eIF4E-eIF4G complex inhibition synergistically enhances the effect of sorafenib in hepatocellular carcinoma

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The clinical efficacy of sorafenib in hepatocellular carcinoma (HCC) is disappointing due to its low response rate and high rates of adverse effects. The eukaryotic translation initiation factor 4F (eIF4F) complex, mainly consisting of eIF4E-eukaryotic translation initiation factor 4G (eIF4G) interaction, is involved in the induction of drug resistance. Herein, we aimed to demonstrate that eIF4E-eIF4G complex inhibition enhanced the effect of sorafenib. The antiproliferation effect of combined treatment was evaluated by MTT assay and colony formation assay. Flow cytometry was used to detect the early cell apoptosis and cell cycle. The specific mechanism was demonstrated using western blot and lentivirus transfection. The combination of sorafenib with eIF4E-eIF4G inhibitors 4E1RCat (structural) or 4EGI-1 (competitive) synergistically inhibited the cell viability and colony formation ability of HCC cells. Moreover, the combined treatment induced more early apoptosis than sorafenib alone through downregulating the Bcl-2 expression. Besides, the coadministration of sorafenib and 4E1RCat or 4EGI-1 synergistically inhibited the expressions of eIF4E, eIF4G and phospho-4E-BP1 in HCC cells while blocking the phosphorylation of 4E-BP1 with lentiviral transfection failed to increase the sensitivity of HCC cells to sorafenib treatment. PI3K-AKT-mTOR signaling was also inhibited by the combined treatment. In a word, eIF4E-eIF4G complex inhibition synergistically enhances the effect of sorafenib in HCC treatment. Anti-Cancer Drugs 32: 822–828 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: 4E1RCat, 4EGI-1, eIF4E, eIF4G, hepatocellular carcinoma, sorafenib

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Introduction
Liver cancer is the sixth commonly diagnosed cancer in both men and women worldwide and the fourth leading cause of cancer death in 2018 [1]. Hepatocellular carcinoma (HCC) contributes 75–85% of primary liver cancer cases [1]. Currently, effective treatments for HCC include liver resection, ablation, chemoembolization and systemic therapy [2]. For late-stage HCC, the multiple kinase inhibitor sorafenib is the first-line systemic treatment and is beneficial to prolong the survival of HCC patients [2–4]. Sorafenib inhibits cancer cells by targeting RAF1, BRAF, vascular endothelial growth factor receptor and platelet-derived growth factor receptor β (PDGFRB) [5]. However, its low response rate (2–3%) and severe adverse effects are always challenging in HCC treatment [3,4].

The dysregulation of cap-dependent mRNA translation is crucial in the development of many human cancers [6,7], and the eukaryotic translation initiation factor 4F complex (eIF4F complex) is the key regulator of cap-dependent mRNA translation [8,9]. eIF4F complex is mainly composed of proteins eukaryotic translation initiation factor 4E (eIF4E) and eukaryotic translation initiation factor 4G (eIF4G) [10]. The formation of eIF4E-eIF4G interaction activates the biological function of eIF4F complex and initiates the cap-dependent mRNA translation. Furthermore, eIF4E-eIF4G interaction is also tightly regulated by eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). The Thr37/46 phosphorylation of 4E-BP1 is activated by PI3K/AKT/mammalian target of rapamycin (mTOR) signaling and releases eIF4E from eIF4E-4E-BP1 interaction for eIF4E-eIF4G complex formation [7,11].

The PI3K/AKT/mTOR signaling pathway is involved in the acquired drug resistance of HCC cells after sorafenib treatment [12–14], and the eIF4F complex is an indicator of both innate and acquired drug resistance in human cancer [15]. Therefore, we hypothesized that interfering with eIF4E-eIF4G interaction could enhance the antitumor effects of sorafenib in HCC cells.
Materials and methods

Cell culture

HCC cell lines HepG2 and Huh7, which were authenticated by short tandem repeat analysis, were gifted from Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle medium (DMEM, Hyclone, SH30022.01, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, 10099-141, Carlsbad, California, USA) and 1% Penicillin/Streptomycin (Beyotime, C0222, Shanghai, China). Cells were maintained in a Series 8000 Direct-Heat CO2 Incubator (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing a temperature of 37°C and 5% CO2. The DMEM solution was exchanged every 2 days, and the cell lines were cultured no more than ten passages.

Antibodies and reagents

The following antibodies were used: Sorafenib (MedChemExpress, HY-2021, San Francisco, California, USA), 4E-BP1 (CST, 9644, Boston, Massachusetts, USA), β-Actin (CST, 3700), AKT (CST, 9272), Bax (CST, 2772), Bcl-2 (CST, 3498), Caspase-3 (CST, 9662), Caspase-8 (CST, 4790), elf4E (CST, 2067), elf4G (CST, 2469), mTOR (Santa Cruz Biotechnology, sc-293133), PI3K p85 (CST, 4060), Phospho-mTOR (Ser2448) (Santa Cruz Biotechnology, sc-293089, Dallas, Texas, USA), Phospho-AKT (Ser473) (CST, 2067), elF4G (CST, 2469), mTOR (Santa Cruz Biotechnology, sc-293133), P13K p85 (CST, 4257).

The following reagents were used: Sorafenib (MedChemExpress, HY-10201, San Francisco, California, USA), 4E-BP1 (MedChemExpress, HY-14427), 4EG1-1 (MedChemExpress, HY-19831), the powders of these reagents were dissolved in a solvent according to the manufacturer's instructions.

Lentivirus transfection

The pGy-lenti-CMV-eGFP lentivirus was constructed by Ecotop Scientific Co., Ltd. (Guangzhou, China) based on the plasmid from Addgene. The gene with 4E-BP1 overexpression was reconstructed based on the plasmid pcDNA3-TORC1R CAR (T/A) gifted from Jin Zhang (Addgene, 64927, Cambridge, Massachusetts, USA). The gene with the mutation of phosphorylation site Thr37/46 of 4E-BP1 was reconstructed based on the plasmid pcDNA3-TORC1 (T/A) gifted from Jin Zhang (Addgene, 64928). According to the manufacturer's instructions, the lentivirus was transfected into Huh7 and HepG2 cells with polybrene (Solarbio, C8470) for 15 min. The colony quantity was counted with Image-J (NIH, Bethesda, Maryland, USA).

Cell apoptosis assay

HCC cells in six-well plates (1000 cells/well) and phosphatase inhibitor cocktail (MedChemExpress, HY-K0021). Protein concentrations were measured with an enhanced BCA protein assay kit (Beytime, P0010). Total proteins were separated using SDS–PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, USA). Then, after blocking with 5% BSA (Beyotime, ST023) in Tris-Buffered Saline Tween-20 (TBST) solution for one hour at room temperature, membranes were incubated with primary antibodies for 16 h at 4°C. Afterward, membranes were incubated with goat anti-mouse secondary antibody (ZSGB-BIO, ZDR-5307, Beijing, China) or goat anti-rabbit secondary antibody (ZSGB-BIO, ZDR-5306) for 1 h at room temperature. Finally, protein expression was detected with ECL-western blotting substrate (Solarbio, PE0010) using an automatic chemiluminescence analyzer (Tanon 6100, Shanghai, China).
The coadministration of eIF4E-eIF4G inhibitors and sorafenib inhibited the proliferation and colony formation of HCC cell lines. (a) The coadministration of sorafenib with 4E1RCat or 4EGI-1 significantly decreased the viability in HCC cell lines. (b,c) The combined treatment of 4E1RCat or 4EGI-1 with sorafenib significantly decreased the colony formation ability in HCC cell lines. The concentrations of sorafenib, 4E1RCat and 4EGI-1 were 8, 25 and 25 µM, respectively. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. All data were displayed as mean ± SD. eIF4F, eukaryotic translation initiation factor 4E; eIF4G, eukaryotic translation initiation factor 4G; HCC, hepatocellular carcinoma.
4EGI-1 enhanced the effect of sorafenib in apoptosis induction in HCC cell lines. (a,b) The combined administration of sorafenib and 4EGI-1 induced more early apoptosis than sorafenib or 4EGI-1 alone. (c) The expressions of apoptosis-related proteins were detected by western blot 48 h later after the coadministration of sorafenib and 4E1RCat or 4EGI-1. (d,e) The cell cycle was not retarded significantly after the combined treatment. The concentrations of sorafenib, 4E1RCat and 4EGI-1 were 8, 25 and 25 µM, respectively. *P < 0.05, **P < 0.01 and ***P < 0.001. All data were displayed as mean ± SD. HCC, hepatocellular carcinoma.
were fixed in 70% ethanol at 4°C overnight and washed twice with PBS. Then, they were stained with PI/RNase staining buffer (BD Biosciences, 550825) at room temperature for 15 min. Cell cycle analysis was conducted by CytoFLEX analysis of flow cytometry (Beckman Coulter, USA) within 1 h. After that, the results were analyzed further with software Flowjo 7.6. During this process, there are three gating choices in total that are FSC-A/SSC-A, PE-A/PE-H and PE-A/Count, respectively.

**Statistical analysis**

The data in this study were repeated at least three times independently and were analyzed using a one-way analysis of variance (ANOVA, Tukey’s multiple comparisons test) for multiple comparisons with Prism 6.0 software. P value <0.05 was set as a significant statistical difference.

**Results**

**4E1RCat or 4EGI-1 enhanced the effect of sorafenib in hepatocellular carcinoma cell lines**

To detect the effect of sorafenib in HCC cells when combined with inhibitors of eIF4E-eIF4G interaction, both structural inhibitor (4E1RCat) and competitive inhibitor (4EGI-1) were used. The MTT assay showed that the combination of sorafenib with 4E1RCat or 4EGI-1 significantly decreased the cell viability than sorafenib, 4E1RCat or 4EGI-1 alone (all P < 0.0001, Fig. 1a, Supplementary Figure S1, Supplemental digital content 1, http://links.lww.com/ACD/A380). After combined with 4E1RCat or 4EGI-1, sorafenib significantly inhibited the colony formation ability than single drug administration in Huh7 (sorafenib vs. sorafenib+4EIRCat, P = 0.0119; sorafenib vs. sorafenib+4EGI-1, P = 0.0143) and HepG2 (sorafenib vs. sorafenib+4EIRCat, P = 0.002; sorafenib vs. sorafenib+4EGI-1, P = 0.002) cell lines (Fig. 1b,c). Furthermore, the combination of sorafenib with 4EGI-1 significantly induced early apoptosis of Huh7 (P = 0.0005) and HepG2 (P = 0.0205) cells (Fig. 2a,b). The expression of Bcl-2 was downregulated by the combined treatment of sorafenib and inhibitors of eIF4E-eIF4G interaction (Fig. 2c). However, there was no significant difference in the cell cycle process after combination treatment (Fig. 2d,e).

**4E1RCat or 4EGI-1 synergistically enhanced the effect of sorafenib by inhibiting eIF4E and eIF4G in HCC cell lines**

Immunoblotting results showed that the expressions of eIF4E and eIF4G were downregulated after the
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combined treatment of sorafenib with 4E1RCat or 4EGI-1 in HepG2 and Huh7 cell lines (Fig. 3a). Besides, the phosphorylation of 4E-BP1(Thr37/46) was deactivated as well (Fig. 3a).

Gene mutation of the Thr37/46 phosphorylation site of 4E-BP1 failed to enhance the effect of sorafenib in hepatocellular carcinoma cell lines
The effectiveness of lentivirus transfection was confirmed by western blot in Huh7 and HepG2 cell lines (Fig. 3b). Huh7 and HepG2 cells with 4E-BP1 overexpression and 4E-BP1 Thr37/46 mutation were treated with sorafenib, but there was no significant difference in the cell viability between 4E-BP1 overexpression and 4E-BP1 Thr37/46 mutation HCC cells (Fig. 3c).

The combination of sorafenib and 4E1RCat or 4EGI-1 synergistically inhibited the PI3K-AKT-mTOR signaling pathway in hepatocellular carcinoma cell lines
When combined with 4E1RCat or 4EGI-1, sorafenib downregulated the expression of PI3K (p85) in Huh7 and HepG2 cells (Fig. 3d). Furthermore, the expression of AKT was inhibited and the phosphorylation of AKT (Ser473) was deactivated (Fig. 3d). Moreover, the downstream target of AKT, mTOR, was downregulated and deactivated at the phosphorylation site Ser2448 by the combined treatment in HCC cells (Fig. 3d).

Discussion
The drug response of sorafenib in HCC patients is about 2.0–3.3%, and the adverse effects of sorafenib limit its widespread clinical application [3,4]. Herein, we found a novel way to enhance the antitumor effect of sorafenib in HCC cells by inhibiting the eIF4E-eIF4G complex.

It is well known that 4E-BP1 and eIF4G competitively bind to the same site of eIF4E, and eIF4E-eIF4G interaction activates the initiation phase of cap-dependent mRNA translation. 4E1RCat is the structural inhibitor and 4EGI-1 is the competitive inhibitor for eIF4E-eIF4G interaction. 4E1RCat interferes with eIF4E-eIF4G interaction by overlapping the binding site of eIF4E. As a result, both eIF4E-eIF4G and eIF4E-4EBP1 interactions are inhibited by 4E1RCat [16]. On the contrast,
the competitive inhibitor 4EGI-1 displaces eIF4G from eIF4E-eIF4G interaction and empties the docking site for 4EBP1 to promote eIF4E-4EBP1 interaction [17,18]. In this study, both the structural inhibitor 4E1RCat and the competitive inhibitor 4EGI-1 synergistically enhanced the antitumor effect of sorafenib in HCC cells (Fig. 4).

eIF4E-4EBP1 interaction can be promoted by 4EGI-1 and the dephosphorylation of 4EBP1 (Thr37/46). In this study, the Thr37/46 phosphorylation of 4EBP1 was deacivated after specific lentivirus transfection in HCC cells and the results showed that the mutation Thr37/46 sites did not affect the drug response of sorafenib to HCC cells. These results enhanced the evidence that it was eIF4E-eIF4G interaction that mediated the synergistic effect of sorafenib combined with 4EGI-1 or 4E1RCat, while eIF4E-4EBP1 interaction had no significant influence in this synergistic phenomenon (Fig. 4).

The PI3K/AKT signaling is also a crucial mechanism mediating the resistance of HCC cells to sorafenib treatment, and AKT inhibition by GDC0068 can enhance the chemosensitivity to sorafenib in advanced HCC [13,19]. Furthermore, the antiapoptotic protein Bcl-2 is upregulated in acquired resistant HCC cells, and inhibiting the Bcl-2 by microRNA-34a potentiates the effect of sorafenib [19,20]. Besides, the knockdown of eIF4E enhances the chemoresistance of cancer cells through Bax/Bcl-2 regulation [21]. Therefore, these findings support that the synergetic efficacy of sorafenib and eIF4E-eIF4G interaction inhibitors might result from the inhibition of PI3K/AKT signaling, eIF4E or eIF4G (Fig. 4).

In conclusion, eIF4E-eIF4G complex inhibitors, 4E1RCat and 4EGI-1, synergistically enhanced the effect of sorafenib in HCC cell lines. These findings provide a potential strategy to increase the response rate of sorafenib in HCC patients. Therefore, the clinical studies of sorafenib and eIF4E-eIF4G complex inhibitors in HCC patients are in plan in our group. Also, the more detailed biological mechanism of this synergistic effect will be explored according to our plan.

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Conflicts of interest

There are no conflicts of interest.

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