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ORIGINAL RESEARCH

Inactivation of Transcriptional Repressor Capicua Confers Sorafenib Resistance in Human Hepatocellular Carcinoma

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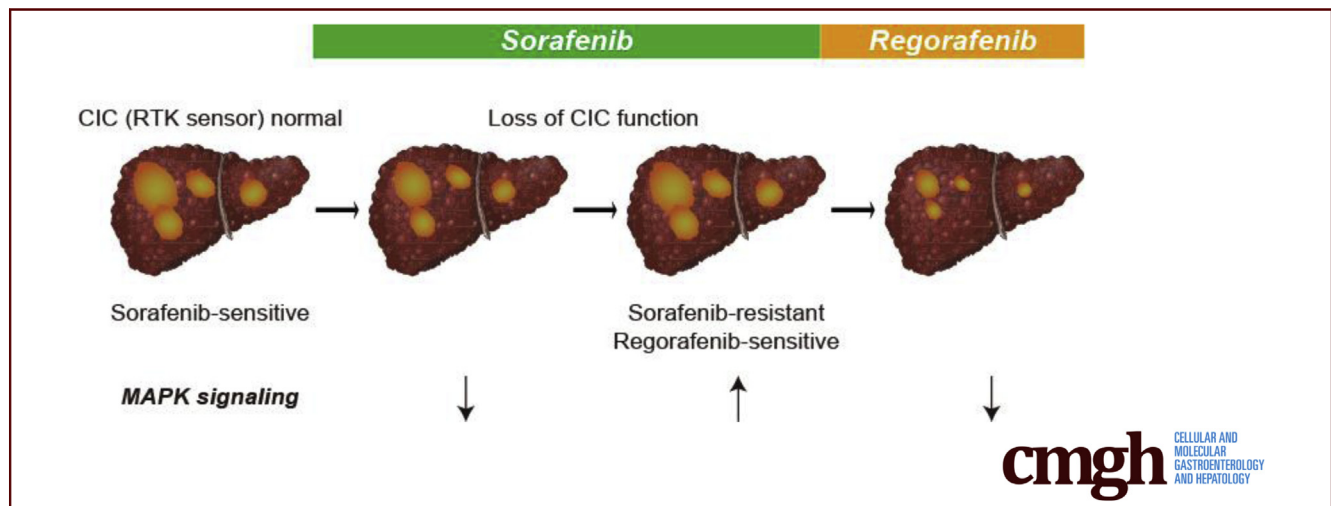
SUMMARY

After long-term exposure to sorafenib, hepatocellular carcinoma (HCC) acquired sorafenib resistance through impairment of the receptor tyrosine kinase sensor Capicua. Regorafenib suppressed sorafenib-resistant HCC growth with impaired Capicua function, showing the utility of sorafenib-regorafenib sequential therapy to prolong overall survival in advanced HCC.

BACKGROUND & AIMS: Sorafenib is a multireceptor tyrosine kinase inhibitor that can prolong overall survival in patients with advanced hepatocellular carcinoma (HCC). Although most HCC patients who receive sorafenib ultimately show disease progression, it still is unclear whether and how HCC cells acquire chemoresistance during sorafenib treatment in human beings.

METHODS: We analyzed surgically resected HCC tissues from a patient who received sorafenib for prevention of HCC recurrence after surgery (Adjuvant Sorafenib for Hepatocellular Carcinoma after Resection or Ablation trial) and established patient-derived HCC cells. Whole-exome sequence analysis was performed to detect mutations in sorafenib-resistant clones. We examined 30 advanced HCC cases immunohistochemically and 140 HCC cases enrolled in the Adjuvant Sorafenib for Hepatocellular Carcinoma after Resection or Ablation trial using microarray analysis to evaluate the association of Capicua Transcriptional Repressor (*CIC*) status with sorafenib treatment response.

RESULTS: We found a *CIC* mutation in recurrent HCC specimens after sorafenib. *CIC* encodes Capicua, a general sensor of receptor tyrosine kinase signaling. HCC cells established from the recurrent tumor specimen showed chemoresistance to sorafenib in vitro and in vivo. Established sorafenib-resistant Huh1 and Huh7 cell lines showed reduced expression of Capicua without mutations. Immunohistochemical analysis showed that HCC patients with low Capicua expression showed poor overall survival. Microarray analysis showed that the *CIC* gene signature could predict the preventive effect of adjuvant sorafenib treatment on HCC recurrence. Intriguingly, although *CIC* knockdown induced sorafenib resistance in HCC cell lines, regorafenib suppressed growth of sorafenib-resistant, Capicua-inactivated HCC cells and inhibited extracellular signal-regulated kinase phosphorylation.

CONCLUSIONS: Evaluation of Capicua status may be pivotal to predict response to sorafenib, and regorafenib treatment could be effective to treat HCC with functional Capicua impairment. (*Cell Mol Gastroenterol Hepatol 2020;10:269–285; https://doi.org/10.1016/j.jcmgh.2020.02.009*)

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the second leading cause of cancer-related mortality worldwide.[1] Sorafenib, a multityrosine kinase inhibitor known to prolong overall survival in patients with advanced HCC,[2,3] has widely been used for the treatment of advanced HCC for 10 years. Because sorafenib targets multiple kinases and HCC is heterogeneous in terms of activated signaling pathways, several studies have tried to identify molecular targets activated by HCC and inhibited by sorafenib. Potential biomarkers such as *FGF3/4* amplification, *VEGFA* amplification, and serum cytokines are reported to correlate with clinical response and survival outcome.[4–8] However, although sorafenib could prolong the median overall survival of advanced HCC patients by approximately 3 months, patients ultimately showed disease progression, suggesting acquisition of sorafenib resistance.

A previous study showed the role of *MAPK14* amplification in HCC with sorafenib resistance.[9] HCC with *MAPK14* amplification showed no response to sorafenib in vivo, with rapid tumor progression even after initiation of sorafenib treatment in mice. Another study showed the role of tumor-initiating cells and insulin-like growth factor/fibroblast growth factor signaling in HCC,[10] consistent with previous findings that tumor-initiating cells show chemoresistance to various molecular targeted agents, including sorafenib.[7,11,12] Although these studies clarified the role of mitogen-activated protein kinase (MAPK) signaling alteration or tumor-initiating cells on sorafenib resistance, it remains unclear whether these molecular events are really responsible for the chemoresistance acquired during sorafenib treatment in human beings. No HCC studies evaluating the mechanisms of sorafenib resistance acquired in human beings have been reported to date.

In this study, we obtained HCC specimens from an early stage HCC patient who underwent surgical resection and was enrolled in the Adjuvant Sorafenib for Hepatocellular Carcinoma after Resection or Ablation (STORM) trial. The patient received sorafenib treatment but developed recurrent HCC 2 years after enrollment and underwent repeat surgical resection. We performed whole-exome sequence analysis to characterize the mutation profiles of HCC tissues before sorafenib treatment (presorafenib) and afterward (postsorafenib) to identify the key genetic events responsible for the acquisition of sorafenib resistance in human beings. We established patient-derived cancer cells obtained from postsorafenib HCC and evaluated the relation between sorafenib sensitivity and the identified molecular events. We also established sorafenib-resistant cells by treating Huh1 and Huh7 HCC cell lines with sorafenib for 3 months to evaluate whether the identified molecular events are experimentally reproducible in cell lines. Lastly, we evaluated the effect of regorafenib, an analogue of sorafenib that potently blocks multiple protein kinases and is reported to prolong overall survival of HCC patients with disease progression on sorafenib treatment,[13] on sorafenib-resistant HCC cells in vitro and in vivo.

Results
CIC *Mutation in Sorafenib-Resistant HCC*

We encountered a patient who was enrolled in the STORM trial who received sorafenib treatment for 2 years after initial surgical resection. Magnetic resonance imaging showed similar arterial enhancement with a defect in the hepatobiliary phase and high-intensity, T2-weighted images in HCC presorafenib and postsorafenib (Figure 1A). The patient underwent surgical resection for recurrence, and we performed whole-exome sequence analysis of the 2 HCC tissues and peripheral blood mononuclear cells (PBMCs) as control. We found 38 and 46 genes mutated in the presorafenib and postsorafenib HCC tissues, respectively (Figure 1B and Supplementary Table 1). Twenty-nine mutations were commonly detected in both the presorafenib and postsorafenib HCC tissues, suggesting that the 2 HCCs originated from the same ancestral clone. Sorafenib treatment might slightly increase tumor mutational burden of postsorafenib HCC, so we examined these mutations in detail. We found that *CTNNB1*, a key driver gene encoding β-catenin, was mutated (*CTNNB1* D32G) in both the presorafenib and postsorafenib HCC tissues. However, we further identified an additional novel *CTNNB1* G34R mutation specifically in the postsorafenib (*CTNNB1*) HCC tissues (Figure 1B and Supplementary Table 1). Noticeably, although both the presorafenib and postsorafenib HCCs showed moderately differentiated histology, nuclear accumulation of β-catenin was somewhat attenuated in the postsorafenib HCC compared with the presorafenib HCC (Figure 1C). This finding suggests that signaling pathways may be activated in postsorafenib HCC in addition to Wnt/β-catenin signaling. We subcutaneously injected single-cell suspensions obtained from the presorafenib and postsorafenib HCC tissues in nonobese diabetic, severe combined immunodeficient (NOD/SCID) mice. We successfully obtained subcutaneous tumors and established patient-derived HCC cells only from] a postsorafenib HCC tissue sample (HCC- sorafenib resistance [SR]). We also established independent patient-derived HCC cells (metastatic tumor of the liver [MT]) as a reference. Compared with
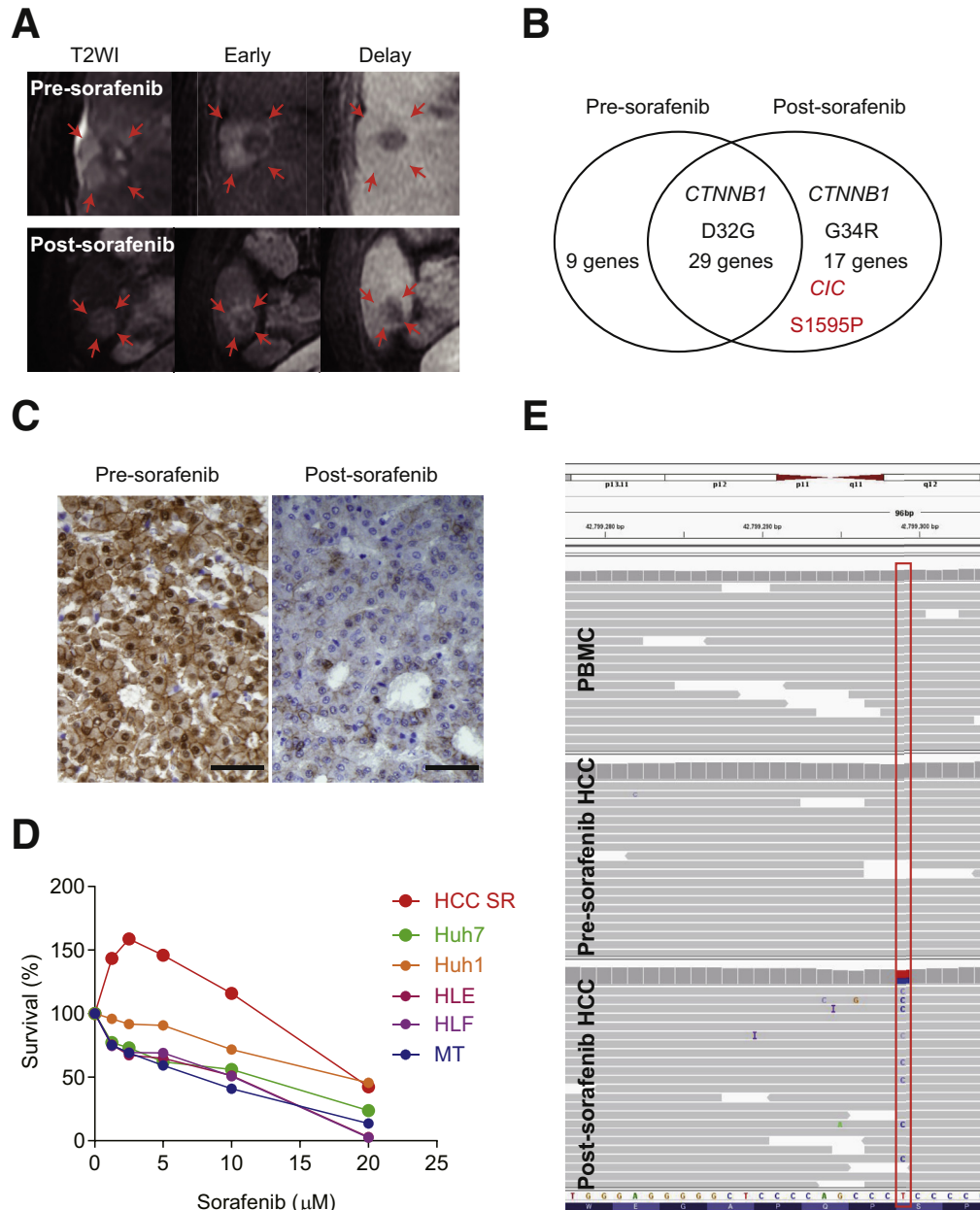
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Figure 1. Analysis of acquired sorafenib resistance in human HCC. (*A*) Magnetic resonance images of HCC tissue from a patient enrolled in the STORM trial. *Upper*: Magnetic resonance images of presorafenib HCC. *Lower*: Magnetic resonance images of recurrent HCC after 2 years of sorafenib treatment. *Red arrows* indicate the margin of the tumors. (*B*) Venn diagram of gene mutations in presorafenib and postsorafenib HCCs. *CTNNB1* D32G mutation was commonly detected in presorafenib and postsorafenib HCCs; specifically, 17 genes were mutated, including *CTNNB1* G34R and *CIC* S1595P, in postsorafenib HCC. (*C*) Immunohistochemistry analysis of β-catenin expression in presorafenib and postsorafenib HCC. *Scale bar*: 50 μm. (*D*) Cytotoxicity assay of HCC-SR, HCC cell lines (Huh1, Huh7, HLE, and HLF), and MT cells (patient-derived cells) treated with indicated concentrations of sorafenib for 48 hours (n = 5, mean values are shown). (*E*) CIC:c.4783T>C (p.Ser1595Pro) mutation detected only in postsorafenib HCC, not in PBMCs or presorafenib HCC, as shown by Integrative Genomics Viewer ver. 2.4.1 (Broad Institute of Massachusetts Institute of Technology and Harvard Medical School, Cambridge, MA). The *red rectangle* indicate the position of CIC S1595P mutation only detected in post-sorafenib HCC.

HCC cell lines (Huh7, Huh1, HLE, and HLF cells) and MT cells, HCC-SR showed clear sorafenib resistance, and sorafenib treatment induced somewhat higher cell proliferation at a concentration of less than 10 μmol/L (Figure 1*D*). We performed whole-exome sequence analysis of HCC-SR and a comparison of the mutation profiles of HCC-SR and

PBMC showed 1192 genes mutated in HCC-SR, indicating that HCC-SR extensively acquired or lost the genetic mutations during cell line establishment. Interestingly, we detected the *CTNNB1* S37C mutation specifically in HCC-SR, but not in presorafenib and postsorafenib HCC, indicating that *CTNNB1* mutation might be a key driver conserved but

evolved in these series of tumors. We also found *Dystonin* nonsense mutation in HCC-SR, although *Dystonin* harbored a missense mutation in postsorafenib HCC. We compared the mutation profiles of 17 genes mutated specifically in postsorafenib HCC with allele frequency exceeding 20% (Supplementary Table 1). Among these 17 genes, we only found a mutation that was detected distinctively in postsorafenib HCC and HCC-SR, but not in presorafenib HCC or PBMCs; this was the *CIC* S1595P missense mutation (Figure 1*E*). *CIC* encodes Capicua, a transcriptional suppressor conserved from *Caenorhabditis elegans* to human beings.[14] Ser[1595] is located in the pro-rich region, closely located in the conserved C1 motif of *CIC*, which plays a fundamental role as a transcriptional suppressor.[14] *CIC* Ser[1595] is reported to be phosphorylated in the liver.[15] The significance of this rare single nucleotide variation (rs745695673, *CIC* S1595P) has not yet been fully clarified.

## Capicua Regulates MAPK Signaling and Sorafenib Sensitivity in HCC

Capicua activity is known to be regulated by receptor tyrosine kinase (RTK) signaling in drosophila and mammals,[16] and it works as a tumor suppressor.[16–18] A recent report suggested the role of Capicua as a tumor suppressor in HCC to inhibit tumor progression by controlling the ETS Variant Transcription Factor 4 (ETV4)–Matrix Metallopeptidase 1 (MMP1) axis.[19] In the presence of RTK inhibitors, Capicua is activated and suppresses the downstream Ras/MAPK target genes.[20] Several studies have indicated *CIC* mutations in various cancers including brain, lung, and gastric cancer.[14] A recent report suggested that the *CIC* mutation resulted in resistance to epidermal growth factor receptor inhibitors in lung cancer.[21] Therefore, taking the earlier-described molecular information into account, we evaluated the role of Capicua on cell proliferation and sorafenib sensitivity in HCC.

We performed knockdown experiments using 2 small interfering RNAs (siRNAs) specifically targeting *CIC* (small interfering RNAs targeting CIC 1 and small interfering RNAs targeting CIC 2) or control siRNAs in Huh7 cells. We confirmed the knockdown of *CIC* both at the messenger RNA (Figure 2*A*) and protein levels (Figure 2*B*). In this setting, MAPK signaling was strongly enhanced by *CIC* knockdown compared with control. *CIC* knockdown resulted in a 2-fold increase of enhanced cell proliferation (Figure 2*C*) and further altered chemosensitivity to sorafenib (Figure 2*D*). We performed similar knockdown experiments using the same siRNAs in patient-derived MT cells (Figure 2*E* and *F*), and found increased ETV4 and MMP1 gene expression (Figure 2*G*). Interestingly, with *CIC* knockdown in HCC-SR cells in which *CIC* function already was impaired, we found no effects of *CIC* suppression on sorafenib sensitivity in HCC-SR cells (Figure 2*H*). Furthermore, when we introduced wild-type *CIC* DNA in HCC-SR cells, sorafenib sensitivity was acquired (Figure 2*I*). All these data suggest that *CIC* regulates the MAPK signaling pathway and that its functional loss confers chemoresistance to sorafenib, which is known to moderately inhibit V-Raf Murine Sarcoma Viral

Oncogene Homolog B (B-RAF) kinase and suppress the MAPK signaling pathway.

Acquired Sorafenib Resistance Correlates With Inactivation of CIC and Activation of the ETV4–MMP1 Axis in HCC Cell Lines

The earlier-described data suggest that inactivation of Capicua may confer sorafenib resistance in HCC. To evaluate whether Capicua inactivation could occur in acquired sorafenib resistance, we generated sorafenib-resistant clones using Huh1 and Huh7 cells by treating these cells with sorafenib (2.5–5 μmol/L) for 3 months in vitro. Established sorafenib-resistant clones (Huh1-SR and Huh7-SR) showed chemoresistance to sorafenib compared with the parental Huh1 and Huh7 cells (Figure 3*A*). We performed whole-exome sequence analysis of parental Huh1, Huh7, Huh1-SR, and Huh7-SR cells (Supplementary Tables 2 and 3), and found no mutations in *CIC* in any of these cell lines. We found an additional 46 and 10 mutations with allele frequency exceeding 20% specifically in Huh7-SR and Huh1-SR compared with Huh7 and Huh1 of the same passages, respectively. These data indicate that the tumor mutational burden might be increased modestly (Huh7) or weakly (Huh1) by 3-month sorafenib treatment in vitro. Intriguingly, we found a reduction in Capicua protein expression with strong phosphorylation of extracellular signal–regulated kinase (ERK)1/2 and AKT Serine/Threonine Kinase (Akt) in sorafenib-resistant clones (Figure 3*B*). Phosphorylation of signal transducer and activator of transcription 3 (STAT3) was slightly suppressed in sorafenib-resistant clones. We evaluated the expression of *MAPK14*, previously reported as a gene related to sorafenib resistance in a mouse HCC model.[9] We found slightly increased MAPK14 in Huh7-SR cells compared with Huh7 cells, and this was statistically significant (Figure 3*C*). We evaluated the expression of *ETV4* and *MMP1*, downstream target genes known to be suppressed by Capicua, in Huh7 and Huh7-SR. We found modest and strong activation of *ETV4* and *MMP1* in Huh7-SR, respectively (Figure 3*C*). We further evaluated the amounts of epithelial cell adhesion molecule (EpCAM)-positive cancer stem cells because a previous report described an increase in EpCAM-positive cancer stem cells.[10] We confirmed the enrichment of EpCAM-positive cells in Huh7-SR cells compared with Huh7 cells (Figure 3*D*). We evaluated the subcellular localization of Capicua in Huh7 and Huh7 SR cells using immunofluorescence analysis. We found that although Capicua was located mainly in the nucleus in both HuH7 and HuH7-SR cells, HuH7-SR showed lower nuclear expression of Capicua (Figure 3*E* and *F*). Furthermore, we found that cytoplasmic Capicua was barely detected in HuH7-SR cells, suggesting the degradation of cytoplasmic Capicua pools in sorafenib-resistant cells. In contrast, HCC-SR cells showed both cytoplasmic and nuclear Capicua expression (Figure 3*G*) irrespective of the loss of Capicua function in terms of sorafenib sensitivity (Figure 1*D*), suggesting that *CIC* mutation might be associated with CIC loss of function and degradation. These data indicate that long-term sorafenib treatment

A

Huh7

B

Huh7

C

Huh7

D

Huh7

E

MT

F

MT

G

MT

MT

H

HCC-SR

I

HCC-SR
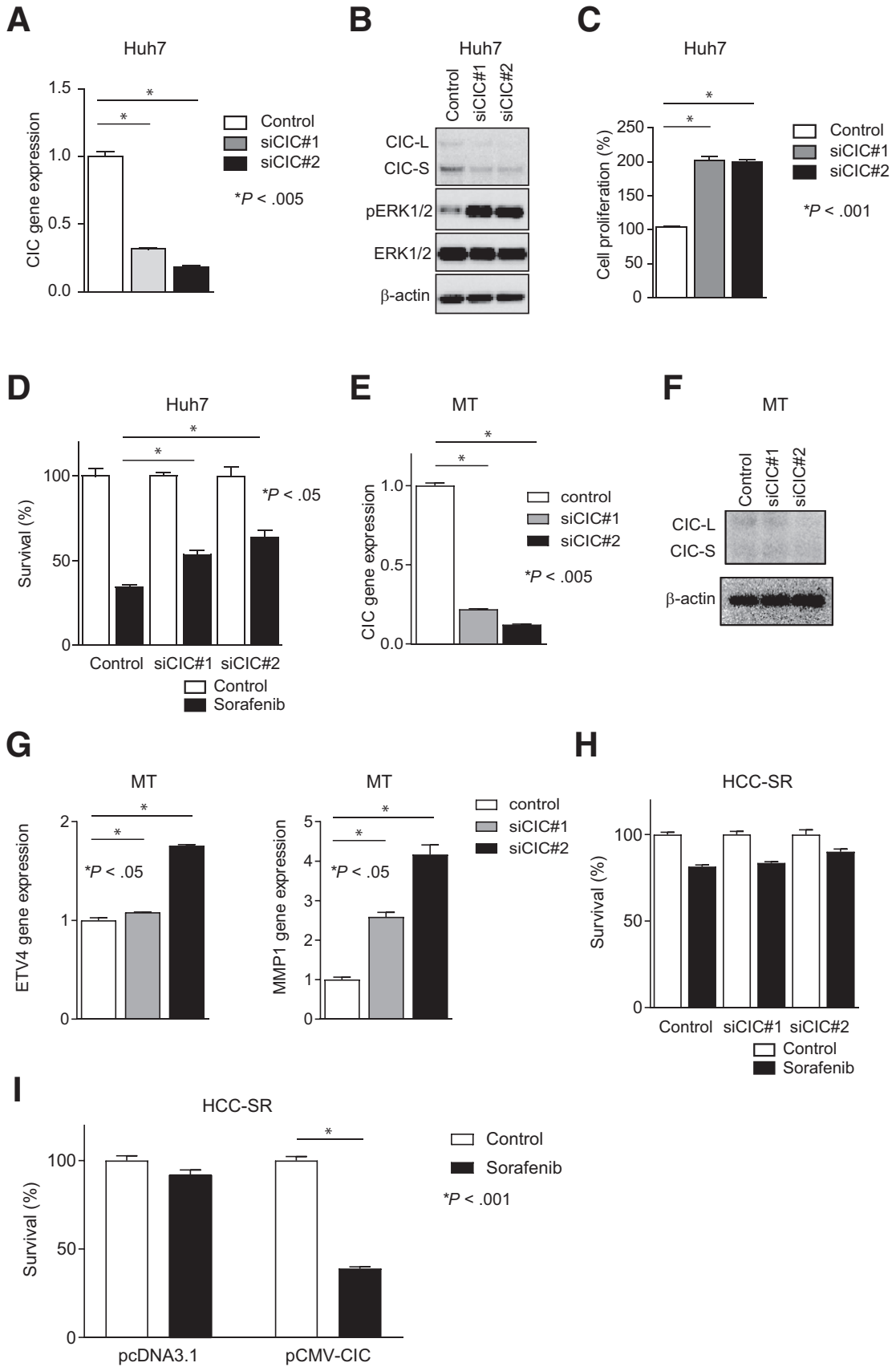
resulted in the acquisition of sorafenib resistance in HCC, and CIC protein expression was down-regulated in sorafenib-resistant HCC.

Capicua Expression Status Correlates With Survival Outcome of Advanced HCC Patients Treated With Sorafenib

The earlier-described data indicated a pivotal role of Capicua in chemoresistance to sorafenib in HCC. To further confirm this finding, we evaluated Capicua expression in HCC tissues obtained from 30 patients with advanced HCC who received sorafenib. All tissue samples were obtained using needle biopsy before sorafenib treatment, and 11 and 19 HCC specimens were classified as CIC-high and CIC-low, respectively, based on immunohistochemistry (Figure 4A). All patients received sorafenib treatment, and the median treatment period was 2.8 months (25%–75% percentile, 1.3–5.5 mo). Analysis of the clinicopathologic characteristics showed that there was no significant difference in terms of age, sex, histology, etiology, liver function, major vascular invasion, extrahepatic metastasis, and tumor markers between CIC-high and CIC-low HCCs (Supplementary Table 4). Kaplan–Meier survival analysis clearly indicated better overall survival of HCC patients regarded as CIC-high compared with those regarded as CIC-low with statistical significance ($P = .021$) (Figure 4B). We evaluated the effect of CIC expression after sorafenib treatment in 3 of these HCCs for which tumor biopsy samples were available. Three samples were regarded as CIC-high before sorafenib treatment, and after the patients showed clinical disease progression, we found that CIC expression was attenuated in all 3 HCC cases (Figure 4C), consistent with our in vitro data. These data supported the notion that evaluation of Capicua expression status using needle biopsy may be useful to predict response to sorafenib in the clinical setting.

Capicua-Regulated Genes Predict the Outcome of Adjuvant Sorafenib Treatment in HCC Recurrence After Surgical Resection or Local Ablation

Although the phase 3 STORM trial comparing sorafenib with placebo as adjuvant treatment did not achieve the primary end point, namely improvement of recurrence-free survival (RFS), a recent biomarker study successfully has shown the potential gene signature that could predict sorafenib benefit.[22] To evaluate if Capicua status could correlate with survival benefit from adjuvant sorafenib treatment, we tried to identify the gene set specific to Capicua protein status because Capicua protein function does not correlate directly with CIC messenger RNA expression levels.[19] Accordingly, we performed microarray analysis to identify genes expressed differentially by CIC knockdown using cell lines. We used Huh7, HLE, and HLF cells that were relatively sensitive to sorafenib compared with Huh1 (Figure 1D). Efficient knockdown of CIC was confirmed in each cell line (Figure 5A). We identified 265 genes expressed differentially between Huh7, HLE, and HLF cells treated with control siRNAs (3 samples) and those treated with 2 siRNAs specifically targeting CIC (6 samples) with statistical significance ($P < .005$). Among them, 112 genes were listed in the probe set of Gene Expression Omnibus 109211 STORM trial data, and we designated them as the CIC gene signature. Gene probes corresponding to CIC were not included in the Gene Expression Omnibus 109211 data. From the STORM trial data, 140 samples were regarded as sorafenib RFS responders (n = 42) or nonresponders (n = 98), based on the expression status of the 146-gene set. This gene set was identified using survival analysis of RFS data of HCC patients treated with sorafenib or placebo, and could discriminate patients benefiting from sorafenib in terms of extended RFS from patients for whom sorafenib had no effect.[22] Hierarchical analysis of these 140 samples using the CIC gene signature clearly distinguished 2 major subtypes, one comprising mainly sorafenib RFS responders (blue box) and the other mostly comprising nonresponders (orange box) (Figure 5B). To further test if the CIC gene signature could predict sorafenib RFS responders or nonresponders, we applied 7 multivariate class prediction algorithms with 100 random permutations. This analysis resulted in a statistically significant prediction of sorafenib RFS responders and nonresponders with accuracy ranging from 87% to 95% and statistical significance ($P < .01$) (Figure 5C). These data showed that CIC status in HCC may affect the response to sorafenib as adjuvant therapy after locoregional treatment.

Figure 2. (*See previous page*). *CIC* status and sorafenib resistance in HCC cell lines. (*A*) Quantitative reverse-transcription polymerase chain reaction analysis of *CIC* gene expression in Huh7 cells treated with control or siRNAs specifically targeting *CIC* 48 hours after transfection (n = 3, means ± SD, Student *t* test). (*B*) Western blot of CIC, ERK1/2, phosphorylated ERK1/2, and β-actin in Huh7 cells treated with control or siRNAs specifically targeting *CIC* 72 hours after transfection. (*C*) Cell proliferation assay of Huh7 cells treated with control or siRNAs specifically targeting *CIC* 72 hours after transfection (n = 5, means ± SD, Student *t* test). (*D*) Cytotoxicity assay of Huh7 cells. Huh7 cells were transfected with control or siRNAs specifically targeting *CIC* 24 hours before treatment. Cells then were treated with sorafenib (10 μmol/L) for 48 hours (n = 5, means ± SD, Student *t* test). (*E*) Quantitative reverse-transcription polymerase chain reaction analysis of *CIC* gene expression in MT cells treated with control or siRNAs specifically targeting *CIC* 48 hours after transfection (n = 3, means ± SD, Student *t* test). (*F*) Western blot of CIC and β-actin in Huh7 cells treated with control or siRNAs specifically targeting *CIC* 72 hours after transfection. (*G*) Quantitative reverse-transcription polymerase chain reaction analysis of *ETV4* and *MMP1* gene expression in MT cells (n = 3, means ± SD, Student *t* test). (*H*) Cytotoxicity assay of HCC-SR cells. HCC-SR cells were transfected with control or siRNAs specifically targeting *CIC* 24 hours before treatment. Cells then were treated with sorafenib (10 μmol/L) for 48 hours (n = 5, means ± SD). (*I*) Cytotoxicity assay of HCC-SR cells. HCC-SR cells were transfected with control plasmid (pcDNA3.1$^+$) or plasmid encoding *CIC* (pCMV6-CIC-Myc-DDK) 24 hours before treatment. Cells then were treated with control (0.1% dimethyl sulfoxide) or sorafenib (5 μmol/L) for 72 hours (n = 3, means ± SD).
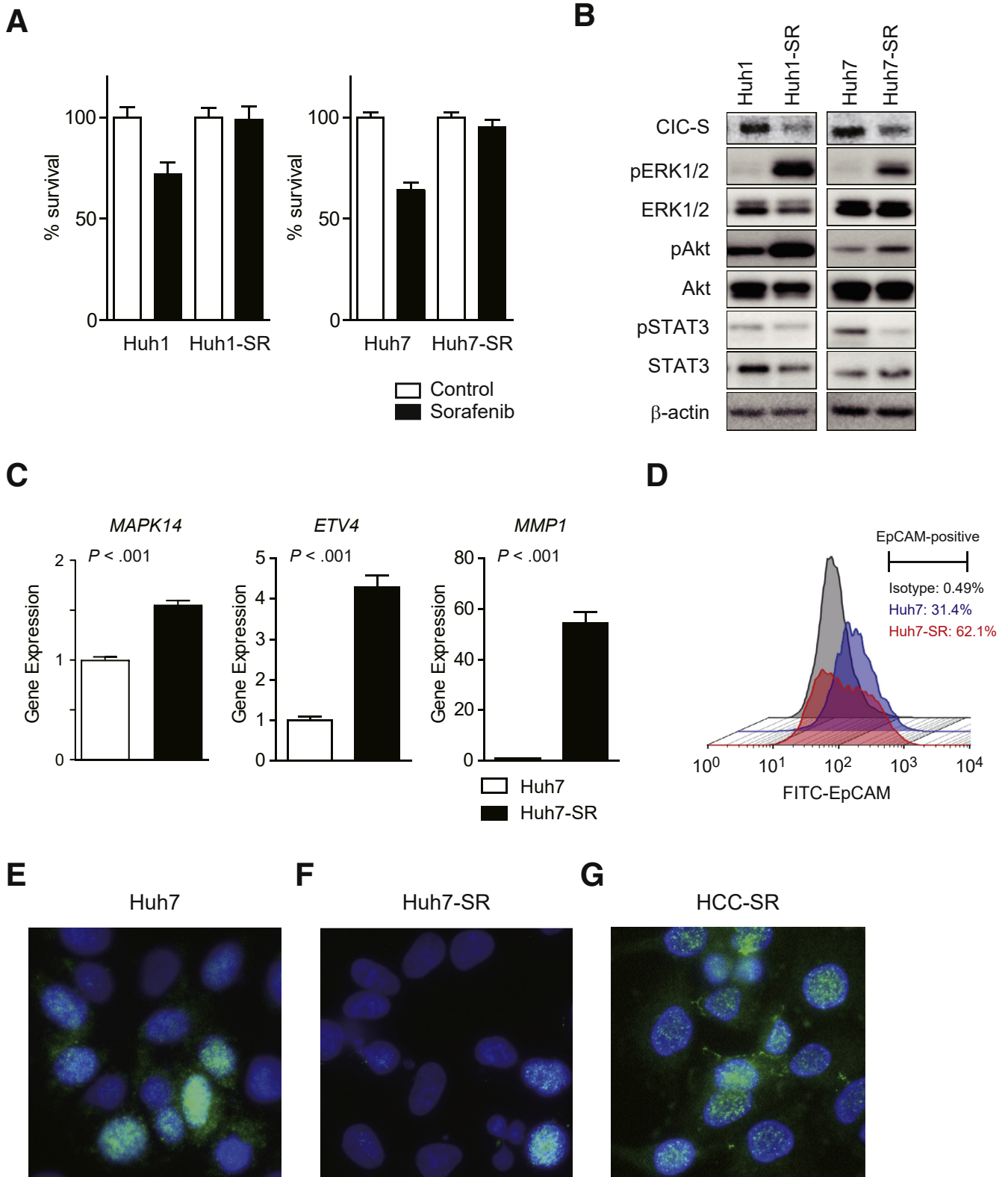
Figure 3. Characterization of sorafenib-resistant HCC cell lines established in vitro. (*A*) Cytotoxicity assay of Huh1, Huh1-SR, Huh7, and Huh7-SR cells treated with control (0.1% dimethyl sulfoxide) or sorafenib (10 μmol/L) for 48 hours (n = 5, means $\pm$ SD, Student *t* test). (*B*) Western blot of CIC, ERK1/2, phosphorylated ERK1/2 (pERK1/2), Akt, phosphorylated Akt (pAkt), STAT3, phosphorylated STAT3 (pSTAT3), and β-actin in Huh1, Huh1-SR, Huh7, and Huh7-SR cells. (*C*) Quantitative reverse-transcription polymerase chain reaction analysis of *MAPK14*, *ETV4*, and *MMP1* gene expression in Huh7 and Huh7-SR (n = 3, means $\pm$ SD, Student *t* test). (*D*) Fluorescence-activated cell sorting analysis of EpCAM expression in Huh7 and Huh7-SR cells. (*E–G*) Immunofluorescence analysis of Capicua in (*E*) Huh7, (*F*) Huh7-SR, and (*G*) HCC-SR cells. FITC, fluorescein isothiocyanate.
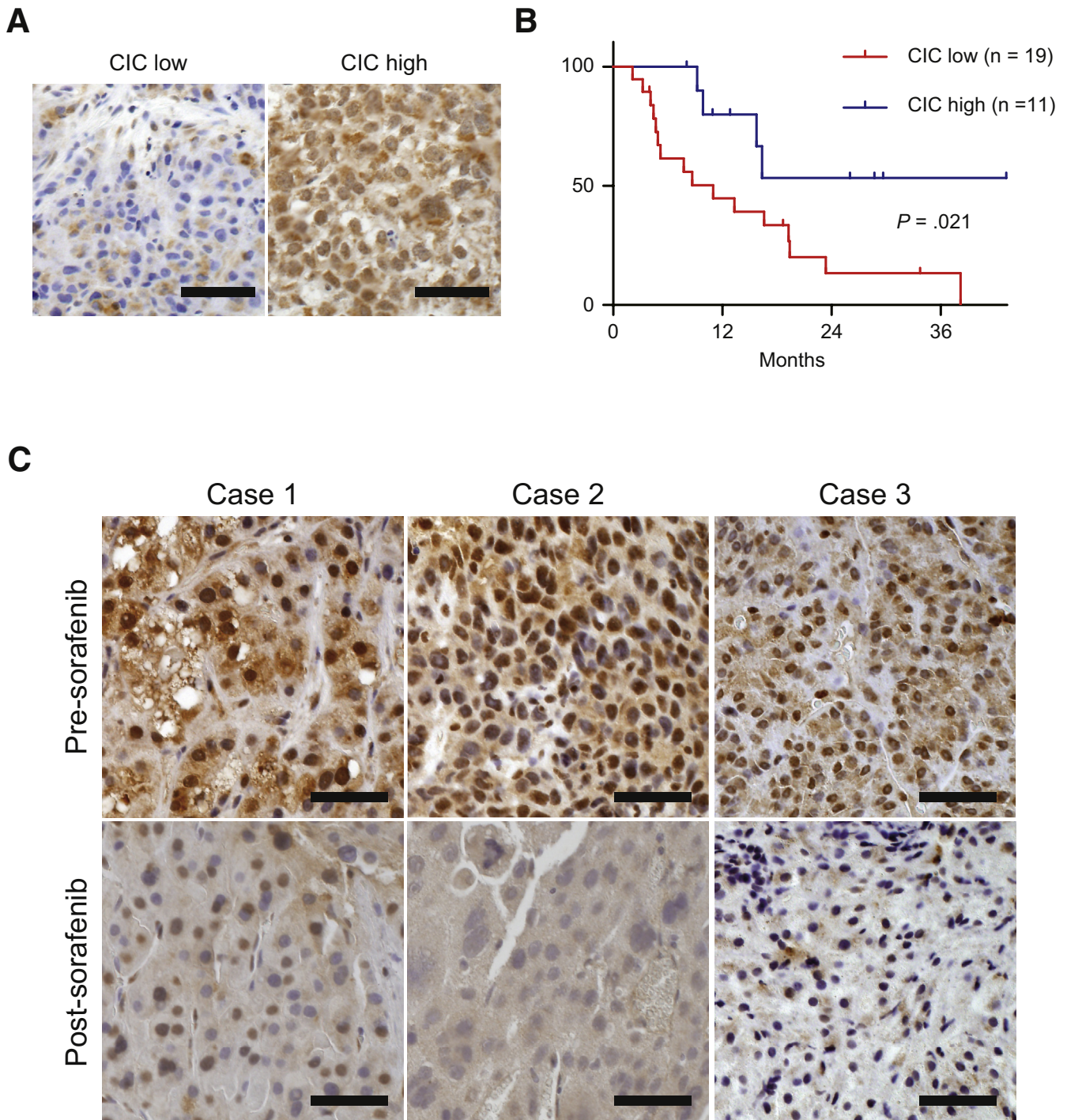
A

B

C

Figure 4. *CIC* status and sorafenib resistance in advanced HCC patients. (*A*) Representative photomicrographs of immunohistochemistry staining with anti-CIC antibodies in HCC specimens obtained from advanced HCC patients. (*B*) Overall survival curves of advanced HCC patients defined by CIC high (blue, n = 11) and low (red, n = 19) by immunohistochemistry. All patients received sorafenib treatment after needle biopsy. (*C*) Representative photomicrographs of immunohistochemistry staining with anti-CIC antibodies in 3 HCC specimens obtained before and after sorafenib treatment from advanced HCC patients. *Scale bars*: 50 μm.

Regorafenib Suppresses MAPK Signaling in Sorafenib-Resistant HCC

Regorafenib is an RTK inhibitor reported to inhibit RAF-1 and B-RAF kinase more strongly compared with sorafenib.[23,24] To evaluate the effect of regorafenib on sorafenib-resistant HCC, we treated Huh1-SR, Huh7-SR, and HCC-SR with control dimethyl sulfoxide (0.1%), sorafenib (10 μmol/L), or regorafenib (10 μmol/L) for 48 hours. We

A

B

Sorafenib RFS Responders ■　　Non-responders ■

C

Performance of CIC gene signature

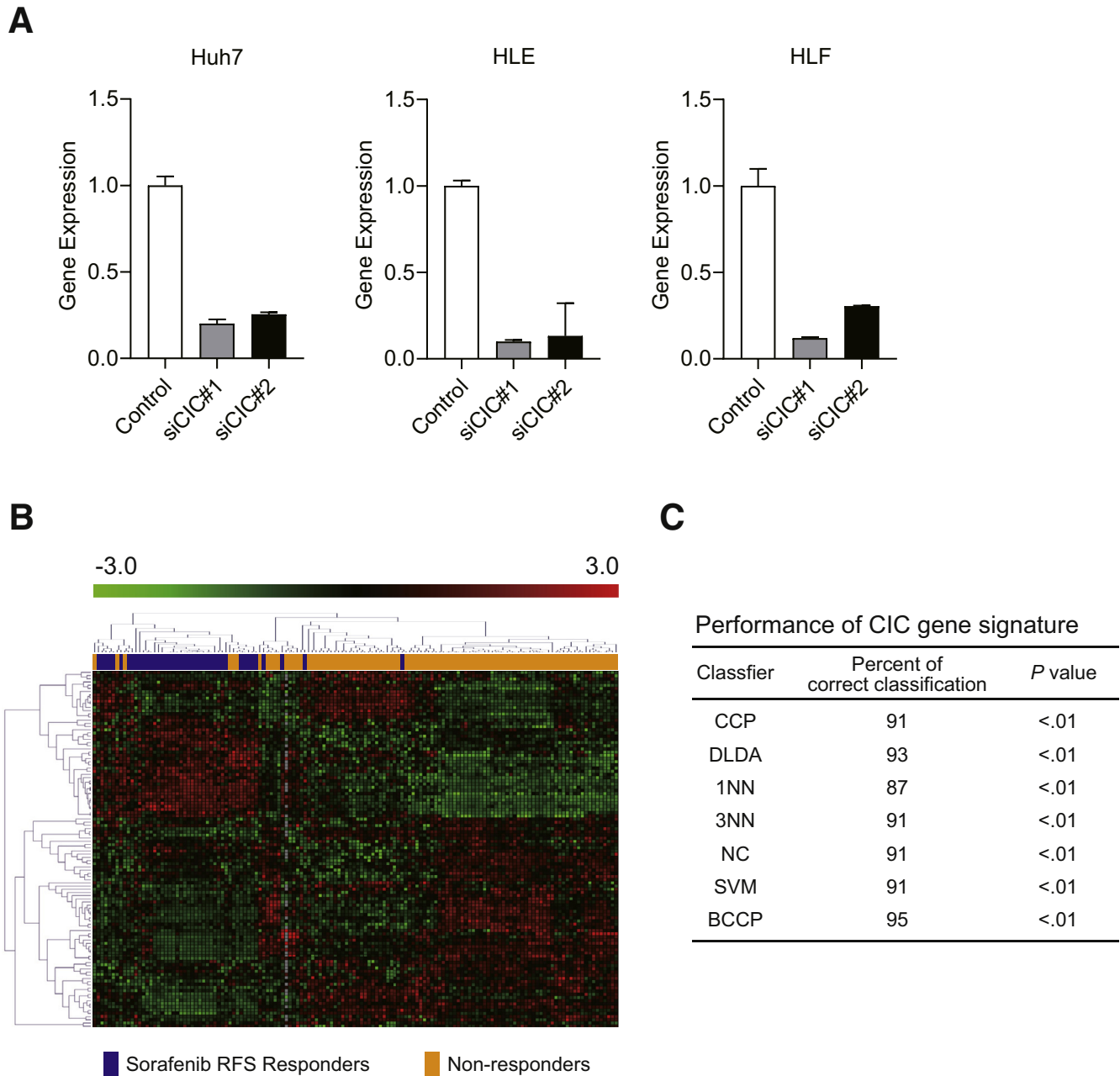| Classfier	Percent of correct classification	P value
CCP	91	<.01
DLDA	93	<.01
1NN	87	<.01
3NN	91	<.01
NC	91	<.01
SVM	91	<.01
BCCP	95	<.01

Figure 5. *CIC* status and HCC recurrence after adjuvant sorafenib treatment. (*A*) Quantitative reverse-transcription polymerase chain reaction analysis of RNA samples used for generation of CIC gene signature in HCC cell lines. Huh7, HLE, and HLF cells were treated with control siRNAs or 2 different siRNAs specifically targeting *CIC*. RNA was extracted 72 hours after transfection and quantitative reverse-transcription polymerase chain reaction analysis for *CIC* was performed in each cell line (n = 3, means ± SD, Student *t* test). (*B*) Hierarchical cluster analysis of HCC. A dendrogram of the 2-way hierarchical cluster analysis of gene expression data from 140 HCC cases of STORM study using the CIC gene signature is shown. Red and green cells show high and low expression levels, respectively. *Blue boxes* and *orange boxes* indicate sorafenib RFS responders and nonresponders, respectively. (*C*) Class prediction analysis of HCC. Seven different class prediction algorithms were used to classify 140 HCC cases enrolled in the STORM trial using the CIC gene signature. All classifier models predicted sorafenib RFS responder or nonresponder classes with accuracy, ranging from 87% to 95% and with statistical significance (*P* < .01). CCP, compound covariate predictor; DLDA, diagonal linear discriminant analysis; 1NN, 1 nearest neighbor; 3NN, 3 nearest neighbors; NC, nearest centroid; SVM, support vector machines; BCCP, Bayesian compound covariate predictor.

found no effect of sorafenib, but a strong effect of regorafenib on the survival of these sorafenib-resistant cells in vitro (Figure 6*A*). We further evaluated the effect of sorafenib or regorafenib on MAPK signaling and found suppression of ERK1/2 phosphorylation by regorafenib, but not by sorafenib or control dimethyl sulfoxide in sorafenib-
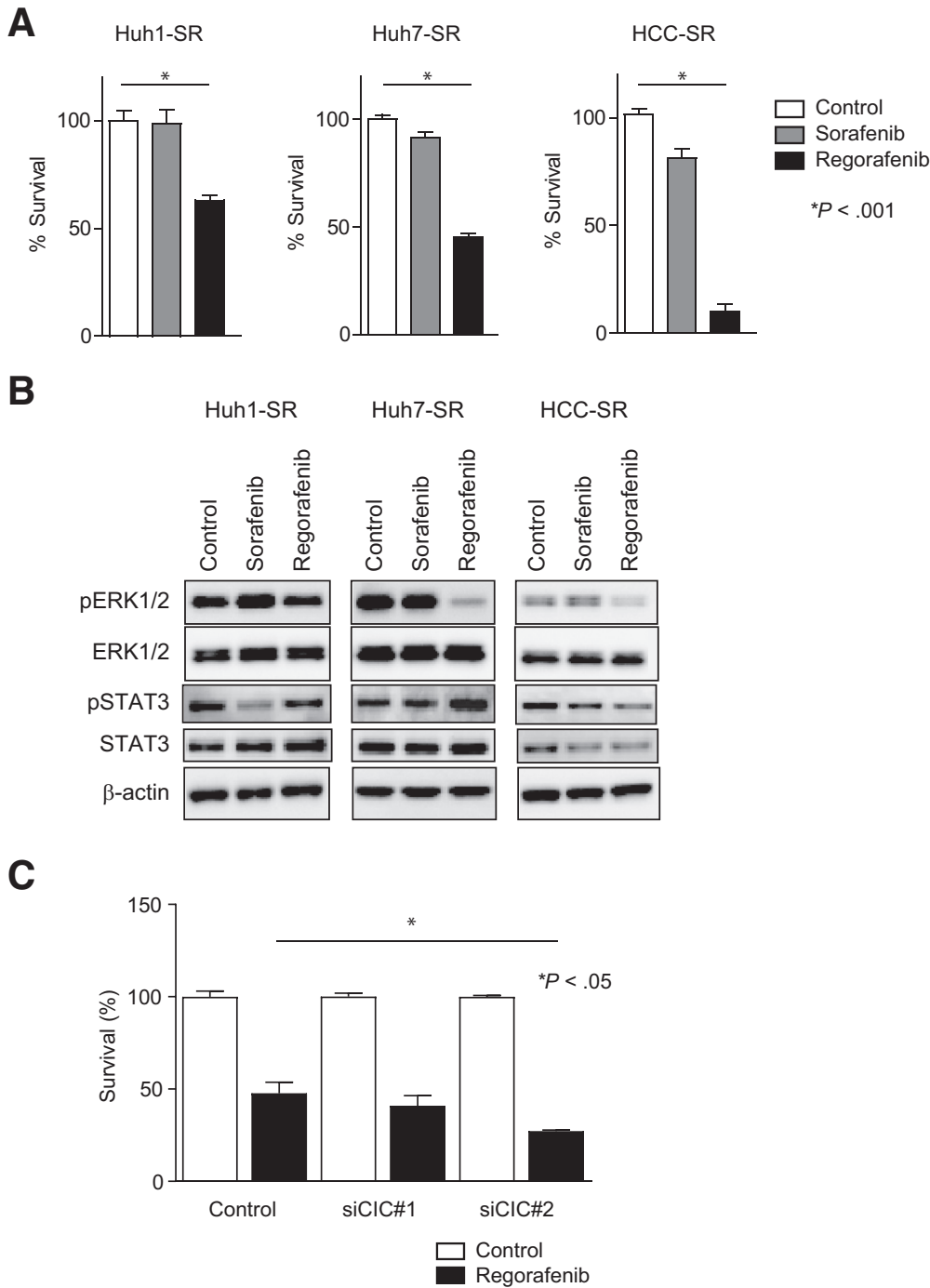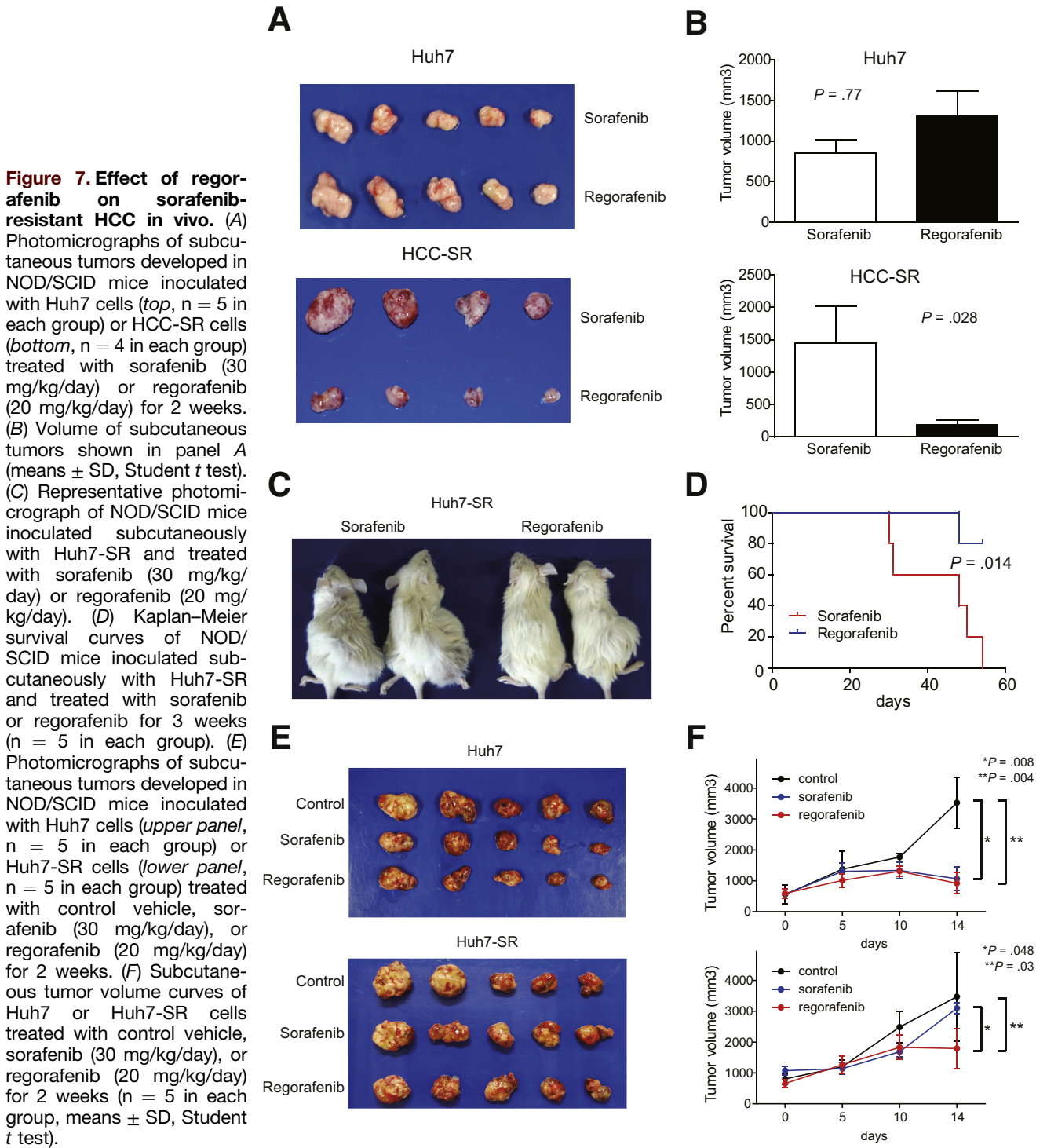
Figure 6. Effect of regorafenib on sorafenib-resistant HCC in vitro. (*A*) Cytotoxicity assay of Huh1-SR, Huh7-SR, and HCC-SR cells treated with control (0.1% dimethyl sulfoxide), sorafenib (10 μmol/L), or regorafenib (10 μmol/L) for 48 hours (n = 5, means ± SD, Student *t* test). (*B*) Western blot of ERK1/2, phosphorylated ERK1/2 (pERK1/2), STAT3, phosphorylated STAT3 (pSTAT3), and β-actin in Huh1-SR, Huh7-SR, and HCC-SR cells treated with control (0.1% dimethyl sulfoxide), sorafenib (10 μmol/L), or regorafenib (10 μmol/L) for 48 hours. (*C*) Cytotoxicity assay of Huh7 cells. Huh7 cells were transfected with control or siRNAs specifically targeting *CIC* 24 hours before treatment. Cells then were treated with regorafenib (10 μmol/L) for 48 hours (n = 5, means ± SD, Student *t* test).

resistant HCC cells (Figure 6*B*). Although a previous report indicated the role of regorafenib on STAT3 signaling inhibition,[25] we could not detect suppression of STAT3 by regorafenib in these sorafenib-resistant cells (Figure 6*B*). We further evaluated the effect of regorafenib on Huh7 cells with *CIC* knockdown and confirmed that regorafenib effectively suppressed the survival of Huh7 cells irrespective of the down-regulation of *CIC* expression (Figure 6*C*).

Regorafenib Inhibits Sorafenib-Resistant HCC Growth In Vivo

Our data suggest that regorafenib suppresses cell growth and the ERK signaling pathway more effectively than sorafenib in sorafenib-resistant *CIC*-inactivated HCC cells in vitro. However, regorafenib is a multiple RTK inhibitor that targets angiogenesis (vascular endothelial growth factor receptors and Tyrosine kinase with Ig and

Figure 7. Effect of regorafenib on sorafenib-resistant HCC in vivo. (*A*) Photomicrographs of subcutaneous tumors developed in NOD/SCID mice inoculated with Huh7 cells (*top*, n = 5 in each group) or HCC-SR cells (*bottom*, n = 4 in each group) treated with sorafenib (30 mg/kg/day) or regorafenib (20 mg/kg/day) for 2 weeks. (*B*) Volume of subcutaneous tumors shown in panel *A* (means ± SD, Student *t* test). (*C*) Representative photomicrograph of NOD/SCID mice inoculated subcutaneously with Huh7-SR and treated with sorafenib (30 mg/kg/day) or regorafenib (20 mg/kg/day). (*D*) Kaplan–Meier survival curves of NOD/SCID mice inoculated subcutaneously with Huh7-SR and treated with sorafenib or regorafenib for 3 weeks (n = 5 in each group). (*E*) Photomicrographs of subcutaneous tumors developed in NOD/SCID mice inoculated with Huh7 cells (*upper panel*, n = 5 in each group) or Huh7-SR cells (*lower panel*, n = 5 in each group) treated with control vehicle, sorafenib (30 mg/kg/day), or regorafenib (20 mg/kg/day) for 2 weeks. (*F*) Subcutaneous tumor volume curves of Huh7 or Huh7-SR cells treated with control vehicle, sorafenib (30 mg/kg/day), or regorafenib (20 mg/kg/day) for 2 weeks (n = 5 in each group, means ± SD, Student *t* test).

EGF homology domains-2) and stromal signaling (fibroblast growth factor receptors and platelet-derived growth factor receptors) as well as oncogenic signaling (RAF-1, B-RAF, rearranged during transfection, and KIT) activated in the tumor microenvironment.[23,24] We therefore evaluated the effect of sorafenib and regorafenib on the growth of sorafenib-naïve and sorafenib-resistant cells in vivo in a subcutaneous xenotransplantation model using NOD/SCID mice. Unexpectedly, we found no differences in the size of tumors comprising sorafenib-naïve Huh7 cells that were treated with sorafenib or regorafenib (Figure 7*A* and *B*). In contrast, we found stronger tumor growth suppression of HCC-SR cells by regorafenib compared with sorafenib (Figure 7*A* and *B*). Similar results were observed in Huh7-
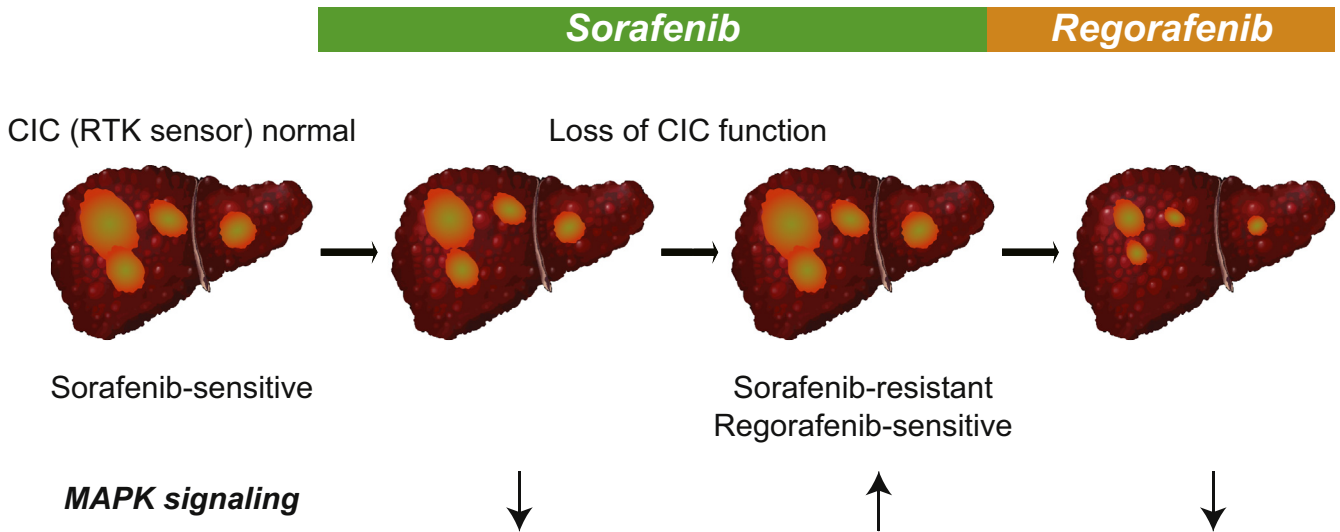
Figure 8. Schematic representation of the status of CIC and the effect of sorafenib–regorafenib sequential therapy in advanced HCC. Sorafenib suppresses HCC growth at least in part by suppressing MAPK signaling in the presence of normal RTK sensor CIC. Continuous exposure of HCC cells to sorafenib results in CIC inactivation and evokes sorafenib resistance. Regorafenib, a more potent inhibitor of MAPK signaling than sorafenib, suppresses the sorafenib-resistant HCC cell growth. Regorafenib can overcome sorafenib resistance irrespective of CIC RTK sensor status modified by exposure to sorafenib. Thus, sorafenib–regorafenib sequential therapy effectively overcomes drug resistance and prolongs overall survival in advanced HCC.

SR cells treated with regorafenib or sorafenib (Figure 7C). The survival benefits of regorafenib treatment were shown clearly compared with sorafenib treatment in Huh7-SR xenotransplanted NOD/SCID mice with a statistically significant difference ($P = .014$) (Figure 7D). We further compared the effect of sorafenib and regorafenib with the control vehicle treatment on sorafenib-naïve Huh7 and Huh7-SR (Figure 7E and F). Sorafenib and regorafenib treatment equally suppressed the growth of sorafenib-naïve Huh7 cells compared with the control cells. In contrast, regorafenib suppressed the growth of sorafenib-resistant Huh7-SR more strongly compared with sorafenib and control vehicle.

Discussion

Sorafenib is a multi-RTK inhibitor used as first-line treatment of advanced HCC patients with clear survival benefits. Our data suggest that long-term sorafenib treatment results in acquired sorafenib resistance in HCC. This resistance, at least in part, is associated with a mutation or reduction of the *CIC* RTK repressor (Figure 8). We found that reduction of *CIC* expression in HCC cells resulted in the acquisition of sorafenib resistance. This study shows the molecular mechanisms of acquired sorafenib resistance in human beings. In addition, we found that regorafenib could overcome sorafenib resistance induced by impairment of the *CIC* RTK repressor in HCC (Figure 8). Our data may explain why regorafenib treatment prolongs the overall survival of HCC patients who have received sorafenib treatment and show disease progression.

Because sorafenib is widely used for the treatment of advanced HCC, biomarkers that could define its chemosensitivity have been studied extensively. We and another group have shown that serum cytokines and growth factors may be related to the chemosensitivity to sorafenib.[6,7] In addition, amplification of *FGF3/4*[4] and *VEGFA*[5] is considered to be linked to the clinical response to sorafenib treatment. In contrast, the existence and mechanism of sorafenib resistance is debatable. A previous study clarified the role of *MAPK14* amplification on sorafenib resistance in HCC.[9] HCC with *MAPK14* amplification showed no response to sorafenib treatment, and *MAPK14* blockade may be a promising approach to overcome sorafenib resistance in such HCCs. Another recent report clarified the role of tumor-initiating cells and insulin-like growth factor/fibroblast growth factor signaling pathway activation in acquired sorafenib resistance in Huh7 cells in vivo.[10] More recently, several studies have indicated the importance of gene/protein expression alterations associated with stemness, autophagy, and microRNAs in acquired sorafenib resistance.[26–31] All of these results were obtained from experiments using cell lines or animal models and were not confirmed in human beings. We would like to emphasize the fact that *CIC* is a molecular event that has been discovered to be functionally altered in acquired sorafenib resistance in human HCC.

We found that HCC-SR and postsorafenib HCC tissues harbored *CTNNB1* S37C and G34R mutations, respectively, suggesting that *CTNNB1* mutations may be related to the acquisition of sorafenib resistance in HCC. Indeed, a recent report suggested that the *CTNNB1* class of HCC belonged to the HCC subtype that might obtain less benefit by sorafenib when used as a neoadjuvant, although its involvement did not reach statistical significance.

We observed that HCC-SR cells not only showed resistance to sorafenib but also showed induced cell proliferation at sorafenib concentrations less than 10 μmol/L. This induced cell proliferation of molecularly targeted agents, such as Mitogen-activated protein kinase kinase/ERK inhibitors in solid tumors, is termed cancer drug addiction.[32] HCC-SR cells might become addicted to sorafenib, which target B-Raf and the ERK signaling pathway.

We found CIC mutation S1595P detected specifically in HCC with sorafenib resistance acquired during a 2-year treatment. Although the significance of this mutation regarding Capicua function has remained unclear, a previous study showed that Capicua Ser[1595] is phosphorylated.[15] Therefore, a missense mutation of CIC S1595P might result in the loss of the phosphorylation site that might affect the function of Capicua. Recent studies have indicated the role of Capicua inactivation on cancer metastasis and resistance to EGFR inhibitors,[20,21] and our data reinforce the clinical significance of CIC mutation on chemoresistance to RTK inhibitors in cancer.

We found that long-term exposure of HCC cell lines to sorafenib resulted in a reduction of Capicua protein expression with an enhanced MAPK signaling pathway, without CIC mutations. A recent study showed the role of Capicua inactivation on MAPK signaling pathway activation[33]; thus, CIC down-regulation with enhanced ERK phosphorylation is reasonable in sorafenib-resistant HCC cell lines. One question that remains to be addressed is how CIC can be down-regulated in HCC cell lines exposed to sorafenib for a long time because previous studies have shown that the short-term exposure of cancer cells to RTK inhibitors resulted in prompt activation of Capicua with a reduction of MAPK signaling target genes.[21] We could not identify the genetic alterations in Huh1-SR and Huh7-SR cells, which could explain the reduction in Capicua after long-term sorafenib exposure. CIC down-regulation in sorafenib-resistant HCC might be acquired through epigenetic, transcriptional, post-transcriptional, translational, or post-translational modification. Future studies are required to clarify the mechanisms of CIC inactivation and sorafenib resistance in HCC.

We found that sorafenib treatment resulted in a mild or modest increase in tumor mutational burden in post-sorafenib HCC (additional 17 mutations after 2 years of sorafenib exposure in human beings), Huh1-SR (additional 10 mutations after 3 months of exposure to sorafenib in vitro), and Huh7-SR (additional 46 mutations after 3 months of sorafenib exposure in vitro), with allele frequency exceeding 20%. These data suggest that exposure to sorafenib may result in clonal selection of sorafenib-resistant cells with a mild to moderate increase in genetic mutations.

Our data clearly show the survival benefit of regorafenib treatment after the acquisition of sorafenib resistance in HCC in mice. A recent phase III randomized study evaluated the effect of regorafenib in HCC patients who showed disease progression on sorafenib treatment (regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment), and clearly proved the survival benefits of regorafenib as a second-line treatment after

sorafenib.[13] Accordingly, regorafenib is approved as a second-line treatment for advanced HCC. Regorafenib is known to be a stronger inhibitor of RAF-1 and B-RAF kinase compared with sorafenib, and our data indicate that regorafenib treatment suppresses the phosphorylation of ERK1/2 and tumor growth even in sorafenib-resistant and CIC-inactivated HCC both in vitro and in vivo. A previous study showed loss of Capicua expression in approximately 45% of HCC,[19] so sorafenib may have no effect on these particular HCC tumors as frontline treatment. Indeed, our data indicated that approximately 60% of advanced HCC patients have loss of Capicua expression, and showed poor overall survival by sorafenib treatment. Regorafenib could overcome sorafenib resistance induced by functional Capicua impairment, and thus regorafenib may be effective to treat such HCCs as frontline treatment.

Regorafenib structurally resembles sorafenib, but differs from it by the addition of a fluorine atom in the central phenyl ring.[34] As a result, regorafenib and its metabolites M2 and M5 show more inhibitory effects on vascular endothelial growth factor receptor-2, platelet-derived growth factor receptor-β, fibroblast growth factor receptor-1, KIT proto-oncogene, receptor tyrosine kinase, RAF-1, and B-RAF.[35] Although the detailed mechanisms of regorafenib's inhibition of the MAPK signaling pathway irrespective of CIC inactivation remain elusive, we speculate that strong inhibition of RAF-1 kinase by regorafenib may explain this effect. Future studies are required, even though our data suggest that Capicua may be a useful biomarker to predict the response to sorafenib. This study paves the way for the development of precision medicine in patients with advanced HCC treated with RTK inhibitors.[36]

Materials and Methods
Patients

HCC tissue samples and PBMCs were obtained from a patient who had undergone resection at the Kanazawa University Hospital in Kanazawa, Japan. The patient subsequently was enrolled in the STORM trial, received sorafenib treatment for 2 years, and underwent surgery for recurrent HCC. Additional HCC tissue samples were obtained from 30 advanced HCC patients. These tissues were obtained using needle biopsy before sorafenib treatment. All patients provided written informed consent, and all tissue acquisition procedures were approved by the Ethics Committee of Kanazawa University.

Cell Culture and Reagents

Four representative HCC cell lines (Huh1, Huh7, HLE, and HLF) were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). These HCC cell lines were cultured routinely in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Sorafenib tosylate and regorafenib hydrate were kindly provided by Bayer (Leverkusen, Germany). Sorafenib tosylate (2.5–5 μmol/L) was supplemented in culture media for 3 months to generate sorafenib-resistant clones in Huh1 (Huh1-SR) and Huh7 (Huh7-SR) cells.

We established HCC cells (HCC-SR) obtained from a patient who was enrolled in the STORM trial to receive sorafenib and eventually developed HCC. Fresh HCC specimens were dissected and digested in 1 mg/mL type 4 collagenase (Sigma-Aldrich Japan K.K., Tokyo, Japan) solution at 37°C for 15–30 minutes. Contaminated red blood cells were lysed with ammonium chloride solution (Stemcell Technologies, Inc, Vancouver, Canada) on ice for 5 minutes. A single-cell suspension was inoculated into the subcutaneous lesions of NOD/SCID mice. The subcutaneous tumors that developed were dissected and digested and then used for cell culture in vitro.

DNA Extraction and Whole-Exome Sequencing

DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The Sure-Select Human All Exon V4 Kit (Agilent Technologies, Santa Clara, CA) was used for whole-exome capture, and the HiSeq 2000 Sequencing System (Illumina, Inc, San Diego, CA) was used for massive parallel sequencing. Sequence reads were mapped against the University of California, Santa Cruz Homo sapiens genome assembly 19 Genome Browser (available from: http://hgdownload.cse.ucsc.edu/goldenPath/hg19/chromosomes). Sequence variations, including single-nucleotide polymorphisms and insertions/deletions, were detected using the Genome Analysis Toolkit software (Broad Institute, Cambridge, MA). Whole-exome sequencing and analysis was performed at Riken Genesis (Tokyo, Japan). To predict the effect of nonsynonymous single-nucleotide substitutions on protein structure, function, and phenotype, we used tools available online, such as SIFT (available from: http://sift.jcvi.org) and Polyphen2 (available from: http://genetics.bwh.harvard.edu/pph2).

Cytotoxicity Assays

For cytotoxicity assays, single-cell suspensions of 2.0×10^3 cells were seeded in 96-well plates and cultured overnight. Then cells were treated with therapeutic reagents and cell density was evaluated at 48 hours after medium change using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. For evaluation of wild-type CIC DNA introduction on sorafenib-resistant HCC-SR cells, a total of 1×10^5 cells were seeded into 6-well plates at 24 hours before transfection. A total of 1 μg of pcDNA3.1$^+$ (Thermo Fisher Scientific, Waltham, MA) or pCMV6-CIC-Myc-DDK (OriGene Technologies, Inc, Rockville, MD) was transfected using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Cells were trypsinized 24 hours after transfection and 2×10^3 cells were seeded onto 96-well plates. Cell density was evaluated 72 hours after medium change as described earlier.

Cell Proliferation Assays

Single-cell suspensions of 2.0×10^3 cells were seeded on 96-well plates 24 hours after transfection of siRNAs and cultured for 72 hours. Cell density was evaluated using the Cell Counting Kit-8 (Dojindo Laboratories).

Western Blot

Whole-cell lysates were prepared using a radio-immunoprecipitation assay buffer. Rabbit polyclonal antibodies to ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, STAT3, phospho-STAT3 (Cell Signaling Technology, Inc, Danvers, MA), CIC (Thermo Fisher Scientific), and anti–β-actin antibody (Cell Signaling Technology, Inc) were used.

RNA Interference

SiRNAs specific to CIC (CIC 1, HSS118259; CIC 2, HSS118260) and negative control (12935200) siRNAs were designed and synthesized by Invitrogen. A total of 2×10^5 cells were seeded onto 6-well plates at 24 hours before transfection. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. A total of 40 nmol/L siRNA was used for each transfection in Huh1 and Huh7 cells.

Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions. Expression of selected genes was determined in triplicate using the 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Each sample was normalized relative to β-actin expression. The following probes were used: ETV4, Hs00383361_g1; MMP1, Hs00899658_m1; and ACTB, Hs999999903_m1.

Microarray Analysis

We purified RNAs from Huh7, HLE, and HLF cells treated with siRNAs specific to CIC (CIC 1 and CIC 2) or negative control 72 hours after transfection. Transcriptome analysis was performed using the GeneChip Human Gene 2.0 ST array (Thermo Fisher Scientific). Differentially expressed genes after CIC knockdown were detected by comparing control and CIC knockdown RNAs using the Welch t test ($P < .005$), performed at Riken Genesis. Microarray data of HCC samples enrolled in the STORM trial were obtained from Gene Expression Omnibus 109211.[22] Class prediction analysis was performed by BRB-ArrayTools (Biometric Research Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) software (version 4.6.0). Hierarchical clustering analysis was performed with the Genesis software (version 1.6) as previously described.[37]

Animal Studies

The study protocol was approved by the Kanazawa University Animal Care and Use Committee, and all procedures were performed in accordance with the guidelines and regulations of Kanazawa University. Six-week-old NOD/SCID mice were purchased from Charles River Laboratories, Inc (Wilmington, MA). We used 1.0×10^5 tumor cells (sorafenib-naïve Huh7, sorafenib-resistant Huh7, and HCC2 cells) suspended in 200 μL Dulbecco's modified Eagle medium and Matrigel (Corning, New York, NY) (1:1), which

then were injected subcutaneously into the flank of each mouse. Treatment of sorafenib or regorafenib against Huh7 and Huh7-SR was initiated when tumor volume reached approximately 1000 mm^3. The incidence and size of subcutaneous tumors was recorded, and mice were euthanized when tumor volume exceeded 4000 mm^3 and were regarded as dead. Sorafenib (30 mg/kg/day), regorafenib (20 mg/kg/day), or vehicle (control) was administered orally 5 days a week for 2–3 weeks 21 days after injection, according to previous studies.[38,39] For histologic evaluation, formalin-fixed paraffin-embedded tumor tissue sections were prepared.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed using EnVision+ Kits (Dako, Carpinteria, CA), according to the manufacturer's instructions. Anti–β-catenin monoclonal antibody clone 14 (BD Transduction Laboratories, San Jose, CA) and anti-CIC rabbit polyclonal antibodies (Sigma-Aldrich Japan) were used to detect β-catenin and Capicua expression in tissues. Nuclear staining intensities to anti-CIC antibodies were evaluated in each sample and defined as CIC high (similar or higher nuclear staining intensities compared with those of adjacent nontumor hepatocytes) or low, respectively. Immunofluorescence analysis was performed using anti-CIC rabbit polyclonal antibodies (Sigma-Aldrich Japan) and Alexa 488 fluorescein isothiocyanate–conjugated anti-rabbit IgG as primary and secondary antibodies, respectively. All images were obtained using a Biorevo BZ-9000 Fluorescence Microscope system (Keyence, Osaka, Japan).

Fluorescence-Activated Cell Sorting

Cultured cells were trypsinized, washed, and resuspended in Hank's balanced salt solutions (Lonza, Basel, Switzerland) supplemented with 1% HEPES and 2% fetal bovine serum. Cells then were incubated with antibodies on ice for 30 minutes. Labeled cells were analyzed by fluorescence-activated cell sorting using a FACSCalibur (BD Biosciences, San Jose, CA). Antibodies used were fluorescein isothiocyanate–conjugated anti-EpCAM monoclonal antibody clone Ber-EP4 (Dako).

Statistical Analysis

The Student t test was used to compare various test groups assayed by quantitative reverse-transcription polymerase chain reaction analysis, cell proliferation assays, and tumor size in vivo. Kaplan–Meier survival analysis with the log-rank test was performed to evaluate the role of Capicua expression status on survival outcome of HCC patients who received sorafenib treatment. Kaplan–Meier survival analysis was performed further to evaluate the survival benefit of regorafenib on sorafenib-resistant HCC in a xenograft model. As a humane end point, mice were euthanized when tumor volume exceeded 4000 mm^3 and they were considered dead. The Mann–Whitney, chi-squared, or Fisher exact tests were used to compare clinicopathologic backgrounds

of CIC high and low HCCs. All analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA).

All authors had access to the study data and reviewed and approved the final manuscript.

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