Activation of extracellular signal-regulated kinase is associated with hepatocellular carcinoma with aggressive phenotypes

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Aim: Sorafenib inhibits multiple kinase signaling pathways, including the rat sarcoma virus (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, and is a promising therapy for hepatocellular carcinoma (HCC). However, the role of ERK activation in HCC remains unclear. This study was designed to investigate the potential link between ERK activation and aggressive HCC phenotypes.

Methods: We evaluated nuclear ERK expression by immunohistochemistry in 154 resected HCC nodules from 136 patients. We then investigated the associations of ERK expression with the clinicopathological characteristics of HCC, c-MET expression, and the molecular subclass biomarkers Ki-67, keratin 19 (KRT19, CK19, or K19), and sal-like protein 4. Multivariate Cox regression analysis was carried out to determine independent prognostic factors for overall survival and recurrence-free survival. The effects of ERK activation by hepatocyte growth factor (HGF) on eight HCC cell lines were further examined.

Results: High-level nuclear expression of ERK was observed in 20 (13%) of 154 nodules and was significantly associated with higher serum alpha-fetoprotein levels (P = 0.034), poorer differentiation (P = 0.003), a higher Ki-67 index (P < 0.001), high-level expression of c-MET (P = 0.008), KRT19 (P = 0.002), or sal-like protein 4 (P < 0.001), and shorter overall survival (multivariate hazard ratio 3.448; P = 0.028) and recurrence-free survival (multivariate hazard ratio 2.755; P = 0.004). HCC cells treated with hepatocyte growth factor showed enhanced cell proliferation together with ERK activation and upregulated KRT19 expression, both of which were inhibited by sorafenib.

Conclusions: High-level ERK activation is associated with a KRT19-positive highly proliferative subtype of HCC with a dismal prognosis. These findings support the key role of the hepatocyte growth factor/c-MET/ERK axis in HCC progression.

Key words: extracellular signal-regulated MAP kinases, hepatocellular carcinoma, keratin 19, molecular targeted therapy, tumor biomarkers

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth leading cause of cancer death in adult men, and the seventh in adult women worldwide.1 A subset of HCC; that is, tumors expressing biliary/stem cell markers (including keratin 19 [KRT19, also known as cytokeratin 19, K19, or CK19] and sal-like protein 4) or those with a high proliferative activity, shows rapid tumor growth and a high metastatic rate,2–4 whereas most HCCs grow relatively slowly and are often treatable even after disease recurrence. Sorafenib, a multikinase inhibitor, is widely used as the first-line treatment for advanced HCC, especially HCC with extrahepatic spread or vascular invasion,5–7 although the antitumor effects are insufficient for controlling large and multinodular HCC, and for preventing tumor
recurrence in the surrounding inflamed liver after treatment. Other types of multikinase inhibitors have also proven effective against unresectable HCC, thereby underscoring the importance of signaling pathways triggered by tyrosine kinase receptors in the progression of HCC, which has a potential for promising new targeting therapies for HCC. However, biomarkers that can predict the response of HCC to these targeted therapies are currently unavailable. Consequently, to refine therapeutic strategies against HCC, it is essential to establish effective markers for tumors susceptible to multikinase inhibitors.

Extracellular signal-regulated kinase (ERK) is a downstream effector of the rat sarcoma virus (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase kinase (MEK) pathway, one of the main pathways targeted by sorafenib. Once activated by phosphorylation, phosphorylated ERK (pERK) translocates into the nucleus where it induces genes involved in cell proliferation and anti-apoptosis. pERK thereby promotes tumorigenesis and tumor growth in various types of cancer cells. Experimental evidence also shows that constitutive activation of cellular ERK1/2 is important for the proliferation and invasion of human HCC cells. The Ras/Raf/MEK/ERK pathway is activated by receptor tyrosine kinases, such as c-MET, which is overexpressed in HCC with high proliferative activity. Among the molecules involved in the Ras/Raf/MEK/ERK pathway, the Raf activation level was significantly associated with reduced overall survival and recurrence-free survival in patients with HCC, although the sample size of the study was relatively small (n = 81). Another study showed that the HCC expression level of pERK1/2 is an independent prognostic factor of overall survival. These lines of evidence suggest the importance of Ras/Raf/MEK/ERK pathway activation in the promotion of HCC progression; however, the clinicopathological and functional role of the ERK pathway in HCC remains to be fully elucidated.

By analyzing 154 surgically resected tumors with information on their histologically annotated molecular subclass, we set out to examine whether ERK activation reflects aggressive HCC phenotypes. Furthermore, we investigated the functional role of the ERK pathway in eight human HCC cell lines by means of hepatocyte growth factor (HGF) or sorafenib treatment.

METHODS

Patients and tissue samples

FROM JANUARY 2003 to December 2010, 205 HCC nodules in 157 consecutive patients were resected at Keio University Hospital in Tokyo, Japan. Of these, 51 HCC nodules in 21 patients met the following exclusion criteria: metastatic or recurrent tumors, pretreated tumors, tumors with massive necrosis, tumors with distinct adenocarcinoma components, liver transplantation cases, and specimens inappropriate for immunohistochemical evaluation. Consequently, 154 HCC nodules in 136 patients were analyzed in the present study. Curative surgery was carried out in 130 patients, and these were included in the survival analysis. This study was approved by the ethics committee of Keio University School of Medicine (approval number: 20040034).

A centralized review of the histopathological HCC diagnoses was carried out by a single pathologist (M.S.) based on the World Health Organization Classification of 2010 or the general rules of the Liver Cancer Study Group of Japan. The fibrosis stage in background liver was scored by the METAVIR (liver fibrosis) classification.

Evaluation of immunohistochemical markers

Immunohistochemical staining was carried out using a Bond-Max automated immunohistochemical staining machine (Leica Microsystems, Milton Keynes, UK). In ERK-positive cells, ERK was mainly detected in the cytoplasm. In some of these cells, ERK was also detected in the nucleus. In the present study, ERK activation was assessed using nuclear expression levels obtained immunohistochemically with anti-ERK1/2 antibody (rabbit; E10; 1:100; Cell Signaling Technology), pERK staining was observed in non-tumorous hepatocytes, which were used as negative internal controls (Fig. 1). In our validation study using an anti-pERK antibody (mouse; E10; 1:100; Cell Signaling Technology), pERK staining was exclusively observed in ERK tumors, whereas no distinct expression of pERK was seen in ERK+ tumors in 10 randomly selected ERK+ cases (Fig. S1). Therefore, we defined ERK+ as representing high-level nuclear expression of ERK (ERKhigh), and ERK+ and ERK− as having low-level nuclear expression of ERK (ERKlow).

The staining intensity of c-MET by monoclonal antibody (rabbit; D1C2; 1:500; Cell Signaling Technology) was assessed based on the modified c-MET expression scoring system used in a previous study: negative (c-MET−) = no staining observed in HCC cells; weak positive (c-MET+) = strong membrane staining in <10% of HCC cells or...
weak membrane staining in any HCC cells; strong positive (c-MET++) = strong membrane staining at least in 10% of HCC cells (Fig. 2). No distinct membrane staining was observed in non-tumorous hepatocytes, which served as negative controls. We defined c-MET++ as c-METhigh, and both c-MET+ and c-MET− as c-METlow. Immunohistochemical scoring of ERK and c-MET for all HCC nodules was carried out by two observers (T.M. and H.T.) using multi-head microscopy.

Cell lines and reagents
Hep3B, HepG2, and PLC/PRF/5 HCC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HLF and Huh-7 HCC cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). KIM-1, KYN-2, and Li7 were established as previously reported. A total of 24 h after plating, cells were treated with sorafenib (Nexavar, 5 μM; Bayer Pharma AG, Berlin, Germany) and/or HGF (10 μM; Sigma, St Louis, MO, USA).

In vitro assays
For Western blotting, anti-ERK1/2 (dilution 1:1000) or anti-pERK1/2 (dilution 1:1000) was used. The results were quantified using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). Cell viability was evaluated using WST assays with WST-1 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) after treatment with different concentrations of sorafenib and/or HGF for 48 h. The results are presented as the ratio of treated viable cells relative to viable control cells. For reverse transcription polymerase chain reaction analysis, total RNA
was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was generated using PrimeScripts RT Reagent Kit (Takara Biotechnology, Otsu, Shiga, Japan). All samples were analyzed in triplicate. The KRT19 mRNA levels were normalized using the glyceraldehyde 3-phosphate dehydrogenase mRNA level in each cell. The nucleotide sequences of primers for polymerase chain reaction were as follows: KRT19 (sense, 5′-GAAGAACCATGAGGAGGAAATCAG-3′; antisense, 5′-TTCAGCATCCITCCCGGTCTT-3′), c-MET (sense, 5′-ACCGGCTGAAGCGTAGTAAAG-3′; antisense, 5′-CGATTGCTCACGTGTTGTC-3′), FGFR4 (sense, 5′-TCTGGAGTCGCCGAAGACTG-3′; antisense, 5′-TATGTAATCATTGCTGGTGGAGC-3′), and GAPDH (sense, 5′-GCACGGTCAAGGCTGAGA-3′; antisense, 5′-ATGGTGTTGAAGACGCAGT-3′).

Statistical analysis
All analyses were carried out using SPSS, version 23.0 (IBM, Armonk, NY, USA). Correlations between categorical variables were tested using the χ²-test and Fisher’s exact test, and those between continuous variables were assessed using the Mann–Whitney U-test. Survival was analyzed using Kaplan–Meier curves and the log–rank test. Two cases of surgery-associated death were excluded from the survival analysis. Hazard ratios were estimated by univariate and multivariate survival analyses using the Cox regression model. Variables with P < 0.10 using the univariate log–rank test were further explored in the multivariate setting. Differences were considered statistically significant at P < 0.05.

RESULTS
Clinicopathological characteristics of ERK-activated HCC

We carried out immunohistochemical assessment of ERK1/2 in 154 HCC nodules from 136 patients. We found that 102 nodules (66.2%) showed nuclear ERK staining (ERK+ or ERK++) in tumor cells,
and that 20 nodules (13.0%) were characterized as ERK++ (i.e. ERKhigh; Fig. 1). ERKhigh HCC nodules showed higher serum levels of alpha-fetoprotein (P = 0.034), poorer differentiation (P = 0.003), and a higher Ki-67 labeling index (P < 0.001). Although not statistically significant, ERKhigh tended to be associated with portal venous invasion and/or intrahepatic metastasis (P = 0.050). ERKhigh was significantly correlated with high-level expressions of KRT19 (P = 0.002) and SALL4, sal-like protein 4; SN, simple nodular; SNEG, simple nodular with extranodular growth; SNIM, small nodular with indistinct margin; vp/im, portal venous invasion and/or intrahepatic metastasis

*P < 0.05.

At a median follow up of 69 months (range 2–154 months), overall survival after resection of ERKhigh HCC was significantly shorter than that of ERKlow HCC (P = 0.015; Fig. 3a). Three years after resection, recurrence-free survival of ERKhigh HCC was also significantly worse than that of ERKlow HCC (P = 0.043; Fig. 3b). Cox regression analyses were used to determine independent prognostic factors of HCC patients in our cohort. According to univariate analysis, nuclear ERK expression

Table 1. (Continued)

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<th>Variables</th>
<th>Nuclear ERK expression</th>
<th>P-value</th>
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<td></td>
<td>ERK&lt;sub&gt;high&lt;/sub&gt;</td>
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<td></td>
<td>ERK&lt;sub&gt;low&lt;/sub&gt;</td>
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<tr>
<td>≥30</td>
<td>9 (45.0%)</td>
<td>5 (3.7%)</td>
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Liver fibrosis (METAVIR)

F0–2                      | 11 (55.0%)              | 79 (59.0%)|
F3–4                      | 9 (45.0%)               | 55 (41.0%)|

†The χ<sup>2</sup>-test.
‡Fisher’s exact test.
§Mann–Whitney U-test.

and that 20 nodules (13.0%) were characterized as ERK++ (i.e. ERK<sub>high</sub>; Fig. 1). ERK<sub>high</sub> HCC nodules showed higher serum levels of alpha-fetoprotein (P = 0.034), poorer differentiation (P = 0.003), and a higher Ki-67 labeling index (P < 0.001). Although not statistically significant, ERK<sub>high</sub> HCC tended to be associated with portal venous invasion and/or intrahepatic metastasis (P = 0.050). ERK<sub>high</sub> was significantly correlated with high-level expressions of KRT19 (P = 0.002) and sal-like protein 4 (P < 0.001), both of which are putative biliary/stem cell HCC markers (Table 1).<sup>34,35</sup> In our exploratory analysis, we carried out c-MET immunohistochemistry in HCC samples (Fig. 2), and found a significant association between c-MET<sub>high</sub> HCC and ERK (P = 0.008; Tables 1, S1). In univariate survival analyses, c-MET<sub>high</sub> HCC was associated with early recurrence (P = 0.043), but was not significantly correlated with overall survival (P = 0.072; Fig. S2).

ERK activation is an independent prognostic factor for HCC

At a median follow up of 69 months (range 2–154 months), overall survival after resection of ERK<sub>high</sub> HCC was significantly shorter than that of ERK<sub>low</sub> HCC (P = 0.015; Fig. 3a). Three years after resection, recurrence-free survival of ERK<sub>high</sub> HCC was also significantly worse than that of ERK<sub>low</sub> HCC (P = 0.043; Fig. 3b). Cox regression analyses were used to determine independent prognostic factors of HCC patients in our cohort. According to univariate analysis, nuclear ERK expression
KRT19 expression ($P = 0.003$), and the Ki-67 labeling index ($P = 0.019$) were associated with overall survival. However, multivariate analysis showed that ERK$^\text{high}$ (hazard ratio [HR] 3.984, 95% confidence interval [CI] 1.290–12.346; $P = 0.016$), KRT19 expression (HR 5.294, 95% CI 1.488–18.868; $P = 0.010$), and tumor diameter $\geq$ 3 cm (HR 3.469, 95% CI 1.307–9.091; $P = 0.012$) were significant prognostic factors for overall survival (Table 2). The prognostic factors for early recurrence within 3 years after resection were ERK$^\text{high}$ (HR 2.755, 95% CI 1.385–5.464; $P = 0.004$), high KRT19 expression (HR 3.096, 95% CI 1.166–8.197; $P = 0.023$), and the presence of venous invasion and/or intrahepatic metastasis (HR 2.436, 95% CI 1.283–4.626; $P = 0.007$) by both univariate and multivariate analyses (Table 3).

HCC cells ERK-activated by HGF showed elevated proliferative activity and KRT19 upregulation

Immunohistochemical analyses showed a positive correlation between ERK activation and c-MET expression. Consequently, we investigated the functional role of ERK in HCC cell lines exposed to HGF, a c-MET ligand that triggers activation of the Raf/MEK/ERK pathway. Sorafenib, a multikinase inhibitor, is universally used to treat HCC. Sorafenib targets Raf and consequently suppresses ERK activation, so we use it to inhibit ERK activity. Although total ERK levels were almost unchanged in HCC cells when treated with HGF and/or sorafenib, pERK levels showed marked change, which indicates altered ERK activity on these treatments (Fig. 4a). Compared with control cells, HCC cells treated with HGF showed increased pERK levels and enhanced cell proliferation (Fig. 4a,b). In contrast, compared with control cells, sorafenib treatment decreased the pERK/ERK ratio in HepG2, Hep3B, Huh7, KIM1, and KYN2 cells. Nevertheless, cell proliferation was decreased in all the cell lines. There was no significant correlation between sensitivity to sorafenib and baseline levels of pERK among the eight HCC cell lines examined ($P = 0.531$ by Spearman’s correlation test; Fig. S3). Furthermore, compared with HGF treatment, treatment with sorafenib with HGF decreased the pERK/ERK ratio only in HepG2, Huh7, KIM1, and KYN2 cells. However, cell proliferation was decreased in all the cell lines (Fig. 4a,b). HepG2 and Hep3B cells treated with HGF showed upregulation of KRT19 at the mRNA level, which was inhibited by sorafenib (Fig. 4c). Expression levels of c-MET and fibroblast growth factor receptor 4 (FGFR4) were shown to vary among the cell lines (Fig. S4). In particular, expression levels of c-MET in HepG2 and Hep3B after HGF treatment were not distinct from those of other cell lines. In contrast, vascular endothelial growth factor receptor 2 (VEGFR2) was rarely expressed in HCC cell lines (data not shown). Expression levels of either c-MET or FGFR4 were not correlated with the pERK/ERK ratio after HGF administration ($P = 0.183$ and $P = 1.000$, respectively, by the Spearman’s correlation test; Figs 4a, S4).
DISCUSSION

ERK IS ACTIVATED by phosphorylation of its tyrosine residues, allowing it to migrate into the nucleus and activate transcription factors that regulate critical cellular biological processes including the cell cycle, apoptosis, and cell differentiation.\textsuperscript{14–16} In the present study, the activation of ERK in HCC tissues was immunohistochemically evaluated based on nuclear expression levels of ERK within tumor cells. ERK-activated (ERK\textsuperscript{high}) HCC was found in 20 (13\%) of 154 HCC nodules, and was characterized by a high serum alpha-fetoprotein level, poor tumor differentiation, and the presence of venous invasion and/or intrahepatic metastasis, which are all known malignant clinicopathological factors in HCC.\textsuperscript{38} Furthermore, patients with ERK\textsuperscript{high} HCC had significantly worse outcomes than those with ERK\textsuperscript{low} HCC, which is consistent with previous studies.\textsuperscript{23} We showed that ERK activation correlates with a higher Ki-67 labeling index in HCC, supporting the pivotal function of ERK facilitating proliferative

\begin{table}
\centering
\caption{Univariate and multivariate analyses of predictors for overall survival}
\begin{tabular}{llllllll}
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Variables & & Univariate analysis & & & Multivariate analysis & & \\
 & & & HR & 95\% CI & \(P\)-value & HR & 95\% CI & \(P\)-value \\
\hline
ERK & & & & & & & \\
Low & 1.000 & & & & & 1.000 & \\
High & 3.179 & 1.249–8.092 & 0.015* & 3.448 & 1.144–10.309 & 0.028* \\
c-MET & & & & & & & \\
Low & 1.000 & & & & & 1.000 & \\
High & 2.217 & 0.930–5.263 & 0.072 & 1.815 & 0.699–4.695 & 0.221 \\
KRT19 & & & & & & & \\
<5\% & 1.000 & & & & & 1.000 & \\
≥5\% & 5.343 & 1.801–15.850 & 0.003* & 5.650 & 1.629–19.608 & 0.006* \\
SALL4 & & & & & & & \\
<5\% & 1.000 & & & & & 1.000 & \\
≥5\% & 2.962 & 0.868–10.109 & 0.083 & 2.472 & 0.384–15.917 & 0.341 \\
Ki-67 index (%) & & & & & & & \\
<30 & 1.000 & & & & & 1.000 & \\
≥30 & 3.303 & 1.217–8.966 & 0.019* & 1.634 & 0.328–8.130 & 0.549 \\
Tumor differentiation & & & & & & & \\
Well/moderate & 1.000 & & & & & 1.000 & \\
Poor & 1.970 & 0.668–5.814 & 0.210 & & & & \\
AFP (ng/mL) & & & & & & & \\
<400 & 1.000 & & & & & 1.000 & \\
≥400 & 2.257 & 0.662–7.692 & 0.193 & & & & \\
PIVKA-II (mAU/mL) & & & & & & & \\
<400 & 1.000 & & & & & 1.000 & \\
≥400 & 1.572 & 0.597–4.149 & 0.359 & & & & \\
Macroscopic growth pattern & & & & & & & \\
SNIM/SN & 1.000 & & & & & 1.000 & \\
SNEG/CM/others & 1.379 & 0.866–2.198 & 0.176 & & & & \\
vp/im & & & & & & & \\
Absent & 1.000 & & & & & 1.000 & \\
Present & 1.943 & 0.657–5.744 & 0.230 & & & & \\
Tumor size (cm) & & & & & & & \\
<3 & 1.000 & & & & & 1.000 & \\
≥3 & 2.203 & 0.903–5.206 & 0.072 & 3.067 & 1.214–7.752 & 0.018* \\
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\end{tabular}
\end{table}

AFP, alpha-fetoprotein; CI, confidence interval; CM, confluent multinodular; KRT19, keratin 19; PIVKA-II, protein induced by vitamin K absence or antagonists-II; SALL4, sal-like protein 4; SN, simple nodular; SNEG, simple nodular with extranodular growth; SNIM, small nodular with indistinct margin; vp/im, portal venous invasion and/or intrahepatic metastasis

*\(P<0.05\)
activity.15,16 Previous studies also showed an important role for ERK in promoting migration and invasion of HCC cells.15,16 These lines of evidence, together with our findings, suggest that ERK activation represents a mechanistically driven prognostic biomarker in patients with HCC.

The ERK pathway is activated by various stimuli, including receptor tyrosine kinase and G protein-coupled receptors.39–41 A key role of the HGF/c-MET axis in activation of the downstream ERK signaling pathway has been identified in a number of cancer types, including HCC.19–21 In our in vitro study, administration of HGF induced ERK activation and promoted cell proliferation. Furthermore, our immunohistochemical analysis showed a significant correlation between high c-MET expression and ERK activation. Therefore, the HGF/c-MET axis might contribute, in part, to activation of the ERK pathway and its resulting cell proliferation in clinical HCC. However, a clinical trial of tivantinib, a c-MET inhibitor, for patients with HCC showed that overall survival was not improved

### Table 3 Univariate and multivariate analyses of predictors for recurrence-free survival

<table>
<thead>
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<th>Variables</th>
<th>Univariate analysis</th>
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<th>Multivariate analysis</th>
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<td>HR 95% CI</td>
<td>P-value</td>
<td>HR 95% CI</td>
<td>P-value</td>
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<td>ERK</td>
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<td>Low</td>
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<tr>
<td>High</td>
<td>1.842 (1.007–3.367)</td>
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<td>2.755 (1.385–5.464)</td>
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<tr>
<td>c-MET</td>
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<td>Low</td>
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<tr>
<td>High</td>
<td>1.704 (1.017–2.849)</td>
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<td>1.318 (0.724–2.398)</td>
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<td>KRT19</td>
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<tr>
<td>≥5%</td>
<td>3.559 (1.692–7.463)</td>
<td>0.001*</td>
<td>3.096 (1.166–8.197)</td>
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<td>SALL4</td>
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<td>&lt;5%</td>
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<td>≥5%</td>
<td>2.632 (1.297–5.348)</td>
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<td>2.132 (0.663–6.849)</td>
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<td>Ki-67 index (%)</td>
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<tr>
<td>≥30</td>
<td>1.961 (1.001–3.846)</td>
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<td>1.582 (0.508–4.926)</td>
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<td>Well/moderate</td>
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<td>Poor</td>
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<td>1.164 (0.438–3.096)</td>
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<tr>
<td>≥400</td>
<td>1.764 (0.840–3.704)</td>
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<td>PIVKA-II (mAU/mL)</td>
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<tr>
<td>≥400</td>
<td>1.508 (0.881–2.584)</td>
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<td>Macroscopic growth pattern</td>
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<td>SNEG/CM/Others</td>
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<td>vp/im</td>
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<td>Absent</td>
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<tr>
<td>Present</td>
<td>2.849 (1.597–5.081)</td>
<td>&lt;0.001*</td>
<td>2.433 (1.282–4.630)</td>
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<td>Tumor size (cm)</td>
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<tr>
<td>≥3</td>
<td>1.552 (0.956–2.519)</td>
<td>0.076</td>
<td>1.096 (0.610–1.972)</td>
<td>0.759</td>
</tr>
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</table>

AFP, alpha-fetoprotein; CI, confidence interval; CM, confluent multinodular; KRT19, keratin 19; PIVKA-II, protein induced by vitamin K absence or antagonists-II; SALL4, sal-like protein 4; SN, simple nodular; SNEG, simple nodular with extranodular growth; SNIM, small nodular with indistinct margin; vp/im, portal venous invasion and/or intrahepatic metastasis.

*P < 0.05.
in patients treated with tivantinib. Recent results from a phase III clinical trial showed the effectiveness of cabozantinib, an inhibitor of multikinases including c-MET, AXL, and VEGF receptors, in advanced HCC. Our in vitro analysis showed that the pERK/ERK ratio was not significantly correlated with c-MET expression in HCC cell lines. Therefore, other pathways besides the HGF/c-MET axis might be associated with ERK phosphorylation. FGF19 and VEGFA are known to be associated with ERK phosphorylation. Huh7 and Hep3B cells, which express relatively low levels of c-MET, but high levels of FGFR4, were reported not to be affected by c-Met inhibitor treatment. Together with findings in these studies and the present study, it was suggested that HCC cells might be highly dependent on specific signaling pathways for ERK phosphorylation. Furthermore, the present study additionally showed that other potent signaling pathways, which require no ERK phosphorylation and are inhibited by sorafenib, might also be associated with cell proliferation in HCC cells. Although multiple signaling pathways are involved in cell proliferation in HCC cells, the HGF/c-MET axis might be an important pathway in a fraction of HCC patients, because c-MET expression was associated with poor prognosis. Taken together, blockade of the HGF/c-MET/ERK signaling pathway would become a therapeutic option for patients with HCC, if adequate patient selection could be achieved.

Identification of aggressive molecular subtypes of HCC based on gene expression and protein expression profiles is increasingly important in clinical oncology. KRT19 expression in HCC is significantly associated with poor prognosis. KRT19 is an immunohistochemical marker for the biliary/stem cell subtype of HCC, based on the hypothesis that KRT19-positive HCC might be of stem cell origin. Intriguingly, we found a significant correlation between ERK activation and KRT19 expression in resected HCCs. Furthermore, enhanced KRT19 mRNA expression was induced in several HCC cell lines by HGF...
administration in vitro. Therefore, the present findings suggest that KRT19-positive HCC cases might be conditioned by the involvement of the HGF/c-MET/ERK axis, in addition to the innate KRT19 expression in HCCs. A recent study showed that KRT19 expression in HCC was regulated by the cancer-associated fibroblast-derived HGF through the c-MET/ERK/AP1 and SP1 axis, which is consistent with the present findings.⁵⁷ These findings might indicate that the HGF/c-MET/ERK axis is partly involved in the development of KRT19-expressing HCC.

The link between high-level activation of ERK and the therapeutic response of HCC to sorafenib is well established.⁵⁸,⁵⁹ However, the sensitivity to sorafenib did not positively correlate with basal pERK levels among the HCC cell lines we examined. These conflicting findings probably result from differences between the human tumor microenvironment and that of conventional 2-D cell culture. Further mechanistic studies utilizing models that closely mimic human HCC, such as patient-derived xenografts or organoid culture models, will hopefully elucidate the role of ERK in predicting the response to sorafenib therapy.

We recognize our immunohistochemical assessment of ERK activation as a limitation. In general, cellular activation of ERK is investigated using pERK levels. However, we found that nuclear immunostaining by pERK antibody was severely limited compared with that of anti-ERK1/2 antibody, suggesting that ERK1/2 proteins phosphorylated on Thr202/Tyr204 were likely unstable during specimen handling, conventional tissue fixation, and paraffin embedding. Nevertheless, our additional validation analyses showed that pERK staining was selectively found in ERK+ + HCC, but not in any ERK− or ERK+ HCCs. Consequently, we considered ERK++ tumors as having high-level ERK activation, and defined ERK+ tumors as having low-level ERK activation to avoid overestimation of the biological activity.

In conclusion, high-level ERK activation is closely associated with a KRT19-positive highly proliferative subtype of HCC with a dismal prognosis. The present results support the findings of previous experimental studies and clinical trials showing the critical role of the HGF/c-MET/ERK axis in the progression of HCC. Hopefully, this investigation will inform translational research to refine kinase inhibitor-based therapeutic strategies for HCC.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Immunohistochemical evaluation of tumor expressions of extracellular signal-regulated kinase (ERK) 1/2 and phosphorylated ERK1/2 (pERK1/2) in hepatocellular carcinoma specimens.

**Figure S2.** Survival analyses according to c-MET expression.

**Figure S3.** Relationship between baseline expression levels of phosphorylated of extracellular signal-regulated kinase (pERK) and sensitivity to sorafenib in eight hepatocellular carcinoma cell lines.

**Figure S4.** Evaluation of expression levels of c-MET and fibroblast growth factor receptor 4 in eight hepatocellular carcinoma cell lines.

**Table S1.** Clinicopathological characteristics stratified by c-MET expression levels in 154 hepatocellular carcinoma nodules from 136 patients