Loss of Tuberous Sclerosis Complex 2 (TSC2) is Frequent in Hepatocellular Carcinoma and Predicts Response to mTORC1 Inhibitor Everolimus

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Abbreviations list: TSC: Tuberous Sclerosis Complex; HCC: Hepatocellular Carcinoma; mTOR: mammalian Target of Rapamycin; S6K: S6 kinase; 4E-BP: eIF4E binding protein.
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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide and hyper-activation of mammalian target of rapamycin (mTOR) signaling plays a pivotal role in HCC tumorigenesis. Tuberous Sclerosis Complex (TSC), a heterodimer of TSC1 and TSC2, functions as a negative regulator of mTOR signaling. In the present study, we discovered that TSC2 loss of function is common in HCC. TSC2 loss was found in 4 of 8 HCC cell lines and 8 of 28 (28.6%) patient-derived HCC xenografts. TSC2 mutations and deletions are likely to be the underlying cause of TSC2 loss in HCC cell lines, xenografts and primary tumors for most cases. We further demonstrated that TSC2-null HCC cell lines and xenografts had elevated mTOR signaling and, more importantly, were significantly more sensitive to RAD001/everolimus, an mTORC1 inhibitor. These preclinical findings led to the analysis of TSC2 status in HCC samples collected in the EVOLVE-1 clinical trial of everolimus using an optimized immunohistochemistry assay and identified 15 of 139 (10.8%) samples with low to undetectable levels of TSC2. Although the sample size is too small for formal statistical analysis, TSC2-null/low tumor patients who received everolimus tended to have longer overall survival than those who received placebo. Finally, we performed an epidemiology survey of more than 239 Asian HCC tumors and found the frequency of TSC2 loss to be approximately 20% in Asian HBV+ HCC. Taken together, our data strongly argue that TSC2 loss is a predictive biomarker for the response to everolimus in HCC patients.
Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths, accounting for 250,000 to one million deaths globally each year (1). The annual number of incidence and death of HCC are virtually identical, illustrating the high mortality rate of this aggressive disease. Due to the relative paucity of symptoms in the early stages and the rapid doubling time of the tumor, more than 80% of HCCs are diagnosed at advanced stages when surgery is no longer an option (2). Two randomized trials of the multi-kinase inhibitor sorafenib in HCC patients showed an improvement in median overall survival of close to 3 months, establishing sorafenib as the only targeted therapy and the standard of care in advanced HCC (3, 4). However, the efficacy of sorafenib is transient and modest and there are no established predictive biomarkers for sorafenib. Thus, there is an urgent unmet need for more effective novel therapies to combat this deadly disease.

Mammalian Target of Rapamycin (mTOR) is a serine/threonine kinase that plays a critical role in regulating protein translation, cell cycle progression, and cellular function (5, 6). Two well-characterized substrates of mTOR are S6K1 and 4E-BP1, and mTOR activity is regulated by the PI3K/AKT pathway in response to activation of receptor tyrosine kinases. mTOR activation is found in tumors with primary genetic abnormalities activating the PI3K/AKT pathway (7, 8) or in tumors responding to mitogenic signals (9, 10). Hyper-activation of mTOR signaling has been identified in approximately 50-60% of HCC (11). Receptor tyrosine kinase activation rather than somatic mutations of PTEN or PI3KCA, which are rare in HCC, is likely to be responsible for activation of mTOR in HCC. Consistent with this notion, activation of AKT has been found in 40-60% of human
HCC samples (12) and is a risk factor for early recurrence and poor prognosis (13). More importantly, recent studies using knockout mice showed that mTOR activation was sufficient to cause HCC in rodent models (14), indicating the pivotal role of mTOR signaling in HCC. The allosteric mTORC1 inhibitor RAD001/everolimus has been tested in a randomized, placebo-controlled phase 3 clinical trial in patients with advanced HCC who failed sorafenib (EVOLVE-1) (15). Overall survival (OS), the primary endpoint, showed non-significant improvement with everolimus treatment versus placebo. The molecular predictors for a small number of patients who benefited from the everolimus treatment are currently being explored.

The Tuberous Sclerosis Complex (TSC) formed by TSC1 and TSC2 functions as a critical negative regulator of mTOR through the GAP (GTPase-activating protein) activity of TSC2 towards the small GTPase Rheb (16). Phosphorylation of TSC2 by AKT or other kinases inactivates TSC2, which leads to activation of mTOR (16). Although the GAP activity of TSC2 is of obvious functional importance to the TSC complex, TSC1 stabilizes TSC2 by preventing ubiquitin-mediated degradation of TSC2 (16). Therefore, inactivation of either TSC1 or TSC2 gene disrupts the TSC1/TSC2 complex and results in unrestrained kinase activity of mTOR. Everolimus is an approved and effective drug for treating neurological manifestations of TSC, a hereditary disorder caused by germline mutations in TSC1 or TSC2. Intuitively, TSC lesions in tumors might be potential predictive biomarkers for response to everolimus treatment. However, this link has not been rigorously examined, in part because the frequency of somatic mutations in TSC1/TSC2 in most types of tumors including HCC is quite low.
In this study, we observed frequent loss of TSC2 in HCC cell lines and demonstrated that TSC2-null cell lines were more sensitive to mTOR inhibition by everolimus. We then confirmed TSC2 loss in patient-derived HCC xenografts and observed remarkable anti-tumor efficacy of everolimus in TSC2-null xenografts. TSC2 mutations and deletions were identified in many of the TSC2-null HCC cell lines and xenografts, providing a genetic mechanism for TSC2 loss. We also developed a TSC2 immunohistochemistry (IHC) assay for identifying TSC2 loss in primary tumor samples. Analysis of TSC2 status in EVOLVE-1 clinical samples suggested that TSC2-null/low tumor patients who received everolimus tended to have longer overall survival than those who received placebo. Based on our TSC2 IHC profiling of 239 tumors, the frequency of TSC2 loss is estimated to be around 20% in Asian HBV+ HCC. Taken together, our data strongly suggest that TSC2 loss may be used as a predictive biomarker for everolimus efficacy in HCC clinical trials.
Materials and Methods

Antibodies and compounds

Rabbit antibodies against TSC1, TSC2, p-S6K1(T389), S6K1, p-4E-BP1(T37/T46), p-4E-BP1(T70), 4E-BP1, p-AKT(S473), AKT, p-CDK2(T14/T15), PTEN and p27 were purchased from Cell Signaling Technology (Beverly, MA). Tubulin antibody (mouse) was purchased from Sigma. RAD001/everolimus, BKM120, MK-2206 and MEK162 were synthesized at Novartis. pcDNA3-Flag-TSC2 plasmid was a gift from Dr. Pablo Garcia (Novartis Institutes for Biomedical Research, Emeryville, California, USA). The TSC2 G305V mutant was generated using the Q5® site-directed mutagenesis kit (New England Biolabs) according to the manufacturer’s instructions. The sequences of mutagenic primers used are: forward, 5'-gccggtgggctacccagagac-3'; reverse, 5'-ggctctctgggtagcccaccggc-3'.

Cell culture

Hepatocellular carcinoma cell lines were part of the Novartis Cancer Cell line Encyclopedia (CCLE) collection (17) and were authenticated by single-nucleotide polymorphism (SNP) analysis in 2012.

Reverse phase protein array (RPPA)

Reverse phase protein array was performed at the RPPA Core Facility at MD Anderson Cancer Center according to standard procedures.

Immunoblotting
Total cell lysates were prepared by lysing cells using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitors and phosphatase inhibitors, followed by centrifugation at 14,000 rpm for 10 min at 4°C. Equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with primary antibodies overnight at 4°C. Secondary antibodies conjugated with either HRP or infrared dyes were used for signal visualization by ECL film method or an LI-COR Odyssey scanner, respectively.

**Reverse transcription and Taqman quantitative RT-PCR**

Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed with Taqman Reverse Transcription Reagents (Applied Biosystems) according to the manufacturers’ instructions. Transcript levels were assessed using the ABI PRISM 7900HT. Quantitative real-time PCR was performed in 12 μl reactions consisting of 0.6 μl of 20X Taqman probe and PCR primer mix, 6 μl 2X Taqman Universal PCR Master Mix, and 5.4 μl diluted cDNA template. The thermocycling conditions utilized were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. All experiments were performed in quadruplicate. Gene expression analysis was performed using the comparative ∆∆CT method with the housekeeping gene, *GUSB*, for normalization. All Taqman primers/probes were purchased from Applied Biosystems (TSC1, Hs01060648_m1; TSC2, Hs01020387_m1; GUSB, Hs99999908_m1; MZB1, Hs00414907_m1).
CellTiter Glo proliferation assay

Cells were seeded in 96 well plates and treated with different doses of everolimus (6 fold serial dilutions starting from 1µM) for 3 days. A CellTiter Glo assay was performed according to the manufacturers' protocols. Everolimus dose response curve and IC$_{50}$ values was calculated using the XLfit add-in in Excel.

Patient-derived HCC xenograft study

This study received ethics board approval at the SingHealth. All mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA.

The establishment of our collection of HCC patient-derived xenografts has been previously described (18) and the indicated xenograft lines were subcutaneously implanted into male SCID mice (Animal Resources Centre, Canning Vale, Western Australia) aged 9 to 10 weeks. For anti-tumor efficacy experiments, mice bearing the indicated tumors (10 mice per group) were orally administrated with either vehicle (5% dextrose) or varying doses of everolimus (0.25, 0.5, 0.75 or 1.0 mg/kg) daily for indicated days. Treatment started when the tumors reached the size of approximately 170-600 mm$^3$ (dependent on the lines or experimental purpose). Tumor growth was monitored and tumor volume was calculated as described (18, 19). At the end of the study, mice were sacrificed with body and tumor weights recorded. The efficacy of everolimus was determined by T/C ratio, where T and C are the median weight of everolimus -treated and vehicle-treated tumors, respectively. T/C ratios less than 0.42 were considered active...
according to the widely-adopted criteria from the Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute.

**DNA sequencing and copy number analysis**

HCC cell lines DNA sequencing and copy number analysis using SNP6.0 array has been previously described (17). Genomic DNA from HCC xenografts and primary tumors were sequenced using the T5 panel targeted sequencing at Foundation Medicine (Cambridge, MA). Multiplex ligation-dependent probe amplification (MLPA) of TSC2 duplications and deletions was performed using TSC2 SALSA® MLPA® probemix P046-B2 (MRC-Holland) according to the manufacturer’s instructions and analyzed using GeneMarker® software from SoftGenetics LLC.

**TSC2 immunohistochemistry (IHC) assay**

HCC cell lines and patient-derived xenografts were collected, fixed with formalin and processed for paraffin embedding. HCC tissue microarrays were purchased from BioChain (catalog # Z7020056 and Z7020057, unrelated HCC TMAs) and FolioBio (catalog# ARY-HH0225). Formalin-fixed paraffin-embedded (FFPE) tissue sections (4 μM thick) were stained on the Ventana Discovery XT automated staining platform. Standard antigen retrieval condition in cell conditioning 1 buffer was used, followed by incubation with rabbit anti-TSC2 antibody (Cell Signaling Technology, catalog # 4308) at a dilution of 1:200 for 32 min at 37°C. Signal was then visualized using Omni Map (OMap) anti-Rabbit HRP and DAB.
TSC2 expression was assessed semi-quantitatively by a pathologist using a histo-score (H-score) methodology based on staining intensity and percentage of tumor cells expressing TSC2. Intensity was scored as 0 (none), 1 (weak), 2 (moderate), or 3 (strong). H-score was calculated as follows: H-score = [fraction of cells with intensity grade 1 (%)] + [fraction of cells with intensity grade 2 (%) x 2] + [fraction of cells with intensity grade 3 (%) x 3].

Clinical study

The EVOLVE-1 (clinicaltrial.gov # NCT01035229) clinical trial design, patient inclusion and exclusion criteria have been published (15). Briefly, 546 patients aged ≥18 years with BCLC stage B or C HCC whose disease progressed during or after sorafenib or who were intolerant to sorafenib were randomized 2:1 to everolimus 7.5 mg/day group or placebo group. Study drug was given continuously until disease progression or intolerable toxicity. CT/MRI scans were performed every 6 weeks and primary endpoint was overall survival (OS).

The study protocol was reviewed and approved by the Independent Ethics Committee (IEC) and the Institutional Review Board (IRB) for each center. The study was conducted according to Good Clinical Practices (GCP) and according to the ethical principles of the Declaration of Helsinki. Archival formalin-fixed paraffin-embedded (FFPE) tissue sections were collected from patients who provided informed consent for biomarker study.
Statistical analysis

For xenograft growth curves, results are expressed as the means ± S.E.M (standard error of mean) from an appropriate number of replicates for each treatment group versus time as indicated in the figure legends. Paired Student’s t tests were used to calculate the P values. For immunoblots, relevant phosphorylation signals were quantified by densitometry and showed in supplementary Tables. Statistical significance was assessed using two-tailed Student’s t tests and described in the Table footnotes.
Results

TSC2 loss in hepatocellular carcinoma cell lines confers everolimus sensitivity

As part of a proteomic effort to comprehensively characterize Novartis’ cancer cell line collection known as the Cancer Cell Line Encyclopedia (CCLE) (17), a Reverse Phase Protein Array (RPPA) platform was utilized to interrogate the expression levels or phosphorylation status of more than 200 cancer-related proteins in a panel of more than 400 cancer cell lines of various lineages. TSC2 protein levels showed a clear outlier pattern in HCC compared to all other lineages and very low expression was observed in five of the nine HCC cell lines (Fig. 1A). The low TSC2 signal in those five HCC cell lines was unlikely to be due to any quality issue of the cell lysate spotted onto the protein chip as the levels of other proteins in these cell lines were normal (fig. S1A). In addition, these TSC2 low HCC cell lines tended to have elevated mTOR signaling, as demonstrated by the trend of elevated phosphorylation of downstream targets S6K1(T389), 4E-BP1(T37/T46) and S6(S240/S244) (16). This observation is consistent with the role of TSC2 as a negative regulator of mTOR signaling.

We then performed immunoblots with eight of the nine HCC cell lines used in the RPPA analysis. TSC2 protein in SNU-886, SNU-878, SNU-398 and Li-7 cells were essentially non-detectable while TSC2-low status for HuH-6 could not be confirmed (Fig. 1B). Those four cell lines were therefore designated as TSC2-null while JHH-7, SNU-761, HuH-7 and HuH-6 were designated as TSC2 wild-type. All eight HCC cell lines had comparable levels of TSC1, the binding partner for TSC2. The phosphorylation of S6K1(T389) and 4E-BP1(T37/T46) showed a trend of elevation in TSC2-null cell lines compared to the TSC2 wild-type group while the phosphorylation of AKT(S473) tended
to be lower, with the exception of HuH-7 (Fig. 1B and Supplementary Table 1). A similar pattern on phosphorylation of S6K1, 4E-BP1 and AKT was observed in the RPPA data (fig. S1A). This is consistent with the previous observation that activation of mTOR and S6K1 caused by TSC2 lesions results in the feedback inhibition of the upstream insulin-like growth factor receptor 1 (IGF1R)/PI3K signaling pathway (20). Since the level of S6K1 and 4E-BP1 phosphorylation in HuH-7 is comparable to that of the TSC2-null group, we hypothesized that it is due to activation of the PI3K pathway upstream of TSC1/2. Indeed, treatment of HuH-7 cells with the PI3K inhibitor BKM120 or AKT inhibitor MK-2206, but not the MEK inhibitor MEK162, reduced S6K1 phosphorylation (fig. S1B). No PIK3CA mutation or PTEN loss was found in HuH-7 cells (data not shown), suggesting that activation of mTOR in this cell line may be a result of upstream activation at the level of growth factor receptors such as IGF1R.

To investigate whether TSC2 loss rendered the HCC cell lines sensitive to mTOR inhibition, we studied the anti-proliferation effect of everolimus on the eight HCC cell lines using a 3 Day CellTiter Glo (CTG) proliferation assay. While the everolimus IC_{50} values of three of the four TSC2 wild-type cell lines (except HuH-7) were more than 2000 nM, the IC_{50} values of the four TSC2-null cell lines ranged from 0.7 nM to 25.1 nM (Fig. 1C and Supplementary Table 2). The maximal growth inhibition by everolimus at 1 µM was approximately 60% for the four TSC2-null cell lines but only about 35% for the three TSC2 wild-type cell lines except HuH-7 (Fig. 1C and Supplementary Table 2). TSC2 wild-type HuH-7 cells were as sensitive as TSC2-null cell lines to everolimus (IC_{50}=1 nM and maximal growth inhibition=66%), possibly due to the activation of
mTOR by upstream PI3K signaling (fig. S1B). Consistent with the IC$_{50}$ values, we observed complete inhibition of S6K1 phosphorylation by 2.5 nM everolimus in all four TSC2-null cell lines (fig. S1C). Taken together, TSC2 expression level is likely to be the main determinant for mTOR activity and everolimus sensitivity in those HCC cell lines. Although we cannot fully rule out other lesions contributing to everolimus sensitivity in the four TSC2-null cell lines, these results strongly suggest that TSC2 loss is a highly penetrant predictive biomarker for everolimus sensitivity in HCC cell lines.

**TSC2 loss in patient-derived HCC xenografts predicts everolimus efficacy**

We had previously established a large panel of patient-derived HCC xenografts for testing the efficacy of various anti-tumor agents (21, 22). To determine whether TSC2 loss also occurred in those HCC xenografts, we performed immunoblots on lysates from 13 randomly selected patient-derived HCC xenograft tumors. As shown in Fig. 2A, four xenografts (HCC05-0411B, HCC19-1010, HCC14-0910 and HCC24-0309) showed no detectable levels of TSC2 and thus were named as TSC2-null xenografts. Consistent with the immunoblot data from cell lines, basal p-S6K1 (T389) was significantly increased in these four TSC2-null tumors (Fig. 2A and Supplementary Table 3). A trend of increased p-4E-BP1 (T70, functionally similar to phosphorylation at T37/T46 shown in HCC cells lines) levels was also observed in three of these four TSC2-null tumors compared with the TSC2 wild-type tumors (Fig. 2A and Supplementary Table 3). Again, p-AKT (S473) levels were relatively high in TSC2 wild-type tumors but significantly reduced in TSC2-null tumors, probably as a result of feedback inhibition of upstream IGF1R signaling by S6K activation. Because p-AKT levels are regulated by PTEN, we then examined the
PTEN levels and found two models with PTEN loss (HCC26-1004 and HCC17-0211).

Taken together, these data suggest that TSC2 loss may occur in HCC tumors and that mTOR signaling is activated in TSC2-null xenografts.

To determine whether TSC2-null HCC tumors display sensitivity to everolimus, we tested everolimus in the four TSC2-null xenografts along with four TSC2 wild-type xenograft (HCC06-0606, HCC25-0809, HCC13-0109 and HCC13-0212) as determined by TSC2 immunoblot in Fig. 2A. Tumor-bearing mice were treated daily with either vehicle or 1 mg/kg everolimus. Each treatment group involved 8 to 10 independent tumor-bearing mice representing the same xenograft line. It is of note that the dose of everolimus used in this study was approximately 2.5 to 5 times lower than doses used in our previous reports (22) and the toxicity associated with daily 1 mg/kg everolimus treatment was well tolerated. As shown in Fig. 2B, everolimus exhibited potent anti-tumor activities in all four TSC2-null xenografts and substantial tumor growth inhibition was observed as early as day 5 after dosing. The growth-inhibitory effects were particularly pronounced in two of the four TSC2-null xenografts (HCC05-0411B and HCC14-0910). In contrast, the same treatment with everolimus had insignificant anti-tumor activities in two wild-type xenografts (HCC06-0606 and HCC25-0809) and only modest anti-tumor activities in the other two TSC2 wild-type xenografts HCC13-0109 and HCC13-0212 (Fig. 2B). These data suggest that the anti-tumor activity of everolimus we observed was not due to general toxicity and that everolimus was selectively efficacious in TSC2-null HCC tumors.
To further establish the link between TSC2 loss and everolimus efficacy in vivo, we screened another 15 patient-derived HCC models by immunoblot and identified 4 additional TSC2-null xenografts (data not shown). The eight TSC2-null xenografts we identified represent 28.6% of the 28 HCC xenografts we screened. We next tested the anti-tumor activity of everolimus at 1 mg/kg dose in the four new TSC2-null models together with additional TSC2 wild-type models. Efficacy of everolimus was evaluated by comparing the final mean weight of tumors in the treatment arms (T) to those in the control arm (C) (T/C ratio), with a value of <0.42 considered as an active response (a widely-adopted criteria from the National Cancer Institute). T/C ratios from the eight models shown in Fig. 2B were also calculated. Among the 20 xenografts we tested (Fig.2C), everolimus was considered to be active in 8 (36.4%) xenograft lines, namely HCC05-0411B (T/C = 0.05), HCC01-0207 (T/C = 0.09), HCC14-0910 (T/C = 0.12), HCC19-1010 (T/C = 0.14), HCC25-0705A (T/C = 0.14), HCC24-0309 (T/C = 0.25), HCC24-1005 (T/C = 0.27), and HCC5-1318(3) (T/C = 0.34). Strikingly, all eight everolimus-sensitive models were TSC2-null. TSC2 wild-type models modestly responded to everolimus with T/C ratios ranging from 0.44 to 0.74, indicating the important role of mTOR signaling to HCC tumor growth even in the TSC2 wild-type setting. With 8 out of 8 TSC2-null models and 0 out of 12 TSC2 wild-type models as everolimus responders, this enrichment is statistically significant at p < 0.01 (Fisher exact test value=8E-06). Interestingly, the two models with PTEN loss only showed modest responses to everolimus (T/C=0.40 for HCC26-1004 and T/C=0.61 for HCC17-0211), suggesting that PTEN loss may not be as good in predicting everolimus response in HCC models as TSC2 loss. This remarkable enrichment of TSC2-null models in everolimus
responders from a relatively large study (20 models with distinct mutation profiles as shown in Supplementary Table 4) strongly argues that TSC2 loss in HCC leads to hyper-activation of the mTOR signaling pathway and robustly predicts everolimus response in xenograft models.

**Tumor growth inhibition by everolimus was through inhibition of mTOR signaling**

As shown in Fig. 2B, the growth-inhibitory effects were particularly pronounced in TSC2-null xenograft HCC14-0910 and HCC05-0411B at the dose of 1 mg/kg everolimus. To determine the degree and dose-dependent effect of everolimus, we tested its anti-tumor activity at the doses of 0.25, 0.5, 0.75 and 1 mg/kg in those two models. As shown in Fig. 3A, tumor stasis was achieved at 0.75 mg/kg everolimus and there was a dose-dependent growth inhibition by the four doses of everolimus in HCC14-0910. Similar results were observed in model HCC05-0411B (data not shown).

We then performed pharmacodynamic analysis by immunoblot at the end of the efficacy study. As shown in Fig. 3B, everolimus caused a dose-dependent decrease in p-S6K1 (T389) and p-4E-BP1 (T70), indicating successful mTOR inhibition in TSC2-null xenograft HCC14-0910. Similar to previous studies (22), everolimus treatment led to a dose-dependent elevation of p-AKT (S473), probably as a result of relieving the feedback inhibition of IGF1R by S6K1 activation. Everolimus treatment also significantly reduced the phosphorylation of CDK2 (T14/T15) and induced p27 accumulation, both of which are well-established markers of cell cycle arrest. Our data are in agreement with previous studies showing that inhibition of mTOR leads to G1-S cell cycle arrest through
increased levels of p27 (23). In addition, as in our previous study (22), the anti-
angiogenic effect of everolimus, measured by microvessel density, was also observed in
HCC14-0910 and other TSC2-null HCC xenografts (data not shown).

**TSC2 loss in HCC is caused predominantly by TSC2 deletions and mutations**

To understand the molecular mechanism responsible for TSC2 loss in TSC2-null HCC
cell lines, we examined the TSC2 mRNA levels by Taqman RT-PCR assay. TSC2 mRNA
levels in all four TSC2-null cell lines were less than 5% of that of JHH-7 cells, which had
the highest level of TSC2 mRNA (fig. S2A). The TSC2 mRNA levels in the other three
TSC2 wild-type lines (SNU-761, HuH-7 and HuH-6) were approximately 50%, 57% and
26% of that in JHH-7 cells, respectively. The mRNA levels of TSC1 were comparable in
all cell lines. The extremely low levels of TSC2 mRNA in TSC2-null cell lines prompted
us to examine the status of TSC2 gene in these cell lines. As shown in Table 1, SNU-886
and SNU-878 cells harbored TSC2 nonsense mutations (R1032* and S1514*,
respectively) and TSC2 wild-type alleles were lost as indicated by the near 100% mutant
allele frequency. SNU-398 cells contain a focal deletion in TSC2 from exon 9 to exon 36.
No mutation or deletion in TSC2 was detected in Li-7 cells, raising the possibility that the
absence of TSC2 mRNA in this cell line may be due to epigenetic silencing of TSC2. The
TSC2 promoter has been found to be methylated in several cancer types including breast
cancer and oral squamous cell carcinoma (24, 25) and DNA methylation abnormalities
have been shown to be a common lesion in HCC cell lines including Li-7 (26). However,
we were unable to observe a robust re-expression of TSC2 transcripts with treatment of
the DNA methylase inhibitor decitabine and/or HDAC inhibitor trichostatin (TSA) while
another known methylated gene MZB1 was successfully induced by those treatments (fig. S2B). Although suggestive, this negative result does not completely rule out the possibility of TSC2 epigenetic silencing in Li-7 cells.

To determine whether TSC2 mutations and deletions are similarly responsible for TSC2 loss in HCC xenografts as in HCC cell lines, we analyzed the exons of both TSC1 and TSC2 (together with 294 other cancer-related genes) using next generation sequencing. As shown in Table 1, among the eight TSC2-null HCC models we previously identified, TSC2 deletions were found in model HCC19-1010 and model HCC24-1005. In addition, a TSC2 splicing mutation c.1444-2A>T was found in model HCC14-0910 at 100% allele frequency and model HCC24-0309 contained a G305V missense mutation at 99% allele frequency. The impacted glycine residue resides within the TSC1 binding domain of TSC2 (amino acid 1-418) and is highly conserved from mouse to man. To test the functional consequence of the G305V mutation, we expressed the TSC2 G305V mutant by transient transfection of HEK293T cells and evaluated its relative expression at both the mRNA level and the protein level. As shown in fig. S2C, the mRNA level of G305V mutant was only about 30% that of wild-type TSC2 mRNA when equal amount of plasmids were transfected, suggesting that the missense mutation may cause reduced mRNA expression. Importantly, there was no detectable G305V mutant TSC2 protein by immunoblot regardless of its mRNA levels (fig. S2D), suggesting that the G305V mutant protein is unstable.
For the four TSC2-null xenografts in which no TSC2 genetic lesions were identified by sequencing, we performed TSC2 multiplex ligation-dependent probe amplification (MLPA). This PCR-based assay measures the copy number of each exon of the TSC2 gene and its neighboring regions and provides a better resolution of TSC2 copy number than the broad sequencing method. Indeed, loss of two upstream regions that possibly contain the TSC2 promoter and duplication of exon 16-23 were identified in HCC5-1318(3) and HCC05-0411B, respectively (Table 1). Exon duplication of TSC2 has been found in TSC patients and the duplicated exons may disrupt the reading frame of TSC2. All together, we have identified genetic mechanisms for TSC2 loss in 6 out of the 8 TSC2-null models shown in Fig. 2C.

It is known that a xenograft tumor may undergo genetic drift when compared to the original tumor and that certain genetic lesions may be enriched for during the model establishment and passaging process. To validate that TSC2 genetic lesions exist in primary HCC tumors, we sequenced DNA samples from 13 randomly selected Hong Kong HCC biopsies using the same T5 panel at Foundation Medicine. As shown in Table 1, a TSC2 splicing mutation and a TSC2 deletion were identified in sample B253 and B266, respectively. Sample B277 contained a point mutation at 92% allele frequency resulting in an A1141T amino acid change. This mutation was also found in Taiwanese TSC patients (27) and therefore it likely inactivates TSC2. In addition, the majority of disease-associated mutations of TSC2 result in a substantial decrease in the TSC2 protein level (28, 29). In summary, TSC2 mutations and deletions exist in primary HCC tumors.
TSC2 immunohistochemistry assay identified TSC2 loss in primary HCC tumors

Since not all TSC2-null cell lines and xenografts had mutations or deletions in the TSC2 gene, it raised the possibility that non-genetic mechanisms such as epigenetic silencing may be responsible for TSC2 loss in HCC. It might also be challenging to predict the functional consequence of TSC2 missense mutations as in the case of the G305V mutation we identified. Furthermore, for tumor suppressor genes like TSC2, loss of heterozygosity or extremely high mutant allele frequency is needed to predict loss of function at the protein level. To overcome these limitations of the sequencing approach, we developed a TSC2 immunohistochemistry (IHC) assay to identify TSC2 loss in HCC tumors. We first validated the IHC assay using TSC2 wild-type and TSC2-null HCC cell lines. As shown in fig. S3A, intense staining was observed in TSC2 wild-type HuH-6 cells and little signal was detected in TSC2-null SNU-398 cells, suggesting that the assay is robust and specific. We further validated the IHC assay using patient-derived HCC xenografts, which are more heterogeneous than the cell lines. Again there was strong staining in TSC2 wild-type xenograft HCC06-0606 but very faint staining in TSC2-null xenograft HCC-24-1005 (fig. S3B).

We then used the TSC2 IHC assay to address whether TSC2 loss in our xenograft models occurred in primary tumors or was acquired during propagation in the mice. As shown in fig. S3C, TSC2 was not detected in the four primary HCC tumors from which the TSC2-null xenografts HCC05-0411B, HCC19-1010, HCC14-0910 and HCC24-0309 were derived. The TSC2 staining in primary HCC sections was from adjacent normal liver tissues and served as positive controls. This data strongly argue that the TSC2 loss
observed in our HCC xenografts was a faithful representation of the primary tumors from which they were derived and that our IHC assay could robustly identify TSC2 loss in HCC clinical samples.

**HCC patients with TSC2-null/low tumors benefited from everolimus treatment**

EVOOLVE-1 was a double-blind, randomized, placebo controlled (2:1 everolimus vs. placebo), multi-center phase 3 study to evaluate the efficacy and safety of everolimus in adult patients with advanced HCC who had progressed on or after sorafenib treatment, or were intolerant of sorafenib treatment. The trial did not meet its primary endpoint of improving OS by everolimus over placebo. The median OS for the everolimus group and placebo group were 7.56 months and 7.33 months, respectively (Hazard ratio: 1.05; 95% CI: 0.86-1.27; p value=0.675) (15). In order to test whether patients with no or low TSC2 expression in their tumors had an OS benefit from everolimus treatment, we quantified TSC2 expression in tumor samples from the EVOOLVE-1 patients using our IHC assay and an H-score scoring system as described in the Methods. Evaluable TSC2 IHC results were obtained for 139 EVOOLVE-1 patients for whose archival formalin-fixed paraffin-embedded tumor samples were available. We defined 15 tumors with H-scores ranging from 0 to 120 (Table 2) as TSC2-null/low. The 120 H-score cutoff was partially guided by the H-scores of TSC2-null patient-derived HCC xenografts (ranging from 0 to 135, data not shown). These included 8 samples (5.8%) with minimal TSC2 expression (H-score=0-60) and 7 samples (5.0%) with low TSC2 expression (H-score=61-120). The seven TSC2-low samples had only some or universal weak staining (1+). Two tumors
with H-score of 90 and 120, respectively, but had substantial (40%) 2+ or even some 3+ intensity staining were annotated as TSC2 wild-type.

The treatment assignment, overall survival (OS) and demographic features of the 15 patients with TSC2-null/low tumors are listed in Table 2. The patients treated with everolimus (n=10) had longer OS than those treated with placebo (n=5). Six of the 10 TSC2-null/low patients treated with everolimus (patient 10-15) had OS ranging from 9.53 to more than 32.72 months, compared with the 7.56 months median OS of the overall everolimus group. The two patients with very short OS (patient 6 and patient 7) were on treatment for only 2 weeks and discontinued from the study due to either complications of the underlying cirrhosis or intolerable adverse events. Another patient with a short survival of 4.63 months (patient 8) only received treatment for 6 weeks and discontinued due to disease progression. In contrast, 4 of the 5 TSC2-null/low patients treated with placebo had OS ranging from 1.25 to 5.59 months, much shorter than the 7.33 months median OS of the overall placebo group. These findings may suggest an association of TSC2 loss/low with more aggressive disease and that TSC2 status may have prognostic value, consistent with a recent report (30). The only patient with an OS of more than 18.23 months was on placebo for only 6 months and after disease progression received an ALK1 inhibitor (PF-03446962) for 2 months, followed by a MET inhibitor. Although the sample size of TSC2-null/low patients is too small to perform formal statistical analysis, the observed OS benefit from everolimus in patients with TSC2-null/low tumors is consistent with our hypothesis that TSC2-null tumors are sensitive to mTOR inhibition.
TSC2 loss is more common in Asian HBV+ HCC than in Caucasian HCC

The frequency of TSC2 loss in EVOLVE-1 samples (5.8-10.8%, Fig. 4A) is much lower than that in Singapore HCC patient-derived xenografts (28.6%, 28 models) and in Hong Kong primary HCC (23.1%, 13 tumors). This is likely due to the heterogeneity of the ethnic makeup of the EVOLVE-1 patients (55% Caucasian, 35% Asian and 10% others). While the overall profile of the 139 patients with TSC2 IHC results reflected this ethnic diversity, the percentage of Asian in the 15 TSC2-null/low tumor patients increased to 53% (fig. S4A). This raised the possibility that the prevalence of TSC2 loss may vary by ethnicity. To investigate this, we segmented the TSC2 H-scores into three groups: 0-60 (likely to be TSC2-null), 61-120 (TSC2-low) and 121-300 (TSC2 wild-type), and compared the H-score distribution of each race in the 139 EVOLVE-1 samples. As shown in Fig. 4A, the percentage of TSC2-null/low in the 48 Asian HCC samples is 18.7%, about 3 times of that of the 78 Caucasian HCC tumors (6.4%). There were only 13 tumors in the group of other ethnicity, which was too few for a meaningful frequency estimate. The racial composition in each of the three TSC2 H-score segments is shown in fig. S4B and the ratio of Asian to Caucasian increased significantly from the high to the low H-score segments. Taken together, these epidemiology data suggest that TSC2 loss in HCC is more common in Asian than in Caucasian population.

To obtain a more accurate frequency of TSC2 loss in the Asian HCC population, we analyzed additional 322 Asian HCC tumors of which greater than 90% were HBV+ in the Tumor Microarray (TMA) format. The H-score range distribution in 239 evaluable cases is shown in Fig. 4B. There were 20.0% samples with an H-score below 60 and 19.7%
samples had an H-score from 61 to 120, indicating minimal to low TSC2 expression. Therefore, we estimated TSC2 loss to be at least 20% in Asian HBV+ HCC. Consistent with the HBV association, seven of the eight EVOLVE-1 TSC2-null/low Asian HCC tumors were HBV+ (Table 2).
Discussion

One major factor limiting the effective treatment of HCC is the high degree of tumor heterogeneity. The same treatment can produce remarkably different responses ranging from complete response to disease progression in patients with indistinguishable tumors at the histopathological level (31). Understanding the molecular factors that may predict patient benefits derived from novel targeted therapies will be crucial for the successful development of HCC treatments. Despite the high frequency of mTOR activation in HCC, the EVOLVE-1 phase 3 trial showed essentially no OS benefit by everolimus in unselected HCC patients (15). The fact that mTOR inhibition was not sufficient to suppress tumor growth in many HCC patients suggests that mTOR dependence cannot solely be predicted by general mTOR activation. Rather, specific lesions in mTOR pathway components such as PTEN or TSC1/TSC2 are more likely to be useful in identifying HCC patients who will most likely benefit from mTORC1 inhibitors. In the present study, we demonstrated the remarkable prediction of anti-growth effect of everolimus by TSC2 loss in both HCC cell lines and patient-derived xenografts. More importantly, the analysis of a subset of EVOLVE-1 patients suggests that those with TSC2-null/low tumors indeed benefited from everolimus treatment. These clinical data extended our preclinical correlations to human and support the utility of TSC2 loss as a HCC patient selection biomarker for mTORC1 inhibitors, as illustrated in fig. S5.

The molecular mechanisms responsible for TSC2 loss in HCC are likely diverse. The TSC2 deletions and mutations we identified in HCC cell lines, xenografts and primary tumors provided genetic explanations for the majority of TSC2 loss we observed. But
TSC2-null HCC cell line Li-7 and TSC2-null xenografts (HCC25-0705A and HCC01-0207) do not harbor any genetic lesions in TSC2 gene, suggesting the existence of non-genetic mechanisms of TSC2 loss in those HCC samples. It has been reported that promoter methylation was responsible for silencing of TSC2 gene in other cancer types (24, 25). In addition, post-translational mechanisms such as phosphorylation and acetylation could lead to TSC2 loss. TSC2 can be phosphorylated by AKT, ERK, AMPK, and GSK3β at distinct sites and these phosphorylation events inactivate TSC2 (16). Both AKT- and ERK-dependent phosphorylation of TSC2 have been shown to disrupt its interaction with TSC1 and destabilize TSC2 (32, 33). Recently, arrest defective protein 1 (ARD1) was shown to acetylate and stabilize TSC2 and loss of heterozygosity (LOH) of ARD1 has been observed in breast, lung, pancreatic, and ovarian cancer samples (34). It is also noteworthy to point out that TSC1 inactivation can lead to TSC2 protein degradation. Although no TSC1 mutations were identified in any of our HCC samples, a TSC1 mutation has been identified by high-resolution characterization of one hepatocellular carcinoma genome (35). The exact mechanisms of non-genetic loss of TSC2 in HCC remain to be determined.

In agreement with our findings, mutations in TSC1/2 as putative biomarkers that predict efficacy of mTORC1 inhibitors have emerged recently from whole-genome or -exome sequencing analyses of tumor samples from patients who had experienced extraordinary benefit from mTORC1 inhibitor treatments. Solit and co-workers showed that TSC1 loss-of-function mutations were present in one metastatic bladder cancer patient with a durable (>2 years) complete response and four with partial response or stable disease...
after receiving everolimus, but in only one of nine patients with disease progression (36). Similarly, Voss and colleagues found inactivating mutations in \textit{TSC1} or \textit{TSC2}, or activating mutations in \textit{MTOR}, in four of six renal cell carcinoma patients who had durable response to either everolimus or temsirolimus, but not in patients who progressed rapidly on the same treatments (37). Recently, \textit{TSC2} (Q1178*) loss of function mutation was identified in one thyroid cancer patient who achieved a complete response that lasted for 18 months when treated with everolimus (38). Despite lacking placebo arms for comparison in those studies, the remarkable clinical efficacy of mTORC1 inhibitors in distinct types of \textit{TSC1/TSC2} mutated tumors (bladder, kidney, thyroid and liver cancer) suggests a strong correlation between \textit{TSC1/TSC2} loss of function and clinical efficacy of mTORC1 inhibitors such as everolimus. This link is consistent with the preclinical observation that the loss of TSC complex gave rise to high levels of constitutive mTOR activation and is not surprising given the large number of signaling inputs regulating mTOR through the \textit{TSC1/TSC2} complex (16). Interestingly, the prevalence of mutations in \textit{TSC1} versus \textit{TSC2} seems to vary among tumor types.

In this study, we also developed a \textit{TSC2} IHC assay to be used with primary HCC samples in the clinic. The IHC approach is superior to the sequencing assay for the cases of missense \textit{TSC2} mutations with unknown functional consequences and it can also capture \textit{TSC2} loss by all types of mechanisms. However, as for all IHC assays, it was challenging to define the H-score cutoff for \textit{TSC2-null} HCC tumors. We used H-score of 60 and 120 as the cutoff for \textit{TSC2-null} and \textit{TSC2-low} tumors, respectively. The H-score cutoff for \textit{TSC2-null} or low need to be better defined by correlating with \textit{TSC2}
mutations, TSC2 levels in immunoblot and more importantly, everolimus clinical response in more patients.

In this study, we identified 15 TSC2-null/low tumors from 139 EVOLVE-1 samples. By analyzing the patient ethnicity of those samples, we found that TSC2 loss is more common in Asian than in Caucasian HCC. The cause of such ethnic difference in TSC2 loss is unknown but may be related to the distinct etiologies of Caucasian HCC (HCV and alcohol) and Asian HCC (HBV). Additional epidemiology studies in non-Asian populations using our IHC assay are warranted and may better inform the broad utility of TSC2 loss as a patient selection biomarker in HCC.

TSC2 mutations were not reported in recent next generation sequencing studies of HCC (39-42) and there is only one report finding a TSC1 mutation in HCC (35). The different patient ethnicity (Caucasian HCC) and HCC etiology (HCV+ for Japan HCC) in those studies may account for the differences between those and our results. In fact, the HCC mutation spectrums in those papers significantly differed from each other. For those studies with Asian HBV+ HCC samples, only a small discovery set was subjected to whole-genome or -exome sequencing and TSC2 mutations may not be represented in their initial discovery set. Lastly, the targeted exon sequencing assay used in our study is more sensitive than the whole-genome or -exome sequencing method for a particular gene. The sequencing depth at TSC2 locus in our assay (median exon coverage ranging from 496 to 1043) is much higher than that expected from the whole-genome or -exome sequencing.
Previously, the challenge of using TSC1/TSC2 lesions as patient selection biomarkers was that somatic \textit{TSC1/2} mutations in cancer are generally rare. \textit{TSC1/2} mutations are estimated to be less than 2\% in several major cancer types including kidney cancer, based on TCGA (The Cancer Genome Atlas) studies and other large-scale deep sequencing efforts (36, 43). Only recently, bladder cancer was found to have a high frequency of PI3K-mTOR pathway alterations with \textasciitilde{}8\% \textit{TSC1} mutations and \textasciitilde{}2\% \textit{TSC2} mutations (44). Identifying tumor types with reasonable frequency of \textit{TSC1/2} mutations will enable TSC status to be used as a biomarker in the clinic for practical reasons. Based on our TSC2 IHC survey of 239 Asian HCC tumors, the frequency of TSC2 loss is estimated to be around 20\% in Asian HBV\(^+\) HCC. China alone accounts for more than 50\% of the world’s HCC cases and about 80\% of HCC cases in China are HBV\(^+\) (45). Therefore, Asian HBV\(^+\) HCC represents nearly half of the total cases of HCC worldwide. This epidemiology data strongly support TSC2 loss as a potential patient selection biomarker for Asian HBV\(^+\) HCC likely to benefit from everolimus therapy and further exploration of the prognostic value of TSC2 loss.

If the high frequency of TSC2 loss can be confirmed and its prognostic significance can be established in larger and more diverse Asian HCC populations, an everolimus clinical trial focused on the Asian patients will be justified and we recommend TSC2 loss defined by IHC as the patient enrollment criterion. Like other predictive biomarkers, it will always be certain that not all TSC2-null tumors will respond to everolimus treatment. It will be important to examine the additional genetic lesions or clinical features of TSC2-
null tumors to enhance the predicating specificity of this biomarker and to enable drug combinations with everolimus to achieve better anti-tumor efficacy, such as PI3K inhibitors as suggested by a previous report (46). In addition, TSC lesion is unlikely to explain all cases of everolimus response in HCC patients and other predictive biomarkers for everolimus responders, such as activating mutations in mTOR or Rheb, remain to be identified.
Acknowledgements

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References:


Table 1. TSC2 deletions and mutations identified in TSC2-null HCC cell lines, xenografts and primary tumors.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample type</th>
<th>TSC2 mutation</th>
<th>Allele frequency</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNU-886</td>
<td>Cell line</td>
<td>p.R1032*</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>SNU-878</td>
<td>Cell line</td>
<td>p.S1514*</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>SNU-398</td>
<td>Cell line</td>
<td>Deletion</td>
<td>-</td>
<td>Exon 9-36 deleted</td>
</tr>
<tr>
<td>HCC14-0910</td>
<td>Xenograft</td>
<td>Splicing</td>
<td>100%</td>
<td>c.1444-2A&gt;T_p.splice</td>
</tr>
<tr>
<td>HCC19-1010</td>
<td>Xenograft</td>
<td>Deletion</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HCC24-0309</td>
<td>Xenograft</td>
<td>p.G305V</td>
<td>99%</td>
<td>Within TSC1 binding domain</td>
</tr>
<tr>
<td>HCC24-1005</td>
<td>Xenograft</td>
<td>Deletion</td>
<td>-</td>
<td>28 exons deleted</td>
</tr>
<tr>
<td>HCC5-1318(3)</td>
<td>Xenograft</td>
<td>p.S211Y</td>
<td>19</td>
<td>2 upstream exon deleted (MLPA)</td>
</tr>
<tr>
<td>HCC05-0411B</td>
<td>Xenograft</td>
<td>Deletion</td>
<td>-</td>
<td>Exon 16-23 duplication (MLPA)</td>
</tr>
<tr>
<td>B253</td>
<td>Tumor</td>
<td>Splicing</td>
<td>54%</td>
<td>c.1443+2/+13del_p.splice</td>
</tr>
<tr>
<td>B266</td>
<td>Tumor</td>
<td>Deletion</td>
<td>-</td>
<td>Exons 41 of 41 deleted</td>
</tr>
<tr>
<td>B277</td>
<td>Tumor</td>
<td>p.A1141T</td>
<td>92%</td>
<td>Reported in TSC patients</td>
</tr>
</tbody>
</table>

TSC2 mutations and deletions in HCC cell lines from South Korea were identified by targeted sequencing of about 2000 genes and SNP6.0 array copy number analysis, respectively. TSC2 mutations and deletions in most Singapore HCC xenografts and in all Hong Kong primary HCC tumors were identified by targeted DNA sequencing at Foundation Medicine, Inc. TSC2 deletions in HCC5-1318(3) and HCC05-0411B were identified by Multiplex Ligation-dependent Probe Amplification (MLPA, noted in the table). Mutant vs. wild-type allele frequencies are provided for TSC2 mutations.
Table 2. Patient profile, treatment arm and survival outcome of 15 EVOLVE-1 trial HCC patients with TSC2-null/low tumors by IHC.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>TSC2 IHC H-score</th>
<th>Randomized treatment</th>
<th>Overall Survival (months)</th>
<th>Race</th>
<th>Gender</th>
<th>HBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>Placebo</td>
<td>1.25</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>Placebo</td>
<td>1.35</td>
<td>Caucasian</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>Placebo</td>
<td>3.61</td>
<td>Other</td>
<td>M</td>
<td>N.A.</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>Placebo</td>
<td>5.59</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>Placebo</td>
<td>18.23+</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>Everolimus</td>
<td>0.76</td>
<td>Other</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>Everolimus</td>
<td>2.27</td>
<td>Other</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>Everolimus</td>
<td>4.63</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>110</td>
<td>Everolimus</td>
<td>5.85</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>Everolimus</td>
<td>9.53</td>
<td>Caucasian</td>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>Everolimus</td>
<td>9.56</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>Everolimus</td>
<td>18.96</td>
<td>Caucasian</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>40</td>
<td>Everolimus</td>
<td>25.00+</td>
<td>Asian</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>Everolimus</td>
<td>28.02</td>
<td>Asian</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>Everolimus</td>
<td>32.72+</td>
<td>Caucasian</td>
<td>M</td>
<td>-</td>
</tr>
</tbody>
</table>

TSC2 status in the tumors was determined by TSC2 IHC performed at PhenoPath Laboratories. Note that patient 6, 7 and 8 were only treated with everolimus for 2-6 weeks due to various reasons described in the paper.
Figure Legends

**Fig. 1. TSC2 loss in hepatocellular carcinoma cell lines confers everolimus sensitivity.** (A) Reverse Phase Protein Array (RPPA) analysis of TSC2 levels in 275 cancer cell lines of various lineages. 5 out of 9 HCC cell lines (name displayed) showed significantly reduced TSC2 protein levels. (B) Immunoblot of eight HCC cell lines with indicated antibodies. TSC2 was undetectable in four cell lines that expressed low TSC2 protein levels as measured by RPPA. p-S6K1(T389) and p-4E-BP1(T37/T46) levels tend to be elevated in these TSC2-null cell lines and HuH-7 cells. (C) mTORC1 inhibitor everolimus IC$_{50}$ (in µM) and percentage of maximal inhibition by 1µM everolimus (Amax) in HCC cell lines as determined in a 3 Day CellTiter Glo (CTG) assay. Everolimus was significantly more potent and tended to be more efficacious in TSC2-null cell lines (red color) than in TSC2 wild-type cell lines (blue color), with the exception of HuH-7. P values were calculated by a two-tailed Student’s $t$ test. Note that both axes do not originate at zero for better visualization of the data. The IC$_{50}$ and Amax values are provided in Supplementary Table 2.

**Fig. 2. TSC2 loss in patient-derived HCC xenografts predicts everolimus efficacy.** (A) Levels of TSC2, p-S6K1(T389), p-4E-BP1(T70) and p-AKT(S473) in patient-derived HCC xenografts. Indicated HCC tumors were subcutaneously implanted in SCID mice. Tumors were collected when they reached 800-1000 mm$^3$ and processed for immunoblotting. Note that HCC05-0411B, HCC19-1010, HCC14-0910 and HCC24-0309 xenografts had undetectable levels of TSC2 protein and increased phosphorylation of mTOR substrate S6K1. (B) Effects of 1mg/kg everolimus on growth rates of HCC
xenografts. SCID mice bearing the indicated tumors were treated with vehicle or 1 mg/kg everolimus. Each treatment arm involved 8 to 10 independent tumor-bearing mice representing the same xenograft line. Mean tumor volume is shown and error bars represent S.E.M. The experiments were repeated at least twice with similar results. P values were calculated by paired Student t tests for the vehicle group versus the everolimus treatment group. The * denotes P<0.01 and P values of the last measurement are shown. Note that tumor stasis or regression was only observed in TSC2-null xenografts. (C) Bar graph showing endpoint HCC tumor weight T/C (treated versus control) at 1mg/kg everolimus dosing. SCID mice bearing indicated tumors were treated with vehicle or 1 mg/kg everolimus daily for 14-18 days. Each treatment arm involved 8 to 10 independent tumor-bearing mice representing the same xenograft line. Efficacy of everolimus was evaluated by comparing the final mean weight of tumors in the treated arms (T) to those in the control arm (C) (T/C ratio). Note that the 8 best responders to everolimus were TSC2-null xenografts and this enrichment was statistically significant at p < 0.01 (Fisher exact test value=8E-06). The * denotes models with PTEN loss as determined by immunoblot in A.

**Fig. 3. Tumor growth inhibition by everolimus was through inhibition of mTOR signaling.** (A) Dose-dependent anti-tumor effects of everolimus in TSC2-null xenografts. Mice bearing TSC2-null xenograft HCC14-0910 were randomized into 4 groups (8 mice per group) for daily dosing of 0.25, 0.5, 0.75 or 1mg/kg everolimus or vehicle. The experimental procedure and data are displayed similarly as in Fig.2 B. P values were calculated by paired Student t tests for the vehicle group versus the everolimus treatment
group at 0.25 and 0.50 mg/kg everolimus doses. The * denotes P<0.01. (B) Dose-dependent effects of everolimus in TSC2-null xenograft HCC14-0910 on decreasing the phosphorylation of mTOR substrates (S6K1 and 4E-BP1) and inducing cell cycle arrest (CDK2 phosphorylation and p27 induction). 2h after the last dose in (A), mice were sacrificed and lysates from vehicle- and everolimus-treated tumors were subjected to immunoblots with the indicated antibodies. Representative blots of two tumors for each dose are shown and the experiment was repeated at least twice with similar results.

**Fig. 4. TSC2 loss is more common in Asian HBV+ HCC than in Caucasian HCC.**

(A) TSC2 IHC H-score range distribution in 139 HCC tumors from the EVOLVE-1 phase 3 clinical trial. H-scores (0-300) were divided into three groups: 0-60 (likely to be TSC2-null), 61-120 (TSC2 low) and 121-300 (TSC2 wild-type). The percentage of each range is marked at the top of the bars. The H-score distribution of each ethnic group in the 139 samples is also displayed. (B) TSC2 IHC H-score range distribution in 239 Asian HCC cases with evaluable IHC staining using duplicate-core TMA. The percentage of each range is marked at the top of the bars. Over 90% of these tumors are HBV⁺.
Figure 1
Figure 2

(A) Western blots showing TSC2 null and TSC2 wild-type models with antibodies against phospho-S6K1(T389), S6K1, phospho-4E-BP1(T70), 4E-BP1, phospho-AKT(S473), AKT, and PTEN. Tubulin was used as a loading control.

(B) Tumor volume over time for TSC2 wild-type (HCC06-0606, HCC19-1010, HCC26-0809, HCC14-0411B, HCC14-0910) and TSC2 null (HCC25-0809, HCC13-0109, HCC13-0212, HCC14-0910) models treated with vehicle or 1 mpk EVE. PTEN null models showed a significant difference in tumor volume compared to TSC2 wild-type models, with P<0.01.

(C) RAD001 endpoint T/C ratio for all models, showing a significant difference between TSC2 wild-type and TSC2 null models, with P<0.01. Total models=20.
Figure 3

A) Days of treatment

B) Everolimus (mg/kg): p-S6K1 (T389), S6K1, p-4E-BP1 (T70), 4E-BP1, p-AKT (S473), AKT, p-CDK2 (T14/15), p27
Figure 4

(A) Percentage of H-score range in different samples:

- **All samples (n=139)**:
  - 0-60: 5.8%, 6.5%
  - 61-120: 1.3%, 5.1%
  - 121-300: 10.4%, 8.3%
  - 121-300: 15.4%, 7.7%

- **Caucasian (n=78)**:
  - 0-60: 87.7%
  - 61-120: 93.6%
  - 121-300: 81.3%

- **Asian (n=48)**:
  - 0-60: 93.6%
  - 61-120: 81.3%
  - 121-300: 8.3%

- **Other (n=13)**:
  - 0-60: 76.9%
  - 61-120: 5.8%

(B) Percentage in Asian HCC (n=239):

- 0-60: 20.1%
- 61-120: 19.7%
- 121-300: 60.2%
Molecular Cancer Therapeutics

Loss of Tuberous Sclerosis Complex 2 (TSC2) is Frequent in Hepatocellular Carcinoma and Predicts Response to mTORC1 Inhibitor Everolimus

Hung Huynh, Huai-Xiang Hao, Stephen L. Chan, et al.

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