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Pivotal Role of mTOR Signaling in Hepatocellular Carcinoma

Augusto Villanueva¹, Derek Y. Chiang^{2,3}, Pippa Newell¹, Judit Peix¹, Swan Thung¹, Clara Alsinet⁴, Victoria Tovar⁴, Sasan Roayaie¹, Beatriz Minguez¹, Manel Sole⁴, Carlo Battiston⁵, Stijn van Laarhoven¹, Maria I Fiel¹, Analisa Di Feo¹, Yujin Hoshida^{3,6}, Steven Yea¹, Sara Toffanin¹, Alex Ramos^{2,3}, John A. Martignetti¹, Vincenzo Mazzaferro⁵, Jordi Bruix⁴, Samuel Waxman¹, Myron Schwartz¹, Matthew Meyerson^{2,3}, Scott L. Friedman¹, and Josep M. Llovet^{1,4}

¹ Mount Sinai Liver Cancer Program. [Divisions of Liver Diseases and Hemato/Oncology; Department of Medicine; Human Genetics, Department of Genetics and Genomic Sciences; Surgical Oncology, Department of Surgery; Department of Pathology], Mount Sinai School of Medicine, New York, NY 10029, USA

² Department of Medical Oncology and Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Boston, MA 02115, USA

³ Cancer Program, The Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

⁴ BCLC Group [Liver Unit, HCC Translational Research Lab and Department of Pathology], IDIBAPS, CIBERehd, Hospital Clínic, Barcelona 08036, Spain

⁵ Gastrointestinal Surgery and Liver Transplantation Unit, National Cancer Institute, Milan 20133, Italy

⁶ Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

Abstract

BACKGROUND—The advent of targeted therapies in hepatocellular carcinoma (HCC) has underscored the importance of pathway characterization to identify novel molecular targets for treatment. Based on its role in cell growth and differentiation, we evaluated mTOR signaling activation in human HCC, as well as the anti-tumoral effect of a dual-level blockade of the mTOR pathway.

METHODS—The mTOR pathway was assessed using integrated data from mutation analysis (direct sequencing), DNA copy number changes (SNP-array), mRNA levels (qRT-PCR and gene expression microarray), and protein activation (immunostaining) in 351 human samples, including HCC (n=314), and non-tumoral tissue (n=37). Effects of dual blockade of mTOR signaling using a rapamycin analog (everolimus) and an EGFR/VEGFR inhibitor (AEE788) were evaluated in liver cancer cell lines, and in a tumor xenograft model.

RESULTS—Aberrant mTOR signaling (phosphorylated-RPS6) was present in half of the cases, associated with IGF pathway activation, EGF upregulation, and PTEN dysregulation. PTEN and PI3KCA-B mutations were rare events. Chromosomal gains in RICTOR (25% of patients) and

Address for correspondence: Josep M Llovet, MD, Division of Liver Diseases, Box 1123, Mount Sinai School of Medicine, Madison Ave 1425, 11F-70, New York, NY 10029, USA, Phone: 212-6599503, FAX: 212-849-2574, E-mail: Josep.Llovet@mssm.edu.

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positive pRPS6 staining correlated with recurrence. RICTOR-specific siRNA downregulation reduced tumor cell viability *in vitro*. Blockage of mTOR signaling with everolimus *in vitro* and in a xenograft model decelerated tumor growth and increased survival. This effect was enhanced *in vivo* after EGFR blockade.

CONCLUSIONS—mTOR signaling has a critical role in the pathogenesis of HCC, with evidence for the role of RICTOR in tumor oncogenesis. mTOR blockade with everolimus is effective *in vivo*. These findings establish a rationale for targeting mTOR pathway in clinical trials in HCC.

BACKGROUND

Hepatocellular carcinoma (HCC) is a major international health problem. It has become the third cause of cancer-related death worldwide and the most common cause of death among cirrhotic patients in Western countries¹, a trend paralleled by an increase in advanced hepatitis C virus (HCV)-related liver disease². Currently only 30% of HCC patients are eligible for potentially curative treatments such as liver resection, transplantation, or local ablation, according to an established therapeutic algorithm³. Systemic chemotherapy is not efficacious in HCC⁴. Recently, regulatory agencies have approved the Raf/VEGFR/PDGFR inhibitor sorafenib, which significantly increased overall survival in patients with advanced HCC in a phase III trial⁵. This positive effect has spurred intensive research to unravel the genomic aberrations and signaling pathways implicated in hepatocarcinogenesis, and to find new therapeutic targets to tackle this devastating disease⁶.

The serine/threonine kinase AKT was isolated as an oncogene transduced by the acute transforming retrovirus⁷. Its essential role in human cancer was established by demonstration of its frequent amplification and overexpression in various cancers. AKT acts as a cytoplasmic regulator of numerous signals related to cell cycling (Cyclin D1), cell survival (Mdm2/p53), cardiovascular homeostasis (eNOS), and cell growth (mTOR), among others⁸. The tumor suppressor PTEN is a negative regulator of the pathway, and its loss activates AKT.

mTOR complex 1 (mTORC1) is a downstream target of AKT comprised of mTOR, a regulatory associated protein of mTOR (RAPTOR) and mammalian LST8/G-protein β -subunit like protein (mLST8/G β L). mTORC1 acts as a central regulator of cell growth and proliferation by activating S6 kinase, which in turn regulates protein synthesis and allows progression from G1 to S phase of the cell cycle⁹. In contrast, mTOR complex 2 (mTORC2), formed by mTOR, RICTOR and PRR5/G β L, is primarily responsible for the activation of AKT through its phosphorylation of serine 473¹⁰. In previous studies, activation of mTOR pathway in HCC ranged from 15 to 41%, and mTOR inhibitors show antineoplastic activity in experimental models of HCC^{11–14}. However, these studies relied exclusively on immunohistochemistry data^{11, 13}, associations with clinical outcomes were analyzed only in transplanted patients¹³, and there was no evaluation of multi-level blockade of mTOR pathway in HCC.

We report herein a significant role of mTOR pathway activation in human HCC, by using an integrative genomic approach and a training-validation scheme in a large cohort of patients. Aberrant pathway signaling was associated with activation of either the IGF and/or EGF cascade, rather than with somatic mutations of their components. mTOR blockade with a rapamycin analog (RAD001, everolimus; Novartis, Basel, Switzerland), decreased tumor growth and expanded survival in experimental HCC xenograft models. This effect was enhanced *in vivo* when administered along with an EGFR/VEGFR kinase inhibitor. Overall, the data supports efforts to target mTOR signaling in liver cancer patients.

METHODS

Human tissue samples

A total of 351 human liver samples obtained either from liver resection or transplantation were analyzed in different sets (Figure 1). All of the samples were obtained from the HCC Genomic Consortium: Mount Sinai School of Medicine, NY (US), Hospital Clinic, Barcelona (Spain) and Istituto Nazionale dei Tumori, Milan (Italy). Upon Institutional Review Board approval and after patient written informed consent was obtained, tissue specimens were collected. First, we used an exploratory set of 77 fresh frozen samples including normal liver (n=10), cirrhosis (n=10), dysplasia (n=17), and different HCC stages (n=40, as previously described^{15, 16}) to assess the mRNA levels of key genes of the mTOR pathway encompassing the entire pathogenic spectrum in HCV-related HCC. A replication set of 78 HCV-related HCC samples (fresh frozen and paraffin-embedded tissue) was used to confirm results in the exploratory set. For the clinical correlations, we selected patients from both sets who underwent liver resection (n=82, mTOR pathway clinical training set); all of them with HCV-related HCC. Finally, to validate the correlations between mTOR pathway and clinical outcome, we constructed a tissue microarray (TMA) with 196 HCC samples containing samples from all etiologies, including HBV and HCV-related liver disease (mTOR pathway clinical validation set, Table 1).

Quantitative real time PCR (qRT-PCR), SNP-array, gene expression microarray and sequencing

RNA extraction, purification, cDNA synthesis and PCR conditions can be found elsewhere¹⁵. Expression of mRNA was measured with Taqman Probes® (Supplementary Table 1) obtained from Taqman Gene Expression Assays® (Applied Biosystems). Ribosomal RNA (18S) was chosen for normalization. Gene expression microarray studies were conducted following procedures provided by the Affymetrix GeneChip Technical Manual.

DNA was extracted from human samples with the ChargeSwitch gDNA Mini Tissue Kit (Invitrogen) and quantified using PicoGreen (Invitrogen). A detailed explanation of the SNP-array procedures can be found elsewhere¹⁷. PCR and sequencing were conducted by GENEWIZ (<http://www.genewiz.com>). PCR primers for PTEN (exons 5 and 7), PI3KCA (exons 9 and 20), and PI3KB (exons 9 and 20) are listed in Supplementary Table 2 (see Supplementary Information for details).

Cell lines and drugs

Three well-characterized human liver cancer cell lines: Huh-7 (p53 mutated, PTEN wt), HepG2 (PTEN wt, N-RAS and β -Catenin mutated) and Hep3B were maintained as adherent monolayers in DMEM supplemented with 10% FBS, 5% L-glutamine and 1% penicillin-streptomycin. Cells were incubated at 37°C in 5% CO₂. AEE788 is an ATP-competitive dual EGFR/VEGFR tyrosine kinase inhibitor, and RAD001 (everolimus) is a soluble derivative of rapamycin that inhibits mTOR activity. Both drugs were provided by Novartis Pharma AG (Basel, Switzerland).

Xenograft model

Athymic female NU/NU mice (6–8 weeks of age) were subcutaneously injected in the right flank with 5×10^6 Huh7 cells. When tumors reached a volume between 100–250 mm³, mice were randomized in 4 groups: placebo (n=9; drug vehicle), AEE788 (n=9; 25 mg/kg), RAD001 (n=7; 5 mg/kg) and a combination of AEE788 (n=9; 25 mg/kg) plus RAD001 (5 mg/kg). Both drugs were administered 3 times/week per gavage until the animal was euthanized. Response was assessed by measuring delay in tumor growth and overall survival. Apoptosis and proliferation in tumors were evaluated using the DeadEndTM Colorimetric TUNEL System

(Promega) and Ki-67 immunostaining with mouse monoclonal anti-human Ki-67 antigen (Clone MIB-1, DakoCytomation), respectively. To assess the blocking activity of AEE788 and RAD001 in vivo, we used immunostaining for p-EGFR (Tyr1068, Cell Signaling) and p-RPS6 (Ser240/244, Cell Signaling), respectively (see Supplementary Information for details).

Immunoblotting, immunohistochemistry and tissue microarray

For inhibitory studies, cells were pretreated for 1 hour with AEE788 (10 μ M), RAD001 (20 nM) or a combination of both. Then, cells were incubated with rh-EGF (40 ng/mL, Invitrogen). Blotting of membranes was performed using the following primary antibodies (all from Cell Signaling Technology, Danvers, MA): EGFR, phospho-EGFR (Tyr1173), Akt, phospho-Akt (Ser473), mTOR, phospho-mTOR (Ser2448), ERK1–2, phospho-ERK1–2 (Thr202/Tyr204), RPS6, phospho-RPS6 (Ser240/244), RICTOR and β -Tubulin (Santa Cruz). Secondary antibodies used were anti-mouse and anti-rabbit (GE Healthcare).

Formalin-fixed, paraffin-embedded sections were used to assess phosphorylated proteins in human tissue. Samples were incubated with anti-pEGFR (Tyr1068, Cell Signaling), anti-pIGF-IR (Tyr1316, provided by Dr. Rubini, University of Ferrara), anti-pAkt (Ser473, IHC specific, Cell Signalling), anti-pmTOR (Ser2448, IHC specific, Cell Signalling), and anti-pRPS6 (Ser240/244, Cell Signaling). Immunoreactivity was independently graded by three liver pathologists (ST, IF and MS) and finally determined based on their agreement. The variables measured were as follows: (1) Intensity of staining (0=absent, 1=weak, 2=moderate, 3=strong); (2) distribution of staining (1=very focal, 2=focal, 3=diffuse) and (3) localization of the staining (membranous, cytoplasmic, or nuclear). Samples were defined as positive for p-EGFR, p-IGF-IR, p-Akt and p-mTOR when intensity of staining was +2 or higher, regardless of distribution. For p-RPS6, samples were defined positive when intensity and distribution were +2 or higher. TMA blocks were constructed using the Advanced Tissue Arrayer ATA-100 (Chemicon International, see Supplementary Information for details).

Cell viability, proliferation and apoptosis assessment

Cells were plated into 12-well (10,000 cells/well) or 24-well (5,000 cells/well) plate for 3 H-Thymidine Incorporation and MTT Assays, respectively. Cells were incubated with increasing concentrations of AEE788 [0.5–10 μ M], RAD001 [1–20 nM], and RNAi (RICTOR and control, Stealth Technology®, Invitrogen). For cell viability studies, after 24, 48 and 72 hours, cells were incubated with tetrazolium reagent for 1 hour. Culture medium supernatant was then removed and N-propyl alcohol was added. Following thorough solubilization, the absorbance (OD) of each well was measured using a microculture plate reader at 570 nm. For proliferation studies, cells were incubated with the drugs for 24 hours and were then labeled for 3 hours with 1 μ Ci/mL 3 H-Thymidine, fixed in 1N hydrochloric acid and lysed in 0.25N NaOH. Thymidine incorporation was measured in a scintillation counter. The same experiments were also conducted under serum-free conditions (24 hours of starvation).

Apoptosis was measured by propidium iodide (PI) staining and fluorescent-activated cell sorting (FACS) in order to analyze the percentage of cells with subdiploid DNA content (sub-G1) characteristic of fragmentation. Huh-7 cells were plated into each well of a 6-well plate (50,000 cells/well). Cells were treated with AEE788 [10 μ M], RAD001 [20 nM] and a combination of both. Forty-eight hours later, cells received a second dose of drug and were lysed 48 later. Harvested Huh-7 cells were pelleted by centrifugation and fixed with 100% ethanol. Samples were then stored at -20°C for 16 hours until FACS analysis was performed. To measure DNA content, cells were pelleted and resuspended in PBS containing 50 μ g/mL propidium iodide and 100 μ g/mL RNase. Data was acquired on FACSCalibur flow cytometer (Becton Dickinson Instrument, San Jose, CA) and analyzed using CellQuest 3.1 software

(Becton Dickinson). Apoptosis was further confirmed by measuring PARP cleavage using immunoblotting with PARP antibody (Cell Signaling).

Transient transfection and reporter assay

Huh7 transfection efficiency was evaluated using the Block-it™ Fluorescent Oligo (Invitrogen). We performed a dual luciferase reporter assay using a human c-fos promoter subcloned into the pGL3 luciferase vector (Promega, Fitchburg, WI) upstream of the luciferase gene (a gift from Dr. Ron Prywes, Columbia University, NY)¹⁸. Briefly, Huh-7 cells were transfected with 1 µg of the c-fos reporter and 5 ng of pRL-TK plasmid driving the Renilla luciferase gene using 2.5 µL of Lipofectamine 2000 (Invitrogen, Carlsbad, California). Cells were incubated with EGF ([40 ng/mL], Invitrogen) for 30 minutes, lysed, and analyzed for firefly and renilla luciferase activity using the Dual Luciferase Kit® (Promega). Luciferase activity was measured with a luminometer and was normalized against the activity of the *Renilla* luciferase gene. RNAi experiments targeting RICTOR were conducted using Stealth® Technology (Invitrogen) following the manufacture's instructions. Downregulation efficiency was evaluated at the protein level using western blot for RICTOR (Cell Signaling).

Statistical analysis

All the qRT-PCR calculations were analyzed by using the ddCt Method following the same methodology as previously described¹⁵. Comparisons between groups were made using either the t-test or the non-parametric Mann-Whitney test for continuous variables, and the Fisher exact test for comparison of proportions. Correlations were calculated either with the Pearson's coefficient or the non-parametric Spearman's coefficient.

SNP arrays were processed using the GenePattern software package¹⁹ with modules based on dChipSNP algorithms. Gene set enrichment analysis (GSEA) was used to assess which gene sets were significantly associated with differential gene expression among tumors with positive staining for pRPS6 (Ser240/244) and gains in RICTOR²⁰. RPS6 and RICTOR differential expressed genes were obtained using the Significance Analysis of Microarrays Package²¹.

For clinical correlations, the probability curves of recurrence and early recurrence were calculated according to Kaplan-Meier and compared by Mantel-Cox test. Protein staining status and median inferred copy numbers for the genes analyzed for each sample were used as covariates for univariate association with recurrence (deaths were censored in this analysis²²). Clinico-pathological variables assessed in the univariate analysis were tumor size, multinodularity/satellites, vascular invasion, differentiation degree, BCLC stage and AFP levels. Molecular variables analyzed were: staining status of p-RPS6, p-Akt, p-IGF-IR, p-EGFR, p-mTOR, gains in RICTOR, mRNA levels of EGF and IGF2. Significant variables ($p < 0.05$) were included in a step-wise Cox regression analysis of recurrence. Early recurrence was defined as within two years of surgical resection²³. All calculations were done by the SPSS package (SPSS 15.0, Chicago, Illinois, see Supplementary Information for details).

RESULTS

Aberrant activation of the mTOR pathway in human HCC

mTOR pathway gene expression alterations, DNA copy number changes and mutation analysis of HCV-related HCC—We conducted an expression study using qRT-PCR in two different human cohorts, exploratory (n=77) and replication (n=78) sets (Supplementary Fig. 1 and Fig. 2). Dysregulation of key growth regulatory genes including *EGF*, *IGFBP3* and *PTEN* was evident in overt HCC. *EGF* was up-regulated, particularly in advanced HCC cases ($p = 0.001$), and the tumor suppressor *IGFBP3* was down-regulated in early and advanced HCC ($p < 0.001$). Also, a subgroup of 9 HCC patients had very high (>500

fold) upregulation of *IGF2*, what justifies the asymmetric distribution of this variable. In both sets, *PTEN* was down-regulated in advanced HCC ($p=0.01$). *RAPTOR* and *mTOR* were coordinately up-regulated in advanced tumors (1.7 and 2.3 mean fold-increase, respectively, $p<0.01$; Pearson's coefficient=0.57, $p<0.001$). These data was consistent with whole-genome microarray transcriptomic analysis that was conducted in parallel (data not shown).

We used SNP-array technologies to assess copy number alterations in nine genes of the mTOR pathway (*PI3KCA*, *AKT1*, *PTEN*, *mTOR*, *S6K*, *IGF2*, *IGFBP3*, *RAPTOR* and *RICTOR*) in 99 HCC fresh-frozen samples and their cirrhotic counterparts. Overall, there were no high-level amplifications (cut-off above 3.4 copies) or deletions (data not shown), and only *RICTOR* showed significant DNA gains. Sequencing analysis showed a very low mutation rate of *PTEN* (1%, 1/102), *PI3KB* (1%, 1/102) and *PI3KCA* (0%).

Activation of mTOR (pRPS6) and correlations with EGF and IGF signaling—To assess the activation status of mTOR pathway, we studied different members of the mTOR cascade at the protein level. Rates of tumoral staining for p-Akt, IGF-IR and p-RPS6 were 31.2% (29/92), 20.3% (16/79) and 47.7% (41/86), respectively; all were significantly higher than surrounding cirrhotic tissue ($p<0.01$, Supplementary Fig. 2). Activation of EGF signaling was present in 48.5% of cases (ns). In contrast to the null positive staining in cirrhotic tissue, 19.2% (15/78, $p<0.01$) of the tumor samples also displayed prominent staining for p-RPS6 in endothelial cells.

Activation of pRPS6 was significantly associated with EGF signaling: p-EGFR (63.1%, 24/38 vs 35.1% 13/37; $p=0.02$) and high EGF mRNA levels (>5 fold; $p=0.01$). Similarly, pRPS6 activation was also consistently associated with positive p-IGF-IR (68.7% 11/16 vs 39.6% 25/63; $p=0.03$, Table 2). All the above suggests a more prominent ligand-dependant mechanism of activation, rather than a mutation-based phenomenon. It has to be emphasized that mTOR signaling activation was identified in different HCC molecular subclasses recently reported based on unsupervised clustering of gene expression microarray data¹⁷. However, there was a significant enrichment of mTOR activation in the *proliferation* subclass, characterized by AKT-mTOR and IGF signaling activation¹⁷.

Outcome implications of mTOR signaling activation—Activation of pRPS6 was associated with moderate/poorly differentiated tumors ($p=0.04$) BCLC B/C ($p=0.003$), and higher levels of AFP ($p=0.012$), whereas gains in *RICTOR* ($p=0.03$) and p-Akt positive staining were more prevalent in larger tumors ($p=0.007$). Also, gains in *RICTOR* were significantly associated with p-mTOR staining ($p=0.01$). There was a clear shift in p-mTOR localization in cirrhotic tissue and HCC. Staining in cirrhosis was predominantly membranous, while it was typically located in the cytoplasm in HCCs ($p<0.01$).

For outcome prediction we used two independent cohorts of HCC patients treated by surgical resection (Table 1), one including 82 HCV-derived HCCs, and a validation set of 196 HCC patients from all etiologies, where 67.3% of tumors showed positive pRPS6 staining (132/196). Overall, most of the patients had well-preserved liver function (98% Child-Pugh class A), early HCC (BCLC class 0-A ~90%) and small size tumors (median size 3.5 cm). Clinical variables such as tumor size, BCLC class, macrovascular invasion, and multinodularity/satellites were significantly associated with recurrence ($p<0.05$). In the independent set of 196 samples, p-RPS6 was an independent predictor of recurrence (HR=1.8, IC 95%: 1.1–2.8, $p=0.01$) along with BCLC staging (HR=1.9, IC 95%: 1.2–3.1, $p=0.004$) and the presence of tumor multinodularity/satellites (HR=2.2, IC 95%: 1.4–3.5, $p=0.001$). The median time to recurrence in p-RPS6 positive and negative patients were of 25 and 50 months, respectively ($p=0.004$, Fig. 3). These results suggest a potential prognostic relevance of mTOR activation in HCC patients.

To compile a specific gene signature associated with mTORC1 pathway activation, we profiled 91 HCC samples using the human U133 plus 2.0 array (Affymetrix). After supervised analysis using the Significance Analysis of Microarrays Package, we found 193 up-regulated and 127 down-regulated genes distinguishing patients according to p-RPS6 staining status (FDR<0.5%). Among them, up-regulation of genes related to NF-Kappa β (*ZDHHC13*), MAPK (MAPK13) pathways, AMPK subunits, and angiogenesis (VEGFB) were most prominent (Supplementary Table 3). As expected, GSEA showed that a gene set formed by 121 genes involved in capping, splicing, editing and modification of mRNA (i.e. mRNA Processing Reactome) was enriched in phospho-RPS6 positive samples (q-value=0.06).

Dysregulation of mTOR Complex 2 (RICTOR) in human HCC—SNP-array analysis showed increased copy numbers in RICTOR in one fourth of cases (25/100), which were significantly associated with mRNA up-regulation ($p=0.03$, Fig. 4A–B). Gains in RICTOR ($p=0.002$, Fig 4C–D) and combined gains in RICTOR and activated RPS6 (9.8%, 8/81) were significantly associated with recurrence ($p<0.001$) in the training set. Also, gains in RICTOR were an independent predictor of recurrence (HR=2.3, IC 95%: 1.2–4.4, $p=0.01$) along with BCLC staging (HR=4.8, IC 95%: 2.7–8.7, $p<0.001$). Supervised analysis of gene expression data show that EGR2, a candidate tumor suppressor gene that interacts with PTEN²⁴, was significantly downregulated in samples with gains in RICTOR. Also, several subunits of AMPK were significantly enriched in these samples. In vitro, RNAi mediated downregulation of RICTOR in Huh7 cells, which harbor gains in RICTOR, induced a 17% reduction in cell viability measured with the MTT assay, when compared with cells transfected with control siRNA (Fig 4E–F, $p<0.05$). Conversely, cell viability in HepG2, a cell line without gains in RICTOR, remained unchanged.

Blockade of mTOR pathway with everolimus and EGFR inhibitors has anti-tumoral effects in experimental models of HCC

The mTOR inhibitor everolimus inhibits growth in HCC cell lines—Everolimus (RAD001) [20 nM] decreased cell viability in Huh7, Hep G2 and Hep 3B at 72-hours up to 36% ($p<0.001$, Fig. 5). Increasing concentrations of an EGFR inhibitor (AEE788) induced a time- and dose-dependent reduction in cell viability of the 3 cell lines. After 72 hours, high concentrations of EGFR inhibitor [10 μ M] reduced cell viability up to 85% ($p<0.006$). Combination therapy did not enhance the effect on cell viability compared with single EGFR inhibitor (Fig. 5C). Everolimus significantly decreased proliferation up to 20% in Huh-7 (Fig. 5D), while the inhibition by the EGFR inhibitor [10 μ M] was more than 90% in the 3 cell lines ($p<0.01$).

We further examined the mechanism of action of the kinase inhibitors in vitro by FACS analysis. The mTOR inhibitor did not induce apoptosis, whereas the EGFR inhibitor [10 μ M] alone and in combination with everolimus significantly increased the percentage of cells in sub-G1 phase up to 38% and 40%, respectively (Fig. 5E). Apoptosis was confirmed by measuring PARP cleavage (Fig. 5F).

Blocking signals through mTOR and EGF pathways in vitro—To elucidate the efficacy of the kinase inhibitors in blocking downstream targets, we measured the effect of both drugs in the phosphorylation status of different proteins of the Akt/mTOR pathway (EGFR, Akt, mTOR, RPS6) as well as ERK1/2 (Fig. 6A). As predicted, EGFR-inhibitor decreased the phosphorylation of EGFR, Akt and ERK1/2 in Huh7 while everolimus significantly reduced the phosphorylation of RPS6. Combination therapy simultaneously blocked both signals. Similar results were obtained in HepG2 and Hep3B lines (data not shown).

We employed a c-fos luciferase reporter as a surrogate of EGF signaling activation, and found a significant decrease in luciferase activity up to 65% in Huh-7 cells treated with EGFR inhibitor [10 μ M] alone and in combination with everolimus after 30 minutes of stimulation with rh-EGF. In accordance with the protein studies, everolimus [20 nM] did not modify the signal from the c-fos reporter (Fig. 6B).

Antitumoral effect of mTOR inhibitor in vivo, and synergistic effect in combination therapy with EGFR inhibitor—Oral administration of an mTOR inhibitor (everolimus; 5 mg/kg), EGFR-inhibitor (AEE788; 25 mg/kg), or placebo (drug vehicle) were well tolerated by tumor-bearing mice without significant weight loss. Everolimus and the EGFR inhibitor induced a significant delay in tumor growth in comparison with control mice (mean volume after 15 days of treatment: 1039 and 1395 mm³, respectively vs 2396 mm³, controls; $p < 0.05$). Combination therapy further reduced the tumor volume to 784 mm³ ($p < 0.05$, Fig. 6C). Complete tumor response was evident in 2 animals in the EGFR inhibitor and combination arms. Overall survival was enhanced in combination therapy (median 27 days), in comparison with each drug alone (median everolimus: 19 days, EGFR-inhibitor: 21 days), or with control mice (median 16 days), ($p < 0.05$, Fig. 6D).

We then examined the impact of blocking mTOR and EGFR signaling on apoptosis and proliferation using TUNEL and Ki-67 staining *in vivo*, respectively (Supplementary Fig. 3). There was a significant reduction in the proliferation index from 80.8 $\pm$ 9.5% in control mice to 52.6 $\pm$ 25% in the everolimus arm ($p < 0.05$) and to 57 $\pm$ 21% in the combination arm ($p < 0.05$). Regarding apoptosis, there was a significant increase in the apoptotic index from 8 $\pm$ 6 apoptotic bodies per 10 high power fields in control mice to 18 $\pm$ 6 ($p < 0.05$) and 16 $\pm$ 7 ($p < 0.05$) in EGFR-inhibitor alone and in combination, respectively.

To evaluate selective inhibition of downstream targets, we assessed the expression p-EGFR and p-RPS6 in tumor sections using immunohistochemistry. Immunohistochemical reactivity of p-EGFR was decreased in mice treated with EGFR inhibitors or combination therapy, whereas p-RPS6 staining was diminished in mice treated with everolimus and those treated with the combination therapy (Supplementary Fig. 3).

DISCUSSION

Recent studies have identified PI3K/Akt/mTOR pathway as a major oncogenic cascade for targeting molecular therapies in cancer²⁵. mTOR signaling has been implicated in the initiation and progression of multiple tumors, such as leiomyosarcomas and gliomas²⁶. We demonstrate herein that mTOR pathway is activated in a subset of patients with early HCC. Activation of mTOR cascade resulted from ligand-dependant signals from EGF and IGF signaling, rather than from a mutation-dependent mechanism, since no high-level amplifications and only marginal mutation rates in the most prevalent hot spots in PTEN, PI3KCA and PI3KB were identified. In fact, in a subset of patients, high levels of EGF could be primarily responsible for RPS6 activation. Coincidentally, down-regulation of the tumor suppressor PTEN was observed in a significant proportion of patients, mostly in advanced stages of the disease. All these results highlight the relevance of mTOR signaling in HCC, a pathway that has been insufficiently explored in human liver cancer.

These data are the first to characterize the status of *RICTOR* status in human HCC. *RICTOR* is part of MTORC2, however its functions and molecular structure are not entirely known²⁷. We found a significant association between gains in *RICTOR* (25% of HCC) and its transcript expression. Intriguingly, gains in *RICTOR* were significantly associated with p-mTOR, which could be a relevant mechanism of MTORC2 activation in human cancer. Genes significantly dysregulated in samples with gains in *RICTOR* (e.g. EGR2 and AMPK subunits) suggests that

mTORC2 activity could influence signaling through the PTEN-mTORC1 axis. In vitro, downregulation of RICTOR by siRNA reduced cell viability of Huh7 cells, which harbor gains in RICTOR.

We speculate that in HCC, other mechanisms besides mTORC2 activation may be responsible for Akt phosphorylation at residue 473, since no correlation between p-Akt staining and gains in *RICTOR* was observed. Membranous localization of p-mTOR was visibly lost in liver cancer; whether this event is due to divergent mTOR complex 1 and 2 cellular localization requires further investigation. Finally, a significant association between gains in *RICTOR* and early recurrence was identified in the training set, a finding that requires further validation. Strikingly, and in accordance with this data, a recent report indicates that mTORC2 is hyperactivated in gliomas and functions in promoting tumor cell proliferation and invasive potential²⁸. Overall, these results suggest a possible role of mTORC2 in human hepatocarcinogenesis and warrant further evaluation of *RICTOR* as a potential new target in HCC therapy.

Apart from the potential role of *RICTOR* as an oncogene in liver carcinogenesis, we describe other mechanisms underlying mTOR pathway activation. We have confirmed dysregulation of key genes of the mTOR pathway (e.g. *IGF2*, *IGFBP3* and *PTEN*) in HCC^{29, 30}, and herein reported dysregulation of other genes that includes mTOR and RAPTOR. Alterations in copy number or somatic mutations of PTEN, PI3KCA and PI3KB were not identified as major mechanisms of mTOR pathway dysregulation, although these genes were only assessed for mutations in certain exons. The infrequent mutation rate of PTEN (<10%) in earlier studies³¹, supports our data, and suggests that other mechanisms apart from missense mutations may be responsible for PTEN downregulation (e.g. promoter methylation³²). There is some controversy regarding PI3KCA mutations, with frequency rates that range from 0%³³ to 35%³⁴ in HCC. We did not identify any mutation in 2 exons of the PI3KCA gene.

The associations found at the protein level between p-EGFR/EGF and p-RPS6 suggest that RPS6 activation in HCC is partly driven through EGF signaling. Interestingly, our cohort showed higher rates of p-RPS6 activation than p-Akt, and similar to previous reports¹³, there was not a significant correlation between both proteins, which reinforces the concept that alternative pathways besides Akt are responsible for the activation of mTORC1 (e.g. LKB1/AMPK signaling³⁵ or direct signals from IGF-IR activation). Interestingly, marker genes significantly up-regulated in RPS6 activated HCC were related to NF- κ B signaling (*ZDHH13*), angiogenesis (*VEGFB*) and MAPK signaling (*MAPK13*), highlighting the complex signaling cross-talk that is developing even at early clinical stages.

The primacy of mTOR activation in early HCC persisted in both cohorts analyzed, but was not associated with specific etiological factors. We found similar rates of p-RPS6 staining as previously described, but p-RPS6 in our cohort was a predictor of poor prognosis¹³. Recent studies conducted by our group have identified, by unsupervised clustering of microarray data, a singular subgroup of HCC patients (*proliferation group*) where activation of IGF and mTOR (p-RPS6) were enriched¹⁷. This group has been previously associated with poorest outcome³⁶. In our series, patients with mTOR activation showed higher levels of AFP, less differentiated tumors, and a higher incidence of recurrence. These data highlight the relevance of this pathway as an attractive target for the development of new anti-tumoral agents.

To address the issue of multi-targeted blockade of mTOR signaling in HCC and neutralization of potential oncogenic loops, we evaluated the effect of a dual-level blockade of mTOR by an mTOR inhibitor (everolimus, RAD001) and an EGFR/VEGR inhibitor (AEE788) in experimental models. The EGFR inhibitor induced high rates of apoptosis in vitro, a phenomenon not observed with the rapamycin analog. Although everolimus has already proved

antitumoral effects in a rat model of HCC¹⁴, our *in vivo* studies in mouse xenografts revealed an additive anti-tumoral effect with survival implications after dual-level blockade of mTOR pathway. Regarding anti-target activity, RAD001 and AEE788 effectively blocked EGFR and RPS6 phosphorylation, respectively, *in vitro* and *in vivo*. Unlike everolimus, the EGFR inhibitor significantly increased apoptosis based on FACS analysis, PARP cleavage and TUNEL staining. Everolimus exerted its antineoplastic activity by inducing G1 arrest and hence inhibiting proliferation. Therefore, this combination of pro-apoptotic and anti-proliferative mechanisms may explain the additive effect obtained in the experimental model. Recent reports demonstrate that rapalogs inhibit mTORC2 activity *in vitro*³⁷, so we cannot exclude additional antitumoral activity related to this mechanism.

The recent breakthrough in the management of HCC by using the multi-kinase inhibitor sorafenib has established the concept of extending survival by using molecular targeted therapies in this otherwise chemo-resistant disease. Sorafenib provides three months survival extension in patients with a base-line median survival of 8 months⁵. These data have stimulated the search for additional combination therapies, recognizing that HCC is a heterogeneous cancer with many genetic aberrations. In the present study we establish that mTOR pathway activation plays a role in HCC and that RICTOR may be a mediator of human hepatocarcinogenesis, although further evidence will be required to characterize RICTOR as a new oncogene in human cancer. Up-stream ligand-dependent mechanisms might also be responsible for mTOR pathway activation, indicating that this cascade offers relevant targets for cancer drug discovery. The combination of rapamycin analogs and EGFR inhibitors provides evidence for a proof-of-concept effect in experimental models of HCC. Consequently, these data provide the rationale to test combination therapies in early clinical trials in human liver cancer that includes RAD001³⁸. These studies should follow the guidelines reported on design and endpoints in clinical trials in HCC²².

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
LGDN	

	low-grade dysplastic nodules
HGDN	high-grade dysplastic nodules
mTORC	mTOR complex
TKR	Tyrosine kinase receptor
AFP	Alpha fetoprotein
RAD001	everolimus

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Figure 1.

Flow chart summarizing the human samples analyzed in the study of mTOR pathway in HCC (n=351). An exploratory set (n=77) intended to find out the expression pattern of key genes of the mTOR pathway in the whole hepatocarcinogenesis process whereas the replication set (n=78) was focused on HCC samples. A clinical training set originated from both exploratory and replication sets was used to generate hypotheses on clinical correlations of mTOR pathway activation (n=82). These hypotheses were validated in an independent clinical cohort (n=196).

Figure 2.

Dot-plot of the mRNA levels of 6 genes analyzed in the replication set (very early/early HCC (n=19) and advanced/very advanced HCC (n=59)): IGF2, EGF, IGFBP3, PTEN, RAPTOR, mTOR. Each dot represents the fold-change in expression of each sample normalized to 1 (mean expression in normal liver).

Figure 3. Kaplan-Meier curves showing the correlation between p-RPS6 staining and recurrence (A)/ early recurrence (B) in the validation set.

Figure 4.

A: Histogram for the copy numbers in 0.5 Mb windows of *RICTOR*. Red bars represent patients with gains in *RICTOR* (DNA copy number cutoff=2.27). **B:** Dot-plot of the mRNA levels of *RICTOR* in the replication set. **C–D:** Kaplan-Meier plots of recurrence (**C**) and early recurrence (**D**) after clustering patients according to copy number changes of *RICTOR*. **E–F:** Downregulation of *RICTOR* in cell lines transfected with siRNA (Western blot) (**E**), and significant decrease in cell viability measured with MTT assay in Huh7 cells following downregulation of *RICTOR*. Graphic shows percentage of viable siRNA-*RICTOR* transfected cells relative to control siRNA for each time point and cell line analyzed (**F**).

Figure 5. Cell viability (**A–C**) and proliferation studies (**D**) in liver cancer cell lines treated with EGFR inhibitor (AEE788) and mTOR inhibitor (RAD001, everolimus). Results are presented as % of viable cells or % of ^3H -Thymidine incorporation (counts per million) in compare to control. Cell cycle profiles (**E**) and PARP cleavage immunoblotting in Huh7 cells incubated with the compounds (**F**).

Figure 6. Results of the immunoblotting for total and phosphorylated forms of key proteins of the mTOR pathway (**A**). Luciferase activity linked to a c-fos reporter in Huh7 cells after incubation with everolimus and EGFR inhibitor (**B**). Tumor volume and mice survival in xenografts treated with EGFR inhibitor and everolimus (**C–D**).

Table 1

Base-line clinical characteristics of the two cohorts analyzed for mTOR pathway clinical correlations.

	mTOR PATHWAY CLINICAL TRAINING COHORT(n=82)	mTOR PATHWAY CLINICAL VALIDATION COHORT(n=196)
Gender (male)	55 (67.1 %)	152 (77.6%)
Age (range)	68 (42–80)	66 (30–83)
Etiology of liver disease		
HCV	82 (100%)	79 (41.8%)
HBV		62 (32.8%)
Alcohol		18 (9.5%)
Other		30 (15.9%)
Cirrhosis Child-Pugh Class A	80 (97.3%)	181 (98.9%)
Diameter of largest tumor		
Median	3.5 cm	3.5 cm
Interquartile range	2.5 – 5.7 cm	2.4 – 5.5 cm
Barcelona Clinic Liver Cancer Stage		
0	9 (11%)	17 (8.8%)
A	58 (70.7%)	159 (82%)
B	7 (8.5%)	14 (7.2%)
C	8 (9.8%)	4 (2.1%)
Ethnicity		
Caucasian	75 (91.3%)	153 (78.5%)
African-American	3 (3.7%)	6 (3.1%)
Asian	4 (5%)	36 (18.5%)
Bilirrubin [mg/dL]	0.8 (0.5–1.1)	0.8 (0.6–1.1)
Albumin [g/dL]	4.1 (3.6–4.4)	4 (3.8–4.4)
Platelet count [x1000/mL]	156 (131–180)	166 (125–213)
AFP [mg/dL]	16 (6–181)	13.2 (4.5–97.3)
Uninodular	68 (82.9%)	167 (84.3%)
Degree of differentiation *		
Well differentiated	20 (24.7%)	29 (26.4%)
Moderately differentiated	44 (54.3%)	65 (59.1%)
Poor differentiated	17 (21%)	16 (14.5%)
Vascular invasion		
None	34 (42%)	131 (69.3%)
Microvascular	39 (48.1%)	54 (28.6%)
Macrovascular	8 (9.9%)	4 (2.1%)
Treatment		
Liver resection	100%	100%
Recurrence	43 (54.4%)	101 (51.8%)
Early recurrence (<2 years)	32 (40.5%)	72 (36.9%)
Follow up (median)	21 months	26 months

* Data in validation set refers to 113 patients

Table 2

Analysis of clinico-pathological and molecular variables associated with p-RPS6 staining in human HCC.

Clinicopathological variables	p-value
BCLC staging B/C	0.003
AFP plasmatic levels	0.01
Platelet count	0.03
Moderately/Poor differentiated tumor	0.04
Vascular invasion	0.17
Multinodularity	0.34
Bilirrubin	0.45
Albumin	0.6
Tumor size	0.9
Molecular variables	
High mRNA levels of EGF	0.01
p-EGFR staining	0.02
p-IGFR1 staining	0.03
p-mTOR staining	0.2
p-Akt staining	0.6