shown to be unique in their mitochondrial characteristics and reliance on mitochondrial respiration for their supply of energy [13,15–17]. Tigecycline is a FDA-approved antibiotic with potent anticancer activities [18–20]. It kills cancer cells via multiple molecular mechanisms. Besides Wnt/β-catenin inhibition and autophagy induction [19,20], tigecycline directly binds to mitochondrial ribosome and inhibits mitochondrial translation [13,18,21].

In this work, we investigated the biological effects of

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mitochondrial translation inhibition using pharmacological (eg, tigecycline) and genetic (eg, EF-Tu knockdown) approaches in RCC. Our work demonstrates that mitochondrial translation inhibition effectively targets renal cell carcinoma (RCC) and significantly sensitizes RCC response to chemotherapy *in vitro* and *in vivo*. Our work also demonstrate that mitochondrial translation inhibition correlates with suppression of PI3K/Akt/mTOR singaling in RCC.

2. Materials and methods

2.1. Cell lines and drugs

Human RCC cell lines 786-0 and A-498 were obtained from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in complete medium containing RPMI 1640 medium (Life technologies, US) and 10% fetal bovine serum (Invitrogen, US). 786-O ρ0 cell line was established according to the method previously described [22]. Briefly, 786-O cells were growing in complete medium containing 2 μg/ml ethidium bromide (EtBr), 1.5 mM sodium pyruvate, 4 mM L-glutamine and 300 μM uridine, (Sigma, MO, US) for 50 days. 786-O ρ0 cell line was confirmed with mitochondrial DNA depletion analysis and thereafter maintained in above media without EtBr. Tigecycline and paclitaxel (Sigma, US) was dissolved in DMSO and stored in an aliquots at −20 °C.

2.2. Measurement of cell proliferation, apoptosis and colony formation

Cell proliferation was determined using CellTiter 96 AQueous One Solution Cell Proliferation assay kit (Promega, US). Apoptosis was determined by flow cytometry. Apoptotic cells were stained with Annexin V-FITC and 7-AAD (BD Pharmingen, US) kit prior to FACS analysis on a MACSQuant Analyser (Miltenyi Biotec, US). The percentage of Annexin V was analyzed using MACSQuantify software. Colony formation was performed using soft agar. A bottom layer of 0.7% Bacto agar and a top layer of 0.3% Bacto agar containing cells and drug were plated. Culture medium was added to the top and replaced with fresh one every three days. After 10–14 days, colonies were stained with 0.4% crystal violet (Sigma, US) and then photographed.

2.3. Western blot analyses

Cells were suspended in RIPA lysis buffer (Invitrogen, US) containing protease inhibitor cocktails (Invitrogen, US). Proteins from whole cell extracts were loaded to sodium dodecyl sulphate-polyacrylamide gels followed by transfer to nitrocellulose membranes and analyzed by western blot. Antibodies used in western blot are against Cox-1, Cox-2, Cox-4, Grp-78, phosphor and total PI3K, Akt, mTOR and S6, and β-actin (Cell Signaling, US). Detection was performed by the enhanced chemical luminescence method (Pierce, US).

2.4. Real time-PCR

RNA were extracted from cells using TRIzol Reagent (Life technologies, US) and used to produce the first-strand cDNA with iScript cDNA Synthesis Kit (Bio-rad, CA). cDNA was added to a SsoFast EvaGreen Supermix (Bio-rad, CA) and amplified via PCR on CFX96 RT PCR system (Bio-rad, CA). The sequence of primers for human Cox-1, Cox-2, Cox-4 and Grp78 are the same as previously described [13]. The sequence of primers sets for human MT-ND6, MT-CO2, and SDHA are the same as previously described [23]. The mRNA levels of all genes were quantified using a comparative CT method with β-actin for normalization.

2.5. Mitochondrial complex activities

Complex I, II IV and V activities were determined using using Mitochondrial Complex I, II, IV and V Activity Assay Kits (Novagen, US) according to manufacture's instructions. The activity levels were indicated by the decrease in absorbance in mOD/min at 340 nm (I), 600 nm (II), 550 nm (IV) and 340 nm (V) in kinetic mode using Infinite M1000 PRO plate reader (Life Sciences, US).

2.6. Measurement of mitochondrial respiration

Oxygen consumption rate (OCR) was performed using the Seahorse XF96 analyser (Seahorse Bioscience, US). Cells seeded on XF96-well plate were equilibrated to the un-buffered medium for 30 min at 37 °C in a CO_2-free incubator prior to being transferred to the Seahorse X96 analyser. We measured the basal OCR, and then sequentially injected oligomycin (OLI, 1 μg/ml), Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 0.4 μM) and Antimycin A and Rotenone combination (A&R, 2.5 μM and 2.5 μM).

2.7. RNAi of human EF-Tu expression

Cells were plated in 12-well plates and transfected with 100 nM non-targeting siRNA or human EF-Tu-specific siRNAs using Dharmafect Transfection Reagent (Dharmacon RNAi Technologies) for 24 h in accordance with the manufacturer's instructions. The target sequences of human EF-Tu-specific ONTARGETplus siRNAs (Dharmacon RNAi Technologies) are 5′-CAG CCA ATG ATC TTA GAG AAA-3′ and 5′-GCT CAC CGA GTT TGG CTA TAA-3'.

2.8. RCC cancer xenograft mouse model

Male Balb/c mice were maintained under specific pathogen free conditions at 22 °C. All procedures with mice were approved by the Animal Care Committee of The First People's Hospital of Jiangxia District Wuhan City. 786-O cells in log phase suspended in PBS were injected into mice. Tumor diameter was measured every 5 days and volume was calculated as (major axis) x (minor axis) x (height) x 0.52. After development of palpable tumor, 20%/80% DMSO/saline, 1 mg/kg paclitaxel, 20 mg/kg tigecycline or combination of paclitaxel and tigecycline were intraperitoneally injected once every alternative day.

2.9. Statistical analyses

All data are expressed as mean and standard deviation. Statistical analyses were performed by unpaired Student's *t*-test with p-value < 0.05 considered statistically significant.

3. Results

3.1. Tigecycline sensitizes RCC cells to chemotherapy in vitro and in vivo

We examined the effects of tigecycline alone and its combination with standard chemotherapy agent paclitaxel on various aspects of RCC cells including growth, colony formation and survival. 786-O and A-498 cells which were often used as *in vitro* models of clear cell and papillary RCC were exposed to drug alone or

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