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Rapamycin enhances cetuximab cytotoxicity by inhibiting mTOR-mediated drug resistance in mesenchymal hepatoma cells

Wei Chen†, Qi-Da Hu†, Xue-Feng Xia, Chao Liang, Hao Liu, Qi Zhang, Tao Ma, Feng Liang, and Ting-Bo Liang*

Department of Hepatobiliary and Pancreatic Surgery; The Second Affiliated Hospital; Zhejiang University School of Medicine; Hangzhou, PR China

†These authors contributed equally to this work.

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The synergistic effect of combined drug therapy provides an enhanced treatment for advanced liver cancer. We aimed to investigate the underlying mechanism of cetuximab sensitization by rapamycin in hepatoma cells. Four hepatoma cell lines, HepG2, HuH7, SNU-387, and SNU-449, were treated with cetuximab or cetuximab plus rapamycin and growth inhibition was evaluated by measuring relative cell viability and cell proliferation. The cell phenotype was determined for each hepatoma cell line by western blot analysis of E-cadherin and vimentin expression and mTOR activation status. To identify the role of mTOR signaling in cetuximab sensitization, we used deferoxamine-mediated hypoxia to induce epithelial–mesenchymal transition (EMT) in HuH7 and HepG2 cells and measured mTOR activity after rapamycin treatment.

Rapamycin significantly increased cetuximab cytotoxicity in hepatoma cell lines with differential sensitivities. Phenotypic differences among hepatoma cell lines, specifically epithelial (HuH7and HepG2) and mesenchymal (SNU-387 and SNU-449), correlated with the efficacy of rapamycin cotreatment, although rapamycin treatment did not affect cell phenotype. We further showed that rapamycin inhibits mTOR in mesenchymal SNU-387 and SNU-449 cells. In addition, the induction of EMT in HuH7 and HepG2 cells significantly decreased cetuximab cytotoxicity; however, rapamycin treatment significantly restored cetuximab sensitivity and decreased mTOR signaling in these cells.

In conclusion, we identified significant differences in rapamycin-induced cetuximab sensitization between epithelial and mesenchymal hepatoma cells. We therefore report that rapamycin cotreatment enhances cetuximab cytotoxicity by inhibiting mTOR signaling in mesenchymal cells.

Introduction

The heavy burden of liver cancer, which is associated with its high incidence and mortality rates, means that new, effective treatments for hepatic malignancies are urgently required.[1,2] Curative surgical procedures, such as tumor resection and liver transplantation, are not available for unresectable or metastatic liver cancer.[3] Currently, sorafenib is the only approved treatment for advanced disease and is widely used in clinical applications as a first-line treatment.[4,5] However, the need for an effective second-line treatment of advanced liver cancer is still unmet, despite the many agents currently under development.[6]

There is increasing evidence for the efficacy of combined therapy for advanced hepatic carcinoma.[7-9] Elucidation of the synergistic mechanisms of specific drug combinations should lead to enhanced antineoplastic effects and prolonged patient survival. Cetuximab, an epidermal growth factor receptor (EGFR) inhibitor, has an inhibitory effect on EGFR-overexpressing tumors, such as non-small cell lung cancer.[10] However, cetuximab was only modestly effective in clinical trials of hepatocellular carcinoma.[11] Our recent publications demonstrated the cooperative effect of cetuximab and the STAT3 inhibitor NSC 74839 via a mechanism involving EGFR and STAT3 signaling pathways.[12]

In the last decade, there has been accumulating evidence that the novel agent, rapamycin, has a critical role in regulating basic cellular functions by inhibiting the mammalian target of rapamycin (mTOR) signaling pathway.[13] In many neoplasms, mTOR signaling is aberrantly high; therefore, rapamycin is a promising general anticancer agent.[14,15]

In this study, we found that rapamycin enhances cetuximab sensitivity by different amounts in different hepatoma cell lines. We investigated the underlying mechanisms cetuximab resistance in liver cancer and provide evidence for the important roles of the cellular phenotype and basal mTOR pathway activity in this process.

*Correspondence to: Ting-Bo Liang; Email: liangtingbo@zju.edu.cn
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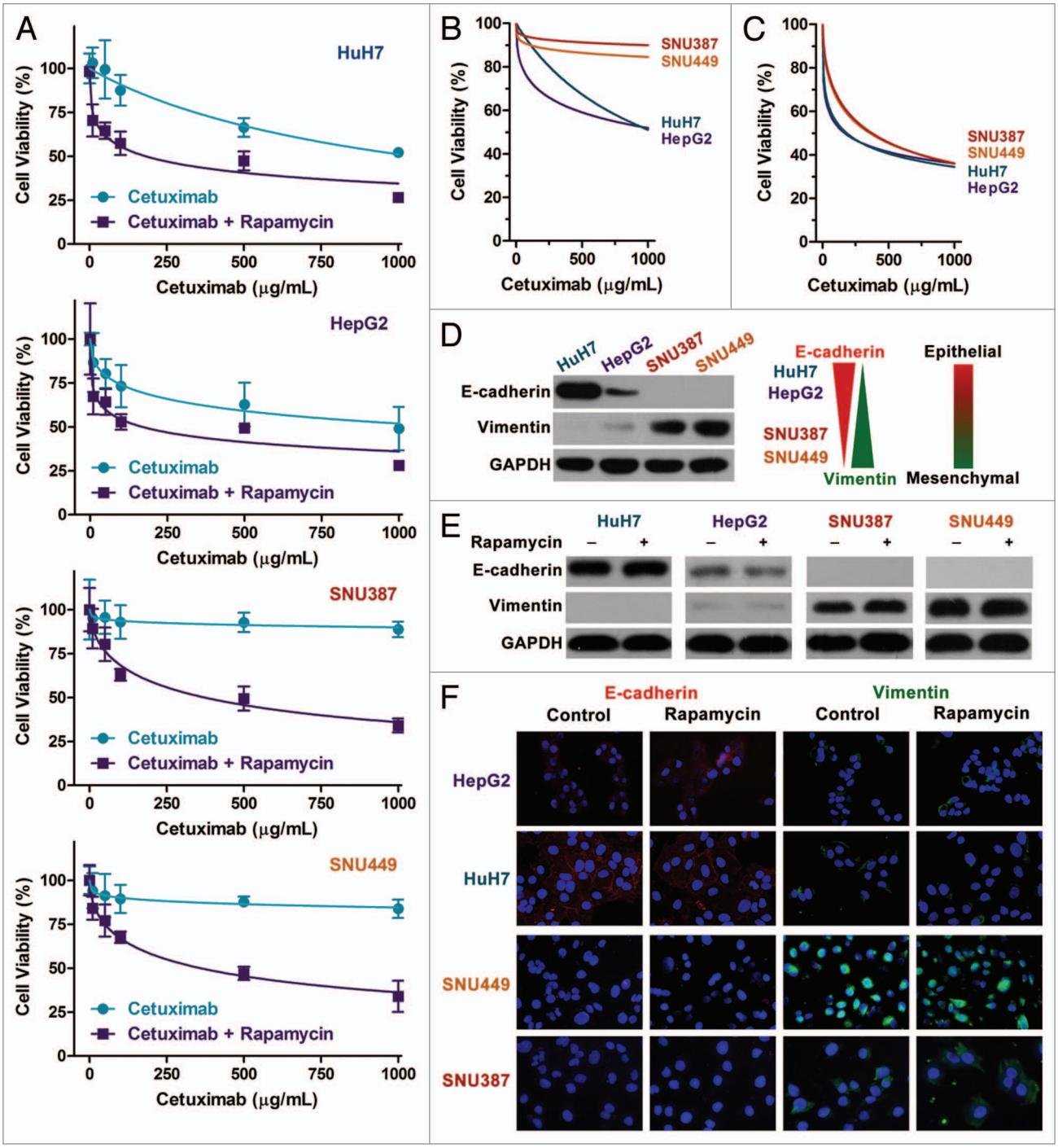

Figure 1. Differential cetuximab sensitization by rapamycin in hepatocellular carcinoma cell lines. (**A**) Cell viability assays show that rapamycin sensitizes HepG2, HuH7, SNU-387, and SNU 449 cells to cetuximab. Cetuximab sensitivity to hepatoma cells (**B**) without or (**C**) with rapamycin cotreatment were obtained from (**A**). (**D**) Western blotting analysis of E-cadherin and vimentin expression characterizes epithelial or mesenchymal phenotypes in different human hepatocellular carcinoma (HCC) cells. Rapamycin treatment does not alter E-cadherin or vimentin expression (**E**) or localization (**F**) in HCC cells.

Results

Rapamycin differentially enhances cetuximab sensitivity in hepatoma cells

Rapamycin significantly increased cetuximab cytotoxicity in HepG2, HuH7, SNU-387, and SNU 449 cells ($P < 0.05$ vs. cetuximab, for all four cell lines; two-way ANOVA followed by Bonferroni post hoc tests; **Fig. 1A**). Cetuximab sensitivity varied among cell lines (**Fig. 1B**). The IC_{50} values were significantly lower in HuH7 and HepG2 cells (1047 ± 148 and 1198 ± 435 μg/mL, respectively) than in SNU-387 and SNU-449 cells ($P < 0.01$, HuH7 or HepG2 vs. SNU-387 or SNU-449; extra

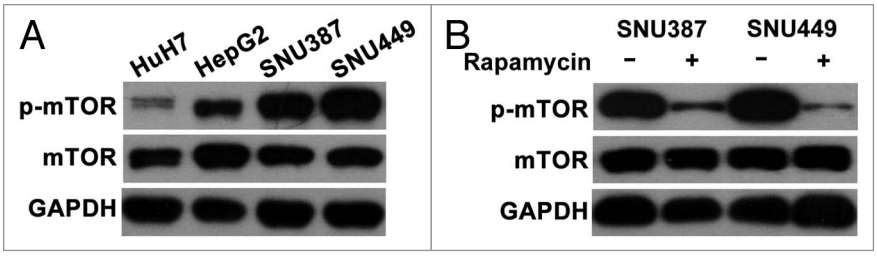

Figure 2. Basal mTOR activity in hepatoma cells. (**A**) Basal mTOR activity (p-mTOR expression) differs between epithelial and mesenchymal cells. (**B**) Rapamycin treatment attenuates p-mTOR expression in SNU-387 and SNU-449 cells.

sum-of-square *F* test). Interestingly, cotreatment with rapamycin reduced the differences in IC_{50} values among hepatoma cell lines (182 ± 29, 169 ± 45, 373 ± 53, and 359 ± 43 μg/mL in HuH7, HepG2, SNU-387, and SNU-449, respectively; **Fig. 1C**). However, the differences in IC_{50} values remained significant ($P < 0.05$, HuH7 or HepG2 vs. SNU-387 or SNU-449; extra sum-of-square *F* test). Furthermore, EdU assay showed that cetuximab sensitivity varied among different cell lines and that cotreatment with rapamycin significantly decreased the cell proliferation in both four cell lines (HuH7, HepG2, SNU-387, and SNU-449) (**Fig. S1A–D**).

Cetuximab sensitization by rapamycin is associated with cell phenotype

We next investigated why rapamycin should induce differential cetuximab sensitization in hepatoma cell lines. We considered the possibility that different cell phenotypes, specifically epithelial (HuH7and HepG2) and mesenchymal (SNU-387 and SNU-449), may cause the different responses of hepatoma cell lines to rapamycin cotreatment.

EMT progression in HCC cells is characterized by the concomitant loss of expression of epithelial cell junction proteins, such as E-cadherin, and gain of mesenchymal markers, such as vimentin.[16] Phenotype marker characterization by western blotting confirmed that the HCC cell lines exhibited different phenotypes (**Fig. 1D**). E-cadherin was primarily expressed in epithelial HepG2 and HuH7 cells but was absent in mesenchymal SNU-387 and SNU-449 cells. In contrast, vimentin expression was higher in mesenchymal cells than in epithelial cells.

Rapamycin treatment has no effect on the phenotype of hepatoma cells

As stated above, the ability of rapamycin to enhance cetuximab cytotoxicity is linked to cell phenotype. However, rapamycin treatment did not alter the phenotype of HCC cells. Cotreatment with rapamycin did not alter levels of E-cadherin or vimentin expression in either epithelial (HepG2 and HuH7) or mesenchymal (SNU-387 and SNU-449) cells (**Fig. 1E**). In addition, immunofluorescence staining showed no change in the localization of E-cadherin and vimentin after rapamycin treatment (**Fig. 1F**).

Rapamycin inhibits mTOR activation in mesenchymal cells

Given that rapamycin specifically antagonizes the mTOR pathway,[13,17] we next investigated the mTOR activation status in epithelial and mesenchymal HCC cells. Epithelial type HuH7 and HepG2 cells showed lower phospho-mTOR (p-mTOR) expression compared with mesenchymal type SNU-387 and SNU-449 cells, although all cells showed similar levels of total mTOR expression (**Fig. 2A**). Furthermore, rapamycin attenuated p-mTOR expression and therefore inhibited mTOR activation in mesenchymal SNU-387 and SNU-449 cells (**Fig. 2B**). Then, we measured the content of AKT and EGFR by western blot. However, results showed that the content of p-AKT and EGFR changed in a different pattern after rapamycin treatment (**Fig. S2**).

Rapamycin restores cetuximab sensitivity to TSC2-silenced cells

To investigate the role of mTOR activation in cetuximab resistance, we next treated TSC2-silenced hepatoma cells with rapamycin. TSC2 suppresses mTOR signaling.[18] We observed that siRNA-mediated TSC2 knockdown promoted mTOR activation, but had no effect on E-cadherin and vimentin expression (**Fig. 3B**). Therefore, TSC2 silencing does not appear to affect cell phenotype. Furthermore, immunofluorescence staining confirmed that no changes in membrane E-cadherin expression and cytoplasmic vimentin expression occurred following TSC2 knockdown (**Fig. 3C**). Interestingly, TSC2 siRNA treatment led to a significant decrease in cetuximab cytotoxicity (i.e., increased resistance) in both HepG2 and HuH7 cells ($P < 0.05$ vs. negative siRNA, for both cell lines; two-way ANOVA followed by Bonferroni post hoc tests; **Fig. 3A**). However, rapamycin treatment significantly attenuated cetuximab resistance in TSC2-silenced cells ($P < 0.001$ vs. TSC2 siRNA, for both cell lines; two-way ANOVA followed by Bonferroni post hoc tests; **Fig. 3A**). As previously observed, there were no changes in E-cadherin or vimentin expression (**Fig. 3B**) or localization (**Fig. 3C**) in both cell lines after rapamycin treatment. These data indicate that rapamycin treatment restores cetuximab sensitivity to TSC2-silenced epithelial type HCC cells.

Rapamycin restores cetuximab sensitivity after hypoxia-induced EMT

We next treated epithelial cells that had undergone EMT with rapamycin to further verify the role of mTOR activation in cetuximab resistance. Deferoxamine (DFO) is a chemical hypoxia-mimetic agent[19] and recent reports have demonstrated that hypoxia can induce EMT.[20-22] We therefore used DFO to induce hypoxia and EMT in HepG2 and HuH7 cells. We observed a significant decrease in cetuximab cytotoxicity in hypoxic HepG2 and HuH7 cells ($P < 0.01$ vs. normoxia, for both cell lines; two-way ANOVA followed by Bonferroni post hoc tests; **Fig. 4A**). Under hypoxic conditions, both cell lines showed increased p-mTOR expression with no change in total mTOR expression (**Fig. 4B**). Moreover, concurrent E-cadherin downregulation and vimentin upregulation confirmed that these cell lines had undergone EMT (**Fig. 4B**). Immunofluorescence staining confirmed a reduction in membrane-associated E-cadherin expression and enhanced cytoplasmic vimentin expression in hypoxic cells (**Fig. 4C**).

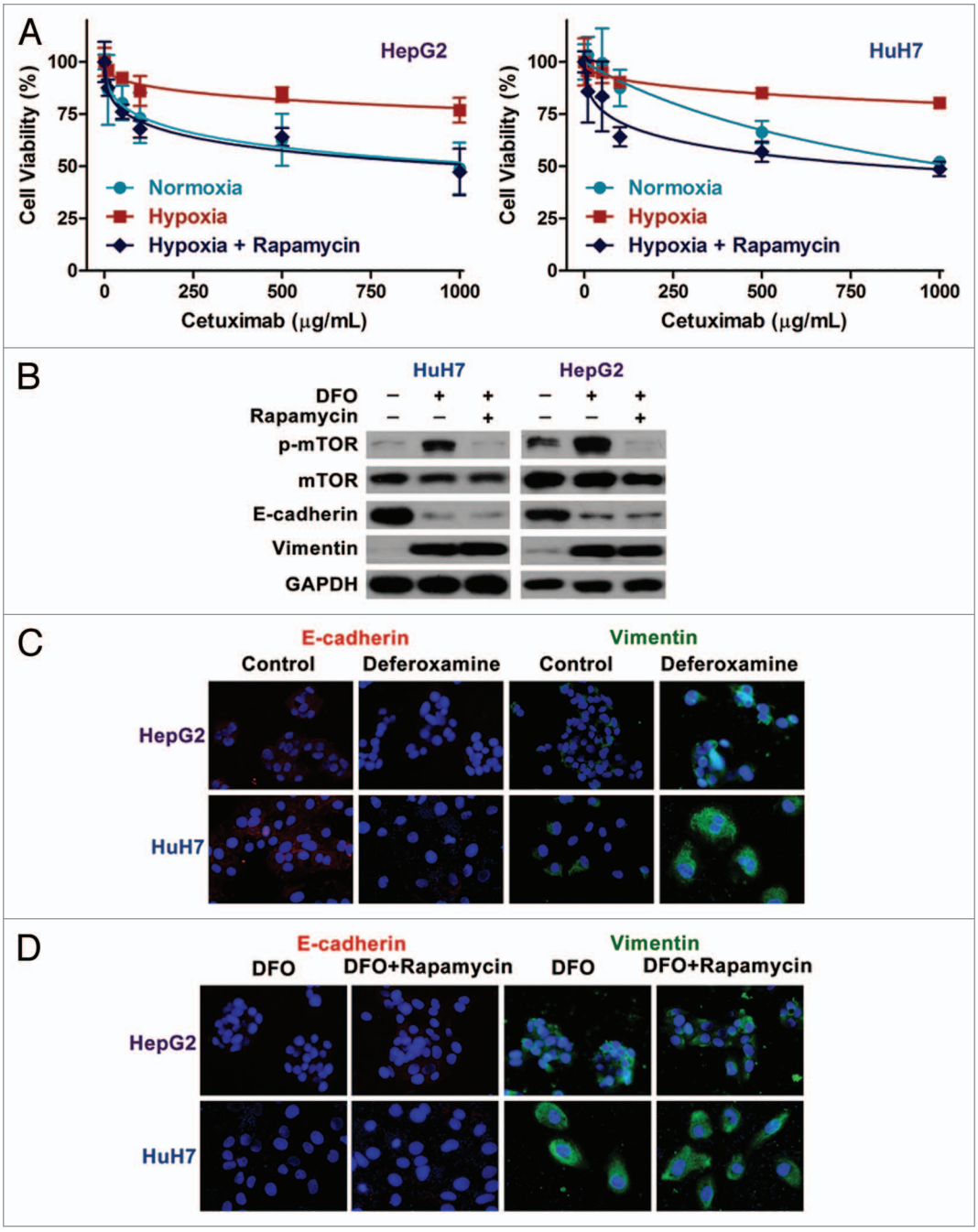

Figure 3. Rapamycin restores cetuximab sensitivity following TSC2 knockdown. (**A**) Cell viability assays show that TSC2 silencing increases resistance to cetuximab and that rapamycin cotreatment restores cetuximab sensitivity. (**B**) Levels of mTOR activity, E-cadherin and vimentin expression, and (**C**) E-cadherin and vimentin subcellular localization in TSC2-silenced HepG2 and HuH7 cells with or without rapamycin treatment.

Similar to our observations in TSC2-silenced cells, rapamycin treatment significantly attenuated cetuximab resistance in hypoxic HepG2 and HuH7 cells undergoing EMT ($P < 0.001$ vs. hypoxia, for both cell lines; two-way ANOVA followed by Bonferroni post hoc tests; **Fig. 4A**). Rapamycin treatment reduced p-mTOR expression in both cell lines (**Fig. 4B**) but had no effect on E-cadherin or vimentin expression, as shown by western blotting (**Fig. 4B**) and immunofluorescence staining (**Fig. 4C**). Therefore, we have shown that rapamycin inhibits EMT-mediated cetuximab resistance under hypoxic conditions.

Discussion

Drug regimens for liver cancer have been extensively explored, but therapeutic strategies remain unsatisfactory.[23,24] Synergistic

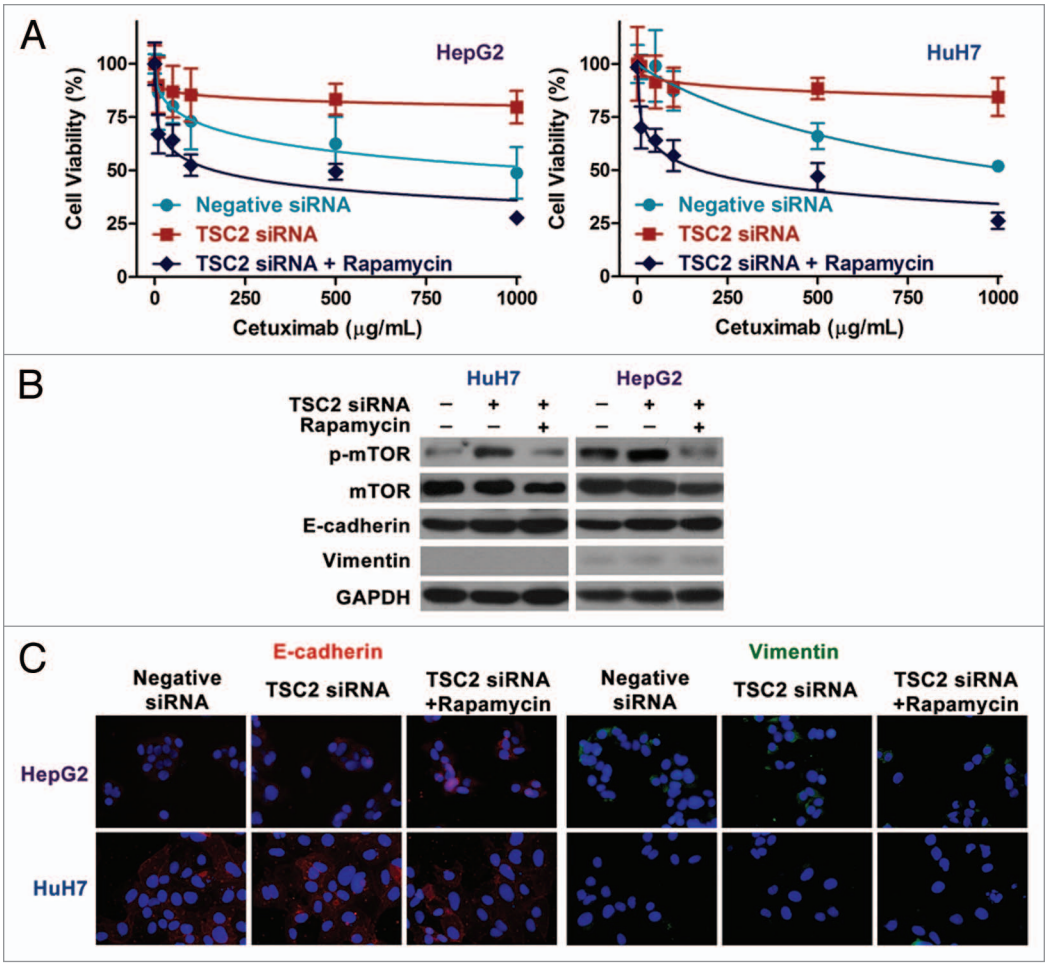

Figure 4. Rapamycin restores cetuximab sensitivity following hypoxia-induced EMT. (**A**) Cell viability assays show that hypoxia-induced EMT increases resistance to cetuximab and that cetuximab sensitivity is restored by rapamycin cotreatment. Levels of mTOR activity, E-cadherin, and vimentin expression, (**B**) and E-cadherin and vimentin subcellular localization (**C and D**) in HepG2 and HuH7 cells undergoing EMT with or without rapamycin treatment.

combination therapy, such as sorafenib plus doxorubicin[7] and capecitabine plus thalidomide,[8] provides a promising approach for the effective treatment of hepatic carcinomas. In this study, we showed that cotreatment with rapamycin significantly enhances cetuximab cytotoxicity. We also observed significant differences in cetuximab sensitization by rapamycin in cell exhibiting two different phenotypes, namely epithelial and mesenchymal. In addition, by comparing cetuximab cytotoxicity in four different HCC cell lines, we showed that epithelial type cells (Hepg2 and HuH7) were more sensitive than mesenchymal type cells (SNU-387 and SNU-449) to cetuximab, with the very large IC_{50} values in mesenchymal cells indicating cetuximab resistance. We investigated the possibility that the primary cause of such disparity is that rapamycin treatment either induces phenotypic transition or triggers a regulatory mechanism.

Regarding the first hypothesis, cells with a mesenchymal phenotype have been reported to show lower sensitivity to chemotherapeutic drugs in several cancers, including hepatic cancer,[16] pancreatic cancer,[25] and breast cancer.[26] The induction of epithelial traits in mesenchymal cells can attenuate differences in of drug sensitivity following cetuximab–rapamycin cotreatment. However, we observed no significant alteration in the phenotype of any cell line following cetuximab–rapamycin cotreatment, i.e., levels of epithelial or mesenchymal biomarkers were unchanged. Therefore, rapamycin enhances cetuximab sensitivity via a mechanism that does not involve phenotypic transition.

As for the second hypothesis, rapamycin is known to mainly inhibit the mTOR pathway[17]; therefore, we considered that differences in cetuximab sensitization may correlate with differences in basal mTOR pathway activity. In support of this hypothesis, we identified differences in mTOR activation status among hepatoma cell lines: epithelial cells exhibited lower p-mTOR expression compared with mesenchymal cells. Furthermore, rapamycin treatment restored cetuximab sensitivity to TSC2-silenced cells. We thus verified that rapamycin inhibition of mTOR regulates cetuximab sensitivity in both epithelial and mesenchymal cells.

Mesenchymal SNU-387 and SNU-449 cells showed increased cetuximab sensitivity and decreased p-mTOR expression after rapamycin treatment. These results indicate that rapamycin inhibition of mTOR signaling is linked to cetuximab sensitization in mesenchymal cells. In addition, the induction of EMT in epithelial HuH7 and HepG2 cells led to the expression of

mesenchymal traits, reduced sensitivity to cetuximab, and upregulated p-mTOR. Furthermore, rapamycin treatment of epithelial cells undergoing hypoxia-induced EMT restored cetuximab sensitivity accompanied by p-mTOR downregulation, but with no effect on cell phenotype. Therefore, our data shows that rapamycin inhibition of mTOR signaling leads to cetuximab sensitization.

Rapamycin has an overwhelming anticancer effect on various types of cancer by inhibiting the mTOR signaling pathway.[13] Rapamycin is also reported to enhance the therapeutic efficacy of carboplatin, camptothecin, cisplatin, doxorubicin, paclitaxel, and vinorelbine.[27-30] Recent reports further indicate that rapamycin shows synergy with cetuximab treatment.[31,32] However, our study provides the first evidence that differences cetuximab sensitivity can be overcome by rapamycin cotreatment. Therefore, rapamycin may have a major role in overcoming cetuximab resistance in mesenchymal cells.

In conclusion, our study shows a significant disparity in cetuximab sensitization by rapamycin between epithelial and mesenchymal cells. We further demonstrated that mTOR activation status is associated with cell phenotype and that mTOR inhibition by rapamycin results in cetuximab sensitization in mesenchymal cells.

Materials and Methods

Cell culture and reagents

The human hepatocellular carcinoma (HCC) cell lines, HepG2, HuH-7, SNU-387, and SNU-449, were obtained from the Shanghai Institute for Biological Science, China. HuH-7 and HepG2 cells were grown in Dulbecco's modified Eagle medium (high glucoseC11995500BT; Gibco) supplemented with 10% fetal bovine serum (FBS; 19003C, Gibco) and 1% penicillin/streptomycin (Sigma). SNU-387 and SNU-449 cells were grown in RPMI 1640 medium (Gibco C11875500BT) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and were used within 3 mo of recovery. Cells were treated with 100 μM deferoxamine (Sigma, D9533) for 4 h to induce hypoxia. Cells were transfected with TSC2 siRNA (Santa Cruz Biotechnology, SC36762) using Lipofectamine 2000 (Invitrogen, 522887) according to the manufacturer's instructions. Transfection medium was replaced with complete medium 6 h after transfection, and cells were incubated for the indicated times. All experiments were performed 24 h after transfection.

Cetuximab was purchased from Merck KgaA and rapamycin was purchased from LC Laboratories (sigma 37094). Deferoxamine was purchased from Sigma. Stock solutions were prepared in dimethyl sulfoxide (DMSO), stored at –20 °C, and diluted in fresh medium for each experiment. To prevent toxicity, the final concentration of DMSO did not exceed 0.5% in any experiment.

Cell viability assays

A cell counting kit-8 (CCK8, CK04; Dojindo) was used to measure relative HCC cell viability after treatment. Cells (8×10^3 cells per well) were seeded into 96-well microplates. After 20 h, the culture medium was replaced by medium containing 10% FBS and the drug concentration indicated. After a further 48 h, 10 μL of CCK-8 solution was added, cells were incubated for a further 4 h, and then absorbance at 450 nm was measured using an MRX II microplate reader (Dynex). Relative cell viability was calculated as a percentage of untreated controls. The half-maximal inhibitory concentration (IC_{50}) was determined by fitting data to the equation:

$$V\% = 100\% \Big/ \left[1 + \left(\frac{[Cetuximab]}{IC_{50}}\right)^{P}\right]$$

where V% is the percentage viability and [Cetuximab] is the concentration (μg/mL) of cetuximab.

EdU incorporation assay

Cells were exposed to EdU (5-ethynyl-2'-deoxyuridine) (Invitrogen) for 2 h at 37 °C. The cells were fixed with 4% formaldehyde for 15 min and treated with 0.5% Triton X-100 for 20 min at room temperature. After washing with phosphate buffered saline for three times, the cells of each well were reacted with 100 μL of 1× Apollo reaction cocktail for 30 min. Subsequently, the DNA contents of cells in each well were stained with 100 μL of Hoechst 33342 (5 μg/mL) for 30 min and visualized under a fluorescent microscope (Leica Microsystems).

Western blotting

Hepatoma cells were seeded into 6-well plates at a density of 2×10^5 cells per well and incubated for 20 h. Cells were then treated with drugs for 48 h, washed with ice-cold PBS and harvested in 100 μL cell lysis buffer (Cell Signaling, 9803) containing protease inhibitors (Cell Signaling, 5871) (Sigma). The protein concentration of lysates was determined using the bicinchoninic acid method (Thermo, 23225) (Pierce). Cell lysate samples (40 μg per lane) were separated using 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, HVPPEA12). Membranes were blocked with Tris-buffered saline/0.1% Tween 20 (TBS/T) containing 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with anti-E-cadherin, anti-vimentin, anti-mTOR (2983s), or anti-phospho-mTOR (Ser2448) (2971s) antibody (1:1000; Cell Signaling). Membranes were washed three times with TBS/T and incubated for 1 h at room temperature with the appropriate secondary antibody conjugated to goat anti-mouse horseradish peroxidase (Cell Signaling, 7074P2) (1:2000; GE Healthcare). Membranes were then washed and immunoreactive bands were developed using an enhanced chemiluminescence reagent (Biological Industries, 20500-120BI) (GE Healthcare) and visualized by autoradiography. Protein loading was normalized using an anti-GAPDH antibody (Proteintech Group 11224-1-AP) (1:5000, Kangchen Biotechnology). Gray-scale analysis of protein bands was performed using ImageJ software (National Institutes of Health).

Immunofluorescence

Hepatoma cells were seeded into 24-well plates and treated as described above. Cells were fixed with 4% formaldehyde for 15 min, washed with PBS, blocked with 5% BSA (Sigma, A2153) for 30 min at room temperature, and then probed with

mouse anti-human vimentin (Abcam, ab8978) or anti-human E-cadherin (Abcam, ab1416) primary antibody (1:100; Cell Signaling,) at 4 °C overnight. Cells were incubated with goat anti-mouse fluorescein isothiocyanate-labeled secondary antibody (1:200 in PBS; Abcam, SC-3764) for 2 h at 4 °C and then washed with PBS. Cells were then incubated for 10 min at room temperature with 4',6-diamidino-2-phenylindole (1:10000; Sigma, D9542) to stain nuclei, washed twice with PBS, and observed using an inverted fluorescence IX81 microscope (Olympus).

Statistical analysis

Statistical calculations were done using Prism 5 (GraphPad). Data are presented as the means and standard deviation (SD). The inhibitory effects of different treatments were compared using two-way ANOVA for repeated measurements (treatment vs. cetuximab concentration) followed by Bonferroni post hoc tests. The best-fit IC_{50} values were compared using an extra sum-of-square F test. For all tests, statistical significance was set at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cbt/article/29113/

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