P21-Activated Protein Kinase Is Overexpressed in Hepatocellular Carcinoma and Enhances Cancer Metastasis Involving c-Jun NH2-Terminal Kinase Activation and Paxillin Phosphorylation

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Abstract

Hepatocellular carcinoma (HCC) is one of the major malignancies in the world. The prognosis of HCC is poor, due to frequent intrahepatic metastasis and tumor recurrence. P21-activated protein kinase (Pak1), a main downstream effector of small Rho GTPases, Rac1 and Cdc42, plays an important role in the regulation of cell morphogenesis, motility, mitosis, and angiogenesis. Here, we show that Pak1 gene was overexpressed in human HCCs. Overexpression of Pak1 in human HCCs was associated with more aggressive tumor behavior in terms of more metastatic phenotype and more advanced tumor stages. In addition, HCC cell line stably expressing Pak1 displayed increased cell motility rates and, conversely, knockdown of endogenous Pak1 expression by small interfering RNA reduced the migration rates of HCC cells. In an established metastatic HCC cell line, we found that Pak1 was overexpressed compared with its primary HCC cell line and this overexpression was associated with higher cell motility. Importantly, we found that c-Jun NH2-terminal kinase (JNK) was activated in HCC cells lines overexpressing Pak1. Inhibition of the JNK activity by chemical inhibitor significantly reduced the migration rates of HCC cells via attenuation of paxillin phosphorylation at Ser178. In conclusion, our results document that Pak1 is overexpressed in HCCs and plays an important role in the metastasis of HCC. The mechanism by which Pak1 induces cancer metastasis may involve activation of JNK and phosphorylation of paxillin. [Cancer Res 2007;67(8):3601–8]

Introduction

Hepatocellular carcinoma (HCC) is a major malignancy worldwide (1) and has high incidences of tumor recurrence and metastasis. Despite improvements in treatment results, the overall prognosis of HCC is still unsatisfactory.

P21-activated protein kinase 1 (Pak1) is a main downstream effector of small Rho GTPases Rac1 and Cdc42 and plays an important role in the regulation of cell morphogenesis, motility, survival, mitosis, and angiogenesis (2, 3). Amplification of Pak1 has been found in several human cancers, including colorectal and breast cancer (4, 5). In addition, emerging evidence has suggested that Pak1 is required for progression and metastasis of breast cancer by mediating growth factor-induced motility and invasiveness (6, 7). More recently, Pak1 expression has been shown to significantly increase in colorectal cancer metastasis to lymph nodes (5). These results suggest that Pak1 is potentially important in carcinogenesis and cancer metastasis.

The molecular mechanism by which Pak1 contributes to carcinogenesis is unclear. Pak1 is believed to be involved in several cell signaling pathways. For instance, activation of Pak1 and its downstream signaling pathways, such as mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB), are believed to be important in carcinogenesis (8). Activation of Pak1 not only activates the NF-κB cell survival pathway but also inhibits apoptosis via phosphorylation of the proapoptotic protein Bad, thereby providing a growth advantage to tumor cells (9, 10). In addition, expression of dominant-inactive form of Pak1 in Rat1 fibroblasts can block Ras-induced cell transformation, indicating that Pak1 plays a role in cell transformation and Ras signaling (11). Furthermore, expression of dominant-active form of Pak1 in breast cancer cells induces the expression of vascular endothelial growth factor, suggesting that Pak1 is also involved in angiogenesis (12).

The role of Pak1 in hepatocarcinogenesis has never been explored. In this study, we sought to examine the expression of Pak1 in human HCCs and characterize its roles in HCC. Our data indicate that Pak1 expression was significantly up-regulated in human HCCs. This overexpression was associated with more aggressive tumor behavior. Pak1 also enhanced the motility of HCC cells as well as the phosphorylation of c-Jun NH2-terminal kinase (JNK) and paxillin. Our findings define a novel Pak1/JNK/paxillin pathway critically involved in the metastasis of HCC.

Materials and Methods

Materials. Anti-Pak1, anti–phospho-Pak1, anti-JNK, anti–phospho-JNK, anti-p42/44 MAPK, anti–phospho-MAPK, anti-Akt, and anti–phospho-Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-Rac1 and anti-Cdc42 antibodies were from BD Biosciences (Palo Alto, CA) and anti-paxillin antibody was from Upstate (Charlottesville, VA). Rabbit anti–green fluorescent protein (GFP) and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–phospho-S178 paxillin antibody was from Abcam (Cambridge, United Kingdom). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Plasmids. The Pak1 construct, pCMV6-Pak1, was a generous gift from Dr. Gary Bokoch (The Scripps Research Institute, La Jolla, CA). Plasmid pEGFP-Pak1 was constructed by subcloning a BamHI/EcoRI fragment containing full-length cDNA of Pak1 into pEGFP vector (Clontech, Palo Alto, CA) via the BglII/EcoRI sites. The Pak1 dominant-inactive mutant was constructed by mutating the lysine at residue 299 to arginine (pEGFP-Pak1 K299R).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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K299R) using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) as per protocol suggested by manufacturer. The mutagenic primer sequence was 5'-GTGGCATTAGCCAGTAGAATTCTC.

Tissue culture and stable clone selection. HepG2 (American Type Culture Collection, Manassas, VA), H2M, and H2P cells (generous gifts from Dr. X.Y. Guan, Department of Clinical Oncology, University of Hong Kong, Pokfulam, Hong Kong; ref. 13) were maintained in DMEM supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 100 units each of penicillin and streptomycin. Cells were transfected with 3 μg DNA constructs using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) as per protocols recommended by the manufacturer. For RNA interference (RNAi) of Pak1, 150 pmol each of Pak1 small interfering RNA (siPak1) and control siRNA (both from Cell Signalling Technology) were separately transfected into HepG2. Forty-eight hours after transfection, G418 at 0.8 mg/mL was added into culture for selection of stable clones.

Reverse transcription-PCR. Total RNA was extracted from tumors and their corresponding nontumorous liver samples from HCC patients using the Trizol reagent (Life Technologies, Inc., Grand Island, NY), and 2 μg each RNA sample was used to prepare cDNA. The semiquantitative PCR primer sequences for Pak1 were 5'-CGTGGCTACATCTCCCATTT (forward) and 5'-TCTCATTAGCCAGTAGATTC (reverse). Quantitative real-time PCR was done using the Applied Biosystems Taqman system (Foster City, CA; Pak1 probe, 5’-CCTCATGACCAGGATCTC (reverse). Quantitative real-time PCR was done using the Applied Biosystems Taqman system (Foster City, CA; Pak1 probe, 5’-CCTCATGACCAGGATCTC (reverse). Quantitative real-time PCR was done using the Applied Biosystems Taqman system (Foster City, CA; Pak1 probe, 5’-CCTCATGACCAGGATCTC (reverse).

Western blot analysis. Human tissue samples and HCC cells were harvested into radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 5 mmol/L sodium fluoride, 1 mmol/L DTT] with freshly added protease inhibitor cocktail (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 2 μg/mL aprotinin, and 2 μg/mL soybean trypsin inhibitor). The cell lysate was cleared by centrifugation at 4°C and the supernatant was stored in small aliquots at −80°C. Normally, 20 μg sample was loaded into each lane, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with respective antibodies.

Transwell cell migration, wound healing, and proliferation assay. The methods for Transwell cell migration, wound healing, and proliferation assay were described previously (14). The best fit curve of growth doubling time for proliferation assay was calculated using GraphPad prism software (GraphPad Software, Inc., San Diego, CA).

Cell spreading assay. Stably transfected cells (1 × 10^5) were seeded in triplicates onto a 12-well plate that had been coated with fibronectin (Calbiochem) at 10 μg/mL for 1 h at room temperature and washed with PBS. Cells were allowed to attach onto the plate. Unattached cells were washed away with PBS and attached cells were trypsinized and counted at different time intervals.

Immunohistochemistry. The method for immunohistochemical staining was described previously (15). Briefly, formalin-fixed paraffin sections were stained for Pak1 and phospho-paxillin (1:500 and 1:1,000 dilutions, respectively) using the streptavidin-biotin immunoperoxidase technique. Membranous and cytoplasmic staining of Pak1 were scored as underexpression (T<NT), normal (NT=T), and overexpression (T>NT) of membranous and cytoplasmic staining of Pak1.

**Figure 1.** Overexpression of Pak1 in primary HCCs. A, box plot, quantitative real-time RT-PCR results of HCCs and corresponding nontumorous samples. ***, P < 0.001, t test. B, top, representative results of Western blot analysis of HCC (T), nontumorous tissue (NT), and normal liver tissue sample (NL); bottom, relative expression (T/NT) of Pak1 after quantification by scanning. C, left, representative immunohistochemical cytoplasmic and membranous staining of HCC (T) and nontumorous liver (NT); right, quantification of immunohistochemical results of 29 HCC cases showing underexpression (T<NT), normal (NT=T), and overexpression (T>NT) of membranous and cytoplasmic staining of Pak1. Membranous (NT) and Membranous (T) were stained for Pak1 and phospho-paxillin (1:500 and 1:1,000 dilutions, respectively) using the streptavidin-biotin immunoperoxidase technique.
Antigen retrieval was achieved by microwave treatment with citrate buffer at pH 6.0 at 95°C for 9 min. The immunohistochemical staining was scored in the following grades according to the percentage of positive hepatocytes: 0, <10% positive; 1, 10% to 30% positive; 2, 31% to 75% positive; and 3, >75% positive.

Clinical HCC samples and patients. Paired samples of primary HCCs and the corresponding nontumorous liver tissues from Chinese patients were collected at the time of surgical resection at The University of Hong Kong, Queen Mary Hospital (Pokfulam, Hong Kong). All specimens were obtained immediately after surgical resection, snap frozen in liquid nitrogen, and kept at −70°C. The diagnosis of recurrence was based on typical imaging findings on computerized tomographic scan or arteriography, and if necessary, percutaneous fine-needle aspiration cytology. Disease-free survival was measured from the date of hepatic resection to the date of death or last follow-up. Overallsurvival was measured from the date when recurrent diseases were diagnosed or, in the absence of detectable tumor, to the date of death or last follow-up. Overall survival was measured from the date of hepatic resection to the date of death or last follow-up.

**Statistical analysis.** Fisher’s exact or χ² test was used for statistical analysis of categorical data, whereas independent t test was used for continuous data. Tests were considered significant when their P values were <0.05.

**Results**

Pak1 was overexpressed in human HCCs. To determine if Pak1 transcript was overexpressed in HCC, paired samples of tumor and their corresponding nontumorous tissues were analyzed using real-time quantitative reverse transcription-PCR (RT-PCR). After normalization with 18S RNA control, 75% (27 of 36) of the HCC samples were found to have a higher expression of Pak1 transcript (>2-folds) in the tumors compared with their nontumorous livers (P = 0.003), and shorter disease-free survival (P = 0.022; Table 1).

**Overexpression of Pak1 enhanced cell migration and cell spreading.** To characterize the effects of Pak1 in HCC, we used HepG2 hepatoma cells to establish stable cell lines overexpressing the enhanced GFP (EGFP) fusion form of Pak1. Ectopic expression of Pak1 in the stably transfected cells (WT-5 and WT-7) was confirmed by anti-GFP antibodies (Fig. 1A). An increased level of Pak1 protein was observed in the tumor tissues compared with the corresponding nontumorous livers. The protein loading was normalized with β-actin control, and a normal liver tissue sample was included for comparison.

### Table 1. Association of overexpression of Pak1 mRNA with clinicopathologic features in human HCCs

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>Pak1 expression</th>
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<tbody>
<tr>
<td></td>
<td>Not overexpressed</td>
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<td>Venous invasion (presence of venous invasion)</td>
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<tr>
<td>Absent</td>
<td>8</td>
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<tr>
<td>Present</td>
<td>1</td>
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<td>Tumor microsatellite (microsatellite mutation)</td>
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<tr>
<td>Absent</td>
<td>9</td>
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<td>Present</td>
<td>4</td>
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<td>Liver invasion (absence of liver invasion)</td>
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<tr>
<td>Absent</td>
<td>10</td>
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<td>Present</td>
<td>2</td>
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<td>Cellular differentiation (Edmondson's grading)</td>
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<tr>
<td>I–II</td>
<td>8</td>
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<td>III–V</td>
<td>1</td>
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<td>Tumor size (cm) (Edmondson’s grading)</td>
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<tr>
<td>≤5</td>
<td>5</td>
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<tr>
<td>&gt;5</td>
<td>4</td>
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<td>Tumor stage</td>
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<tr>
<td>I–II</td>
<td>7</td>
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<tr>
<td>III–V</td>
<td>2</td>
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<td>Tumor encapsulation (absence of tumor encapsulation)</td>
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<tr>
<td>Absent</td>
<td>7</td>
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<tr>
<td>Present</td>
<td>6</td>
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<tr>
<td>Disease-free survival (mo)</td>
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<tr>
<td>Median</td>
<td>Not reached</td>
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<tr>
<td>Mean</td>
<td>43.1 ± 7.7</td>
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<tr>
<td>Overall survival (mo)</td>
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<tr>
<td>Median</td>
<td>Not reached</td>
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<tr>
<td>Mean</td>
<td>49.2 ± 5.9</td>
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</table>
To address the mechanism by which Pak1 enhanced migration, we did the cell spreading assay. Cells were allowed to spread onto the fibronectin-coated plate and the number of cells attached against time was determined. We found that the two stable clones required significantly longer time for attachment compared with the EGFP control cells (Fig. 2C).

Expression of Pak1 down-regulated stress fiber formation. We tested if Pak1 regulated the actin polymerization to increase the migration rates. The WT-5 stable clone was serum starved and followed by treatment with lysophosphatidic acid (LPA) to stimulate the formation of stress fibers and focal adhesion complexes. As revealed by phalloidin staining, much fewer stress fibers were formed in the WT-5 cells compared with the control (Fig. 2D). The focal adhesion complex was fewer, but larger, in the LPA-stimulated WT-5 stable clone than the control. These results suggest that Pak1 enhances migration by down-regulating stress fiber and focal adhesion complex formation.

Knockdown of endogenous Pak1 by siRNA reduced HCC cell migration. To confirm the role of Pak1 on cell migration, we used RNAi to specifically knockdown endogenous Pak1 in HCC cells. The specific knockdown of endogenous Pak1 protein using siRNA in HepG2 cells was confirmed with immunoblotting (Fig. 3A). In Transwell assay, HepG2 cells transfected with Pak1 siRNA migrated much more slowly (~50%) than those treated with control siRNA, suggesting that loss of Pak1 retarded the migration of HCC cells (Fig. 3A). To further examine if the Pak1 kinase activity was essential for the HCC cell migration, the WT and a dominant-inactive form of Pak1 (Pak1 K299R) were transfected into HepG2 cell for Transwell assay. As compared with the GFP control, the WT form of Pak1 promoted the migration of HepG2 cells, whereas the dominant-inactive form inhibited their migration (Fig. 3B).

In addition, we examined Pak1 expression in a pair of primary and its corresponding metastatic HCC cell lines (i.e., H2P and H2M cells, respectively; ref. 13). H2P and H2M cells were derived from a HCC patient with intrahepatic metastasis, and H2P cells were isolated from the primary cancer, whereas H2M cells were isolated from its occlusive tumor venous thrombus. The expression of Pak1 at both mRNA and protein levels was much higher in H2M than in H2P cells, indicating that Pak1 may play a role in the metastasis of HCC (Fig. 3C). To explore if Pak1 regulates the H2M cell migration, we did the Pak1 siRNA knockdown experiment in H2M cells. The knockdown of Pak1 expression with two different siRNAs in the metastatic H2M cells remarkably reduced the migration rate of the cells (Fig. 3D).

Overexpression of Pak1 had no effect on the proliferation rate of HCC cells. Overexpression of Pak1 has been reported to increase cell proliferation in breast cancer by up-regulating the expression of cyclin D1 (17). However, the role of Pak1 in the progression of HCC remained elusive. To address if overexpression of Pak1 promoted cell growth, cell proliferation assay was done. The effect of Pak1 on HCC cell proliferation was mild, and the doubling time of Pak1 stable cell lines, WT-5 and WT-7, (~26 h) was only slightly shorter than that of the EGFP control (~30 h; Fig. 4). In addition, we found no significant difference of cyclin D1 expression between the control and Pak1-overexpressing HCC cell lines (Fig. 4).

Phosphorylation of Pak1 and activation of its downstream targets in HCC. To understand how Pak1 induced HCC cell migration, we examined the activity and downstream signaling of Pak1 in human HCCs. Three pairs of human HCC samples with Pak1 overexpression (Fig. 5A, cases 216, 217, and 220) and an HCC with normal expression of Pak1 (Fig. 5A, case 211), based on the result of RT-PCR, were randomly selected for the analysis of phosphorylation of Pak1 and its downstream substrates. Pak1, but not its upstream regulators, Cdc42 and Rac1, were overexpressed in all three selected samples. In addition, phosphorylation of Pak1 was obviously increased in human HCCs with Pak1 overexpression compared with the corresponding nontumorous liver tissues, suggesting that the activity of Pak1 may be up-regulated in HCCs (Fig. 5A). We observed that one of the well-defined downstream effectors of Pak1
Phosphorylation was increased with increasing dosage of HGF in H2M cells, and this was similar for the phosphorylation of JNK and paxillin at S178 (Fig. 6A), suggesting that they may regulate in a similar pathway.

Activation of Pak1 in HGF-treated H2M cells is likely to be regulated by activation of Cdc42, but not by Rac1, which had a high basal activity in H2M cells (Supplementary Fig. S1). To further delineate the signaling pathway, we used Pak1 siRNA and JNK inhibitor SP600125 to specifically inhibit the activity of Pak1 and JNK, respectively. Inhibition of JNK activity almost completely abolished the phosphorylation of paxillin S178, whereas knock-down of Pak1 expression substantially reduced JNK and paxillin S178 phosphorylation (Fig. 6B).

To confirm the role of S178 paxillin phosphorylation in HCC, we examine our human HCCs with immunohistochemical staining. Our result showed that the expression of S178 paxillin in Pak1-overexpressing human HCCs was substantially increased in the tumor cells, localizing to the nuclei (Fig. 6C). Taken together, these findings suggest a novel pathway in which Pak1 induces phosphorylation of JNK, which in turn phosphorylates paxillin at S178, thus promoting HCC cell migration.

Discussion

In this study, we examined the role of Pak1 in HCC. Pak1 was frequently (~75%) and significantly overexpressed in human HCCs. Importantly, Pak1 overexpression significantly correlated with more aggressive tumor behavior in terms of venous invasion, poorer cellular differentiation, more advanced tumor stage, and shorter disease-free survival. These data have established that up-regulation of Pak1 occurs in HCCs and is associated with a metastatic phenotype.
We have shown that Pak1 was overexpressed at both mRNA and protein levels in our human HCC samples. The mechanism of this overexpression is currently unclear, but it has been reported that chromosome region 11q13-14, which contains the Pak1 gene, is frequently amplified in HCCs (19). The possibility that the enhanced Pak1 expression is due to aberration in promoter activity and/or protein turnover cannot be completely ruled out. In this study, we have documented that not only was the Pak1 overexpressed, the Pak1 activity was also increased in HCCs, as revealed by the phosphospecific antibody that recognizes the activated form of Pak1.

Several lines of evidence here have implicated that over-expression and activation of Pak1 is causally associated with HCC metastasis through the stimulation of cell mobility. First, we have shown that overexpression of Pak1 in human HCCs was associated with a more metastatic phenotype and a shorter disease-free survival. Second, the expression of Pak1 at both mRNA and protein levels was significantly higher in H2M cells derived from a metastatic HCC than in H2P cells derived from the corresponding primary human HCC. Third, HCC cell lines stably expressing Pak1 had higher migration rate, whereas those with knockdown of endogenous Pak1 using siRNA had significantly reduced cell migration rates.

To investigate the underlying mechanism, we have shown that the ability of HCC cells to form stress fibers was attenuated by overexpression of Pak1. Furthermore, inhibition of JNK abolished Pak1-induced cell migration in HCC cells. Recently, it has been shown that phosphorylation of paxillin at serine residue 178 by JNK promotes cell migration (18). In this study, we showed that JNK was phosphorylated and activated in both clinical samples and HCC cell lines stably expressing Pak1. Moreover, we showed that paxillin phosphorylation at S178 by JNK was important for Pak1-mediated migration of HCC cells. Consistent with this notion, we also noticed that the basal phosphorylation level of S178 paxillin in H2M cells was high (Fig. 6B, lanes 1 and 2) and correlated with the highly metastatic property of the cells. With immunohistochemical staining, there was a substantial increase in the S178-phosphorylated paxillin in Pak1-overexpressing human HCCs. Although paxillin is phosphorylated by other kinases [e.g., focal adhesion kinase (20) and ERK (21)], JNK is the only kinase having been reported to phosphorylate paxillin at S178. The possibility that paxillin S178 is the target of other kinases is currently under investigation, but our data have provided evidence that JNK is involved in the regulation of paxillin phosphorylation in HCCs.
established the first evidence that paxillin S178 phosphorylation is associated with metastasis of HCC.

It has been reported that the main upstream regulators of Pak1 (i.e., Rac1 and Cdc42) are up-regulated in breast cancer (22). To address the issue of Pak1 activation in HCCs, we have examined the expression of Rac1 and Cdc42 in clinical samples but observed no significant difference of these regulators at protein level. Although the best evidence is to examine the activity state of these small G-proteins by GTP loading activity pull-down assay, it is still a technical challenge to use clinical samples for this assay. Pak1 has been reported to be activated via several other Cdc42/Rac1–independent mechanisms, such as interaction with lipid/sphingosine (23), filamin A (24), and adaptor protein Nck (25) and activation of phosphatidylinositol 3-kinase/Akt (26) and heterotrimeric G-protein (27).

Particularly, we have observed that, with immunohistochemical analysis, some of the Pak1-overexpressing human HCCs displayed intense membranous staining, implying that Pak1 can be targeted to plasma membrane for activation in HCC. Consistent with this finding, we observed that the overexpressed EGFP-Pak1 protein in Pak1-stable cell line also localized mainly in cytoplasm and plasma membrane, as different from the EGFP control, in which GFP was present in both nucleus and cytoplasm (Supplementary Fig. S2). Thus, we would like to propose that the cytoplasmic accumulation of Pak1 due to gene overexpression may enhance the translocation of Pak1 onto the plasma membrane, leading to the activation of Pak1 as well as the increase in cell migration.

In this study, we observed little difference in the cell proliferation rates between cells stably expressing Pak1 and the parental cells. Although Pak1 has been shown to promote proliferation of breast cancer cells (17), it is possible that Pak1 will have different roles in the development of HCC. In fact, consistent with our in vitro finding in HCC cell lines, Pak1 overexpression was not associated with tumor size in our human HCCs. Thus far, our findings implicate that the major role of Pak1 in HCC is to promote cell migration.

In conclusion, we have shown that Pak1 is overexpressed in human HCCs and HCC cell lines and that Pak1 overexpression plays an important role in cancer metastasis. The mechanism by which Pak1 induces cancer metastasis may involve activation of JNK and phosphorylation of paxillin.

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