Discussion

Epithelial cells specifically develop highly elaborate arrays of non-centrosomal MTs, which are considered to play important roles in the development and maintenance of epithelial polarity (Bacallao et al., 1989; Grindstaff et al., 1998; Müsch, 2004). However, the mechanisms by which these higher-order assemblies of non-centrosomal MTs are constructed remain largely unknown (Bartolini and Gundersen, 2006; Bellett et al., 2009). In the present study, we identified a novel MT-binding protein, MTCL1, which plays essential roles in crosslinking non-centrosomal MTs in the apicobasal MT bundles running along the apicobasal axis of epithelial cells.

Many MAPs, including +TIPs, induce MT bundle formation when overexpressed in cultured cells. Therefore, caution is required when defining bona fide MT-crosslinking proteins (Bartolini and Gundersen, 2006; Chapin et al., 1991; MacRae, 1992). In such situations, several nonmotor MAPs, such as NuMA, PRC1 and TPX2, have been established as genuine MT-crosslinking proteins on the basis of their cellular localizations and loss-of-function phenotypes (Manning and Compton, 2008; Mollinari et al., 2002; Radulescu and Cleveland, 2010; Subramanian et al., 2010). The present results have established that MTCL1 is a new MT-crosslinking protein. First, immunofluorescence microscopy of endogenous MTCL1 revealed its intermittent association with the lateral sides of MT filaments and preferential accumulation to MT bundles in various cellular contexts. Second, siRNA-mediated depletion of MTCL1 impaired the development of the apicobasal MT bundles in polarized epithelial cells. Third, MTCL1 had a direct MT-binding region in its N-terminal region that exhibited intermittent colocalization with MTs. Significantly, this MT-binding region did not induce MT bundling unless it was ligated to the subsequent coiled-coil motifs, not only when it was overexpressed in cells but also *in vitro*. Because the MTCL1 coiled-coil region is likely to form two-stranded structures, and did indeed show oligomerization activity, these results indicate that, like NuMA, MTCL1 forms dimers that hold the two N-terminal MT-binding domains together, and thereby crosslinks MTs (Fig. 8F, top) (Radulescu and Cleveland, 2010). The proline-rich region between the N-terminal MT-binding region and the subsequent coiled-coil motifs (Fig. 3A) might function as a hinge that enables the N-terminal region to move flexibly. Here, we also identified another MT-binding region in the C-terminus of MTCL1. At the moment, it is not clear how this MT-binding region contributes to the MT-crosslinking activity of MTCL1.

Our rescue experiments demonstrate that the N-terminal MT-binding region is indispensable for the development of the apicobasal MT bundles in epithelial cells. Furthermore, the MT regrowth assays suggested that MTCL1 is involved in the accumulation of non-centrosomal MTs at the lateral cortex. Previous studies have revealed the molecular mechanisms involved in the initial step for the development of this epithelial-cell-specific non-centrosomal MT array, including nucleation, release, transport or guidance, and capture of either end of MTs (Bellett et al., 2009; Hotta et al., 2010; Jaulin and Kreitzer, 2010; Mogensen et al., 2000; Mogensen et al., 2002; Oriolo et al., 2007). In this context, our results supplement these previous studies by providing the molecular basis of the late steps regulating the higher-order array of the apicobasal MT bundles. During the development of epithelial polarity, MTCL1 gradually accumulates at the sublateral cortex (Fig. 2D) in a manner dependent on its MT-binding activity through the N-terminal region (Fig. 5C). However, once MTCL1 achieves this sublateral localization, it is maintained there even in the absence of MTs (Fig. 7A,B). This might indicate the presence of unknown structures in polarized epithelial cells that anchor MTCL1 to maintain its sublateral localization. Recently, filamentous GTPases (septins) were shown to guide the directionality of MT plus-end movement and support MT remodeling in polarizing epithelial cells (Bowen et al., 2011). It will be interesting to examine whether the septin scaffold provides the structural anchoring sites for MTCL1 at the sublateral cortex of polarized epithelial cells.

In the present study, we have demonstrated that MTCL1 is a novel binding partner of PAR-1b. MTCL1 knockdown cells mimic several defects of PAR-1b knockdown cells, and our rescue experiments demonstrated that the interaction with PAR-1 is essential for the complete activity of MTCL1 to facilitate epithelial polarization. Although we did not show the data here, PAR-1b specifically phosphorylates a certain serine residue of MTCL1 in the vicinity of its binding site. However, as far as we examined, the phosphorylation did not directly regulate the MT-regulatory activity of MTCL1. As we demonstrated here, even a deletion of the PAR-1b-binding region did not impair the MT-bundling activity, dimerization activity and subcellular localization of MTCL1. Although we cannot completely exclude the possible involvement of another site of phosphorylation by PAR-1, these results reduce the possibility that PAR-1b regulates MTCL1 activity. Instead, the present results favor the notion that MTCL1 assists the recruitment of the PAR-1b MT-destabilizing kinase to coordinate the assembly and dynamics of MTs in the apicobasal MT bundles (Fig. 8F, bottom). This idea is consistent with our observation that the MT stability in the apicobasal MT bundles changed dynamically during the reconstruction process of the structures (Fig. 7A). It is also supported by a recent study demonstrating that the coordination between stable and dynamic MTs is important for normal elongation of MT bundles (Bitan et al., 2012). Interestingly, a similar scaffold function for PAR-1 was suggested for septins that interact with PAR-1-related kinases in yeast (Spiliotis, 2010). Although future studies are required, the hypothesis that MT-bundling factors recruit PAR-1 to regulate MT dynamicity provides an important conceptual framework to clarify the molecular basis of non-centrosomal MT organization in various polarized cells. It should also be clarified in future studies why PAR-1b, as well as MTCL1 knockdown, commonly induces a decrease in cell height and increase in cell area. Although MT disruption in polarized MDCK cells did not significantly affect the cell height (Fig. 7), our present results demonstrate that the morphological defects in MTCL1 knockdown cells were dependent on the N-terminal MT-binding region of MTCL1. Therefore, correct MT organization could be tightly coupled with normal development of the epithelia-specific morphology specifically during the cell polarization processes.

Recently, public databases have been adopting the name 'SOGA2' for MTCL1, which is probably because a recent paper that named its paralog molecule suppressor of glucose by autophagy (SOGA) (Cowerd et al., 2010). The paper argued that the 'MTCL1 paralog' was secreted as 80 kDa and 25 kDa peptides after excision of an 'internal peptide sequence' and mediated adiponectin-dependent suppression of glucose production by inhibiting autophagy. However, the feature of this

putative internal peptide sequence in the MTCL1 paralog is not at all conserved in MTCL1 and, as we demonstrated here, full-length MTCL1 is expressed intracellularly at the expected molecular mass and interacts with MTs. Furthermore, as far as we examined, the full-length cDNA encoding mouse SOGA (GenBank number NM_001164663) is also expressed intracellularly as the expected full-length protein with a molecular mass of 180 kDa, and shows a similar MT-bundling activity to MTCL1 through the conserved KR-rich region in the C-terminus (A.S., unpublished data). Therefore, we strongly claim that the name SOGA2 should not be adopted for the protein we identified in this study, and that it should be called MTCL1 based on the results presented here.

Materials and Methods

Molecular biology

The human PAR-1b cDNA (NM_017490) was reported previously (Suzuki et al., 2004). The cDNA of human KIAA0802 (AB018345) was obtained from Kazusa DNA Research Institute (Chiba, Japan), and that of full-length mouse MTCL1 (AK147205) was purchased from Danaform (Kanagawa, Japan). To purify SBP-tagged N-terminal fragments of MTCL1 (N1 and N4) from human HEK293T cells, the nucleotides of the mouse cDNA were codon-optimized for human cells (Genescript, Piscataway, NJ). The cDNAs were subcloned into appropriate expression vectors: pEGFP (Takara Bio Inc., Shiga, Japan); pGEX (GE Healthcare Japan, Tokyo, Japan); pSRD-derivative vectors (pSRHA or pSRHis) (Masuda-Hirata et al., 2009); pCAGGS-V5 and pCAGGS-Flag-SBP (Yamashita et al., 2010). In some cases, the cDNAs were subcloned in pEB6CAG (Tanaka et al., 1999), pEB-Hyg (Wako Pure Chemical Industries Ltd, Osaka, Japan) or pOS-Tet14-SBP (Cong et al., 2010), all of which are Epstein–Barr virus (EBV)-based extrachromosomal vectors carrying a replication origin (oriP) and replication initiation factor (EBNA-1) sufficient for autonomous replication in human and canine cells. Site-directed mutations were introduced using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), followed by sequencing verification.

Antibodies

The anti-PAR-1b polyclonal antibody (pAb) was reported previously (Suzuki et al., 2004). The anti-MTCL1 pAb was raised by immunizing rabbits with the C-terminal fragment of KIAA0802 (C7: 1322–1420) fused with GST, followed by affinity-purification. Other antibodies were purchased from commercial sources as follows: anti-KIAA0802 (W19), anti-tyrosinated tubulin rat monoclonal antibody (mAb) (YL1/2), anti-β-catenin pAb (H102), anti-ZO-1 rat mAb, anti-GFP mAb (B2), anti-SBP mAb (SB19-C4) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-α-tubulin mAb (DM1A), anti-acetylated tubulin mAb (Sigma-Aldrich, St. Louis, MO); anti-V5 mAb, anti-RFP pAb, anti-ZO-1 mouse mAb (Life Technologies Corporation, Carlsbad, CA); anti-E-cadherin mAb (BD Biosciences, San Jose, CA); anti-V5 pAb (Bethyl Laboratories, Montgomery, TX); anti-GFP pAb (MBL, Aichi, Japan); anti-Na^+/K^+ ATPase mAb (Millipore, Billerica, MA); anti-HA rat mAb (3F10) (Roche Applied Science, Indianapolis, IN).

Cell culture, transfection, and establishment of pEBV stable transformants

HeLa-K cells were a generous gift from Dr S. Tsukita (Osaka University, Osaka, Japan). MDCK II, HeLa-K and HEK293T cells were cultured in Dulbecco's modified Eagle medium (Life Technologies Corporation) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 μg/ml) and 1 mM glutamine. Plasmid transfections were performed using the Lipofectamine 2000 reagent (Life Technologies Corporation) or an electroporation system, NEPA21 (Nepa Gene Co. Ltd, Chiba, Japan) for MDCK II cells and Attracten (Qiagen, Valencia, CA) for HeLa-K cells, according to each manufacturer's instructions. Heterogeneous stable transformants of MDCK cells were established using pEB-based vectors as previously described (Masuda-Hirata et al., 2009).

RNAi experiments

The RNAi target sequences for canine MTCL1 were designed based on the cDNA sequence (Genbank number XM_547667) as follows: 5′-GGCACGGCUACAGAUCAAU-3′ (#1); 5′-CCUGUGACGAGUGCAAGUU-3′ (#2); 5′-GUUCAGCACGGGAAAACCU-3′ (#3). To establish MTCL1-depleted cells, MDCK cells were transfected with pEB6-Super-gfp encoding an shRNA sequence for canine MTCL1 (#1 or #2), and selected with neomycin (Masuda-Hirata et al., 2009). In some cases, we used pEB6-super-AcGFP1-Nuc, which was newly generated by replacing EGFP in pEB6-Super-gfp with AcGFP-Nuc in pAcGFP1-Nuc (Takara Bio Inc.). For rescue experiments, cells were co-transfected with pEB-Super-gfp expressing an shRNA for canine MTCL1 (#1) and pEB-Hyg expressing various mutants of human MTCL1, and subjected to selection using 800 μg/ml geneticin (Life Technologies Corporation) and 200 μg/ml hygromycin (Wako Pure Chemical Industries Ltd). As a nonsilencing RNAi oligonucleotide, Allstars negative control siRNA (Qiagen) was used. The nonsilencing control sequence used for pEB-super-gfp was described previously (Suzuki et al., 2004).

Immunoprecipitation

Cells were solubilized in lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT) containing a cocktail of protease and phosphatase inhibitors (Roche Applied Science) for 30 minutes at 4℃, briefly sonicated, and centrifuged at 15,000 ***g*** for 30 minutes. The resulting supernatants were mixed with protein-A– or protein-G–Sepharose (GE Healthcare) conjugated with appropriate antibodies. To purify SBP-tagged proteins, streptavidin-conjugated Sepharose (GE Healthcare) was used, and the absorbed proteins were eluted with an appropriate buffer containing 10 mM biotin.

MT-binding assays

For pull-down assays, purified proteins dissolved in BRB buffer (80 mM PIPES-KOH pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) were incubated with taxol-stabilized MTs (final concentration: 0.5 mg/ml) at 37℃ for 30 minutes, and subjected to centrifugation (50,000 ***g***) at 22℃ for 30 minutes on a cushion of 40% glycerol in BRB buffer. When MDCK cell extracts were subjected to the assays, cells (1×10^6) were transfected with 60 pmol MTCL1-targeting siRNA (#1) using NEPA21, and homogenized in BRB buffer containing 1 mM DTT and 100 mM KCl at 4℃ before mixing with taxol-stabilized MTs as described above. For MT-bundling assays, SBP-tagged proteins were purified from HEK293T cells, mixed with taxol-stabilized Rhodamine-labeled MTs at a molar ratio of 4:1 at 37℃ for 30 minutes, and gently spotted onto glass slides for immediate examination under fluorescence microscopy.

Immunofluorescence microscopy

Unless otherwise noted, MDCK cells were seeded onto #3460 Transwell filters at 2.5×10^5 cells/cm^2 and cultured for 3–4 days to polarize the cells sufficiently. HeLa-K cells were seeded at 3×10^4 cells/cm^2 immediately before transfection, and subjected to fixation on the next day. The cells were fixed with cold methanol for 10 minutes at −20℃ (for MTs and MTCL1) or 2% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. To stain the detergent-resistant subpopulation of PAR-1b, cells were pretreated with PGEM buffer (100 mM PIPES-KOH pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 15% glycerol) containing 0.5% Triton X-100 for 1 minute before fixation. To observe the MT regrowth process, MDCK cells were treated with 33 μM nocodazole for 2 hours on ice followed by 1 hour at 37℃, and washed with cold medium three times before addition of warm medium and incubation at 37℃. At appropriate times after nocodazole removal, cells were subjected to pretreatment with PGEM buffer containing 0.5% Triton X-100 for 30 seconds followed by methanol fixation. The fixed cells were subjected to standard immunostaining procedures as described previously (Suzuki et al., 2004), using appropriate secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555 or Alexa Fluor 647 (Life Technologies Corporation). For visualization of F-actin, cells were labeled with Rhodamine–phalloidin (Life Technologies Corporation). Most of the samples were examined at room temperature using an AxioImager Z1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CSU10 disc confocal system (Yokogawa Electric Corporation, Tokyo, Japan), an Orca II CCD camera (Hamamatsu Photonics, Shizuoka, Japan), and a 63× 1.4 NA Plan Apochromat or 100× 1.46 NA objective. Images were acquired using MetaMorph software (Molecular Devices, Sunnyvale, CA) and processed with ImageJ software to obtain appropriate brightness and contrast. To obtain images with higher resolutions, another confocal microscopy system (LSM710; Carl Zeiss) with a 100× 1.46 NA objective was used.

Author contributions

A.S. supervised and performed experiments, analyzed data and wrote the paper, Y.S., M.A. and Y.A. performed main parts of experiments. K.Y., M.I., K.S., A.Y. performed some experiments, H.H. and N.A. performed mass spectrometric analyses, T.M. and I.H. helped MT pull down assays, S.O. supported the experiments and provided helpful suggestions.

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Alternative mTOR Signal Activation in Sorafenib-resistant Hepatocellular Carcinoma Cells Revealed by Array-based Pathway Profiling

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Running title: mTOR signal activation in sorafenib-resistant HCC (49 characters and spaces)

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Abbreviations: **AFP**, α-fetoprotein; **Chr**, chromosome; **EGF**, epidermal growth factor; **ERK**, extracellular signal-regulated kinase; **HCC**, hepatocellular carcinoma; **IC50**, half maximal (50%) inhibitory concentration; **p-RPS6 S235/236**, ribosomal protein S6 phosphorylated at the serine 235/236 residue; **MAPK**, mitogen-activated protein kinases; **NCBI**, National Center for Biotechnology Information; **RPPA**, reverse-phase protein array; **mTOR**, mammalian target of rapamycin; **RSK**, 90-kDa ribosomal protein s6 kinase; **S6K**, 70-kDa ribosomal protein s6 kinase; **SHARP**, Sorafenib HCC Assessment Randomized Protocol.

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Summary

Sorafenib is a multi-kinase inhibitor that has been proven effective for the treatment of unresectable hepatocellular carcinoma (HCC). However, its precise mechanisms of action and resistance have not been well established. We have developed high-density fluorescence reverse-phase protein arrays, and used them to determine the status of 180 phosphorylation sites of signaling molecules in the 120 pathways registered in the NCI-Nature curated database in 23 HCC cell lines. Among the 180 signaling nodes, we found that the level of ribosomal protein S6 phosphorylated at serine residue 235/236 (p-RPS6 S235/236) was most significantly correlated with the resistance of HCC cells to sorafenib. The high expression of p-RPS6 S235/236 was confirmed immunohistochemically in biopsy samples obtained from HCC patients who responded poorly to sorafenib. Sorafenib-resistant HCC cells showed constitutive activation of the mammalian target of rapamycin (mTOR) pathway, but whole-exon sequencing of kinase genes revealed no evident alteration in the pathway. p-RPS6 S235/236 is a potential biomarker that predicts unresponsiveness of HCC to sorafenib. The use of mTOR inhibitors may be considered for the treatment of such tumors.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide (1). Advanced HCC is often unmanageable by local treatments (surgical resection, ethanol injection, radiofrequency ablation, chemoembolization), but no systemic chemotherapy with conventional cytotoxic agents had been shown to be effective until a landmark phase III clinical trial (Sorafenib HCC Assessment Randomized Protocol; SHARP) revealed significant survival prolongation in patients treated with sorafenib (BAY 43-9006; Nexavar) (2). Furthermore, it has been reported that some patients showed remarkable tumor shrinkage after short-term administration of sorafenib (3). Based on these results, sorefenib monotherapy has been employed as the current standard first-line treatment for unresectable HCC. However, not all HCC patients show the desired therapeutic benefits of sorefenib. The overall survival prolongation of unselected patients in the SHARP trial was limited to 2.8 months (2), and an objective tumor response was observed only in a small (0.6-2%) proportion of patients (2, 4). Given the relatively high cost and occasional severe adverse events (diarrhea, hand-foot skin reaction, hypertension, and others) (2, 4), there is an urgent need to identify a predictive biomarker that could exclude advanced HCC patients who are unlikely to benefit from sorafenib therapy.

Sorafenib is a multi-kinase inhibitor that blocks tumor cell proliferation and angiogenesis

through inhibition of c-RAF and b-RAF as well as many receptor tyrosine kinases, including vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR3, platelet-derived growth factor receptor-α (PDGFR-α), Fms-related tyrosine kinase 3 (FLT3), RET and c-KIT (5). In view of this broad inhibitory spectrum, the precise mechanisms underlying the anti-tumor activity remain elusive. To date, factors that have been identified to be correlated with the efficacy of sorafenib include phosphorylated extracellular signal-regulated kinase 1 (p-ERK) (6), serum des-γ-carboxyprothrombin (DCP) level (7), phosphorylated c-Jun protein (8), and fibroblast growth factor-3/-4 (FGF3/4) gene amplification (3), but their clinical utility as predictive biomarkers has not been established.

In the present study, we developed a new technique: high-density florescence reverse-phase protein array (RPPA), and used it to search for a biomarker that would identify patients in whom sorafenib would be effective, employing a large library of phosphorylation site-specific antibodies. RPPA represents an emerging technology for proteomics, and is well suited for profiling of phosphorylated proteins. It involves micro-format dot immunoblotting of lysates from tissues or cells (9), allowing simultaneous monitoring of the expression of a particular phosphoprotein in hundreds to thousands of samples under identical conditions in a highly quantitative manner (10). In this study we profiled the activation status of 180 key signaling nodes across a panel of 23 HCC cell lines and identified *de novo* activation of mTOR

signaling in sorafenib-resistant HCC cells.

Experimental Procedures

Cell Lines and Antibodies.

Cell lines used for generating the cancer cell line RPPA are listed in Supplementary Table S1 and were maintained according to their suppliers' recommendations. Recombinant EGF was obtained from R&D Systems (Minneapolis, MN). A total of 180 phosphorylation site-specific antibodies and their dilutions used for RPPA analysis are listed in Supplementary Table S2. The specificity of each antibody was verified by immunoblotting or had been previously described by other investigators.

RPPA.

Cells were collected by scraping and stored at -80°C until use. Cell lysates were prepared with RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with phosphastase (Thermo Scientific) and protease (Sigma, St. Louis, MO) inhibitor cocktails. Protein concentrations of lysates were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). The lysates were serially diluted two-fold four times and printed in quadruplicate onto ProteoChip glass slides (Proteogen, Seoul, South Korea) using a robotic spotter (Genex Arrayer; Kaken Geneqs Inc., Chiba, Japan).

The RPPA slides were incubated overnight with primary antibodies. Following tyramide signal amplification (Dako Cytomation, Glostrup, Denmark), streptavidin Alexa Fluor 647 conjugate (Life Technologies, Carlsbad, CA) was applied to the slides (11). Fluorescence images were captured by an InnoScan 700 microarray scanner (Innopsys, Carbonne, France) and

quantified using Mapix software (Innopsys). After background subtraction, values relative to γ-tubulin were subjected to quantile normalization (12) to ensure uniform distribution of values for each slide in a set of slides. Unsupervised hierarchical clustering, using the Euclidean metric and Ward's method, was conducted with R 2.13.0 (http://www.r-project.org/index.html). The signaling components of the mTOR and MAPK pathways were selected based on KEGG pathway maps (http://www.genome.jp/kegg/kegg3a.html) and used for clustering analyses.

Immunoblot Analysis.

Immunoblot analyses were performed using the NuPAGE Bis-Tris or Tris-Acetate electrophoresis system (Life Technologies, Carlsbad, CA) as described previously (13). All antibodies except for an anti-p-RSK (S380) antibody (R&D Systems) were obtained from Cell Signaling Technology (CST; Danvers, MA). Signals were detected with the ImageQuant LAS 4010 system (GE Healthcare, Giles, UK) and quantified using the ImageQuant TL software package (GE Healthcare).

Growth Inhibition Assay.

Sorafenib, RAD001 (everolimus), and SL0101 were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). CI-1040 and AZD8055 were from Selleck Chemicals (Houston, TX). Stock solutions of the chemicals were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until use. Cells were seeded into 96-well cell culture plates in triplicate at a density of 3,000 cells per well. On the following day, serially diluted drugs were added, and 72 hours later cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI). Relative cell viability was calculated as a percentage of a control treated with 0.1% DMSO after background subtraction. All experiments were repeated at least three times. The data were modeled using a four-parameter log-logistic nonlinear regression

curve fit with a sigmoid dose response. These curves were drawn using R 2.13.0 (http://www.r-project.org/index.html), and IC_{50} values were calculated accordingly.

Immunohistochemistry.

Formalin-fixed, paraffin-embedded sections of needle biopsy samples obtained from nine HCC patients before administration of sorafenib at Wan Fang Hospital and the Taipei Medical University Hospital were immunostained with anti-p-RPS6 Ser235/236 (#2211, CST) or anti-RPS6 (#2217, CST) antibody, as described previously (13). The stained slides were evaluated by pathologists and classified according to the percentage of positively stained cells (0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100%) and the intensity of staining (0, absent; 1, weak; 2, moderate; and 3, strong). A specimen was defined as being positive when either the percentage of positively stained cells or the intensity of staining was 3 or higher. The use of clinical materials was approved by the respective institutional review boards.

Kinome Sequencing.

Genomic DNA was extracted from 20 HCC cell lines using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's protocol. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Three micrograms of genomic DNA was used to construct libraries for sequencing. The quality of the constructed libraries was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All the exon and 5'- and 3'-flanking sequences (200 bp) of 511 kinase genes were captured using a customized SureSelect Target Enrichment System (Agilent Technologies) according to the Illumina Paired-End Sequencing Platform Library Prep Protocol Version 1.0 (Agilent Technologies). Captured DNA fragments (~300 bp) were sequenced using a Genome

analyzer IIx sequencer (Illumina, San Diego, CA). Base calling was performed using the Illumina Pipeline (v1.4) with default parameters. Only paired end (2×75 bases) sequence reads that passed the quality control were mapped to the human reference genome build hg19 (UCSC hg19) using BWA (http://bowtie-bio.sourceforge.net/)(14) with default parameters. Sequencing artifacts were eliminated using Picard MarkDuplicates (http://picard.sourceforge.net/). Variants were called with SAMtools (http://samtools.sourceforge.net/) (15) and annotated using Annovar ENREF_25 (16). The final set of novel variant calls was identified using the following thresholds: SNP quality ≥228, coverage ≥20 reads, frequency ≥10%, and not deposited in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) (version 135).

Evaluation of synergistic drug combinations.

The synergistic interaction of drug combinations was evaluated using the Chou-Talalay median-dose effect method (17) with CompuSyn software (http://www.combosyn.com/). AZD8055 and CI-1040 were mixed at the ratio of their IC_{50} values (1:300). The mixed solution was 2-fold serially diluted five times and added to the cells. Combination Index (CI) values were calculated at the points causing 50%, 75% and 90% reduction of cell viability. CI values equal to 1, >1 and <1 indicate additive, antagonistic and synergistic interactions, respectively.

Results

Generation of the High-density RPPA and Phosphoprotein Profiling.

We constructed a RPPA onto which lysates of 95 cell lines derived from 8 different types of cancer (listed in Supplementary Table S1) cultured in the presence and absence of 10% fetal calf

serum (FCS) for 17 hours and A431 cells untreated or treated with 200 ng/ml epidermal growth factor (EGF) for 10 minutes were randomly plotted. Each lysate was serially diluted (1:1, 1:2, 1:4, and 1:8) and spotted in quadruplicate (16 spots per lysate). This level of high-density spotting (3072 samples per array slide) was achievable because of the highly hydrophobic surface of the array slides, preventing diffusion of the protein samples.

By applying 180 phosphorylation site-specific antibodies (listed in Supplementary Table S2), we determined the activation status of signaling proteins (Fig. 1A). A lysate of A431 cells treated with EGF was included as a positive internal control. A431 cells carry amplification of the *EGFR* (EGF receptor) gene. We confirmed that a >6-fold increase in the signal intensity of ERK1/2 proteins phosphorylated at the threonine 202/tyrosine 204 residue (p-ERK1/2 T202/Y204) was detectable after treatment with EGF (Fig. 1B).

To further verify the data obtained by RPPA, lysates of representative cell lines were electrophoresed and blotted with the same antibodies. In the RPPA analysis, EBC1, SNU-398, and Alexander cells showed a high signal intensity for anti-p-RPS6 S235/236 antibody exceeding the average plus 2SD for 96 cell lines, whereas TOV112D, KOSC-2, and HSC60 cells showed a signal intensity below the average (Fig. 1C, right). The results we obtained by immunoblotting were consistent (Fig. 1C, left).

The glass slides that we used for construction of the RPPA were free of any

auto-fluorescence noise. The use of fluorescent dyes and original signal enhancement significantly increased the sensitivity of signal detection. In fact, MET protein with a high level of phosphorylation (p-MET T1234/1235) in Okajima cells was detectable by immunoblotting, whereas the MET protein with a relatively low level of phosphorylation in SW948 and OVKATE cells was undetectable (Fig. 1D).

p-RPS6 S235/236 Correlates with the Sensitivity of HCC Cells to Sorafenib.

The cancer cell protein array contained 23 HCC cell lines, which exhibited a wide variety of sensitivity to sorafenib (Fig. 2A, upper). SNU-449 was the most sensitive, with a half maximal (50%) inhibitory concentration (IC50) of 0.172 μM. It was approximately 70-fold more sensitive than the least sensitive cell line, SNU-387 (IC50, 12.68 μM). We then compared the IC50 value of each HCC cell line with the phosphorylation level of 180 signaling nodes. Spearman's correlation coefficient analysis (Supplementary Table 3) revealed that p-RPS6 S235/236 had the highest positive correlation ($r = 0.58$, $P = 0.0044$), followed by p-RPS6 at the serine 240/244 residues (p-RPS6 S240/244) ($r = 0.55$, $P = 0.0070$). 90-kDa ribosomal S6 kinase 2 (RSK2) protein phosphorylated at the serine 227 residue showed the third most significant correlation. RSK2 is one of enzymes that phosphorylate RPS6 (18).

Consistent with the RPPA data, intense signals for p-RPS6 S235/236 were detected in the

sorafenib-resistant cell lines by immunoblotting (Fig. 2A, lower). The quantified immunoblot data correlated well with those of RPPA ($r^2 = 0.733$), thus confirming the precision of RPPA (Fig. 2B). p-RPS6 S235/236 and p-RPS6 S240/244 exhibited different phosphorylation patterns among several cell lines (e.g., HLF, KIM1, JHH-1, and JHH-2) (Fig. 2A, lower), suggesting that phosphorylation of S235/236 and S240/244 residues may be mediated by distinct regulatory processes.

p-RPS6 S235/236 is a Potential Predictor of Response to Sorafenib.

We next evaluated whether high levels of p-RPS6 S235/236 were indicative of HCC resistance to sorafenib in clinical samples (Supplementary Table S4). Expression of p-RPS6 S235/236 was examined in biopsy specimens collected from nine HCC patients prior to sorafenib treatment (400 mg bid). Eight patients showed intense staining for p-RPS6 (Fig. 2C). Four patients (Cases 4, 6, 7, and 9) with p-RPS6-positive tumors discontinued sorafenib treatment due to disease progression within 2.3 months. Four patients (Cases 2, 3, 5, and 8) died due to disease progression after starting sorafenib treatment. In contrast, the remaining patient (Case 1), whose tumor was negative for p-RPS6, received sorafenib for 24 months and survived for 27 months. In this particular patient, tumor regression was confirmed by computed tomography scans performed three months after sorafenib administration and remained stable for another three

months. In addition, the α-fetoprotein (AFP) level dropped from 1,621 to 314 ng/ml and remained low for 10 months. These results provide preliminary evidence that that high expression of p-RPS6 S235/236 might be useful for predicting which patients are unlikely to respond to sorafenib. As biopsy is not performed routinely before sorafenib treatment, we were unable to further validate the clinical significance of p-RPS6 S235/236 by examining additional cases.

mTOR Pathway Activation in Sorafenib-resistant Cells.

Given the association between p-RPS6 and sensitivity of HCC cell lines to sorafenib, we assessed the effects of sorafenib on p-RPS6 in representative sorafenib-sensitive and -resistant cell lines. The sorafenib-sensitive HUH-6 and HuH-7 cell lines demonstrated substantial dose-dependent decreases in p-RPS6 levels following treatment with sorafenib (Fig. 3A). Sorafenib also diminished the phosphorylation of downstream molecules in the MAPK pathway, ERK and RSK, in a dose-dependent manner (Fig. 3A), implying that the reduction of p-RPS6 in sorefenib-sensitive cells was likely attributable to blockade of the MAPK pathway (Fig. 3B).

In contrast, the phosphorylation of RPS6 S235/236 in sorafenib-resistant cell lines, especially JHH-2, SNU-423, and SNU-387 cells, was insensitive to the same sorafenib treatment (Fig. 3C). The level of p-RPS6 in JHH-1 and SNU-182 cells decreased to some extent after

sorafenib treatment, but a high concentration (10 μM) of sorafenib was necessary in order to suppress the phosphorylation of RPS6 completely (Fig. 3C), reflecting that the regulation of p-RPS6 in sorafenib-resistant cell lines is different from that in sensitive cell lines. RPS6 is also known to be phosphorylated by 70-kDa ribosomal S6 kinases (S6K) downstream of mTOR (Fig. 3B) (19, 20). We therefore speculated that the mTOR pathway might be alternatively activated in sorafenib-resistant cell lines. In fact, we found that sorafenib-resistant cells had a high level of p-S6K1 (Fig. 3C), while p-S6K1 was barely detectable in sorafenib-sensitive cells (Fig. 3A).

The phosphorylation of ERK in JHH-1 and SNU-182 was suppressed to some extent by sorafenib, but the low level of p-RSK and high level of p-S6K1 indicate that the main regulator of RPS6 phosphorylation was mTOR signaling rather than MAPK signaling.

Absence of Genetic Alterations in the MAPK and mTOR Pathways.

RAF kinases are ones of the known targets of sorafenib, but sorafenib-resistant JHH2, SNU-423, and SNU-387 cells exhibited sustained activation of molecules located downstream of RAF (ERK and RSK), even in the presence of sorafenib (Fig. 3C), suggesting sorafenib-insensitive activation of the MAPK pathway.

To clarify the molecular mechanism driving the activation of the mTOR and MAPK pathways in sorafenib-resistant cells, we sequenced the entire exons of 511 kinases (listed in

Supplementary Table S5) in 20 HCC cell lines using a so-called next generation sequencer. Supplementary Table S6 lists all of the 322 genetic alterations that were not deposited in the dbSNP database. Due to the unavailability of normal counterparts, we were unable to determine whether these alterations were somatic. Eight kinds of DNA alterations were evident in the known mTOR and MAPK pathway genes (Table 1). b-RAF V600E, found in SK-Hep1 cells, is a known driver mutation frequently observed in malignant melanoma (21). Two kinds of alterations were identified in the ATP-binding [S72A (JHH-7)] and AGC-kinase C-terminal [K335T (huH-1, SNU-475 and SNU-185)] domains of the RSK1 genes. Three kinds of alterations were found in the proline-rich domain [A420V (11 cell lines including SNU449) and V422I (HUH-6)] and catalytic [P267L (SK-Hep1, JHH-4, Kim1 and JHH-1)] domains of the S6K2 gene.

These 8 alterations were validated using a conventional sequencing method (Table 1), but no genetic alteration was specific to sorafenib-resistant cell lines. Infrequent alteration of the mTOR and MAPK pathway genes in HCC has been demonstrated by conventional sequencing analysis of surgical samples (22), and this was consistent with the present comprehensive sequencing data. The aberrant activation of the mTOR and MAPK pathways in sorafenib-resistant HCC cells was likely attributable to complex interplay between other oncogenic and anti-oncogenic pathways, or post-translational modifications.

mTOR Inhibitors Repress the Proliferation of Sorafenib-resistant Cells.

The marked inhibition of p-RPS6 S235/236 following exposure to an mTOR inhibitor, everolimus, at a concentration as low as 2 nM (Fig. 3D) confirmed that activation of the mTOR pathway is responsible for the sorafenib-insensitive phosphorylation of RPS6 S235/236 in sorafenib-resistant cells. Consistently, sorafenib-sensitive cells tended to be more sensitive to another mTOR inhibitor, AZD8055, than sorafenib-sensitive cell lines (Fig. 3E), indicating that sorafenib-resistant cells are dependent for growth on constitutive activation of the mTOR pathway.

Synergy of mTOR and MAPK Inhibitors.

Although JHH-1 and SNU-182 cells showed resistance to sorafenib, their ERK phosphorylation was dose-dependently attenuated by sorafenib (Fig. 3C), indicating that the MAPK pathway in these cells still retained some sensitivity to inhibition of RAF or other unknown MAPK-pathway kinases. In fact, sorafenib augmented the down-regulation of p-RPS6 S235/236 by everolimus (Fig. 4A). It is noteworthy that the low level of p-RPS6 S235/236 in SNU-182 cells sustained in the presence of 2 μM everolimus was completely abrogated by the addition of 3 μM sorafenib.

The phosphorylation of RPS6 S235/236 in JHH-2, SNU-423 and SNU-387 cells was

insensitive to sorafenib (Fig. 3C). However, the active MAPK pathway in these cell lines seems to lie downstream of RAF kinases. An MEK inhibitor, CI-1040 (Fig. 3B), enhanced the attenuation of p-RPS6 S235/236 by everolimus in JHH-2 and SNU-423 cells (Fig. 4B), and a RSK inhibitor, SL0101, enhanced the attenuation of p-RPS6 S235/236 by everolimus in JHH-2 and SNU-387 cells (Fig. 4B).

Marked inhibition of p-RPS6 S235/236 by combined blockade of the MAPK pathway downstream of RAF and the mTOR pathway prompted us to examine the effect of this drug combination on HCC cell growth. CI-1040 had no inhibitory effect on the growth of SNU-423 cells at a concentration of 0.1 μM (Fig. 4C), but was able to enhance the growth-inhibitory effect of AZD8055 (Fig. 4D). Synergy between AZD8055 and CI-1040 was confirmed by Chou-Talalay median dose effect analysis (17) (Supplementary Table S8). The combination index (CI) values at 50%, 75%, and 90% growth inhibition were 0.783, 0.804, and 0.827, respectively, where values of <1 are defined as synergistic effects. These results suggest that HCC patients refractory to sorafenib with a high level of p-RPS6 S235/236 might be treatable with an mTOR inhibitor in combination with drugs that block the MAPK signaling pathway.

To further provide a rational basis for synergistic targeting of the mTOR and MAPK pathways in HCC, we performed unsupervised hierarchical cluster analysis of 23 HCC cell lines based on their phosphorylation status of signaling components in the mTOR and MAPK