Rho Kinase Phosphorylation Promotes Ezrin-Mediated Metastasis in Hepatocellular Carcinoma

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Abstract

During progression of hepatocellular carcinoma, multiple genetic and epigenetic alterations act to posttranslationally modulate the function of proteins that promote cancer invasion and metastasis. To define such abnormalities that contribute to liver cancer metastasis, we carried out a proteomic comparison of primary hepatocellular carcinoma and samples of intravascular thrombi from the same patient. Mass spectrometric analyses of the liver cancer samples revealed a series of acidic phospho-isotypes associated with the intravascular thrombi samples. In particular, we found that Thr567 hyperphosphorylation of the cytoskeletal protein ezrin was tightly correlated to an invasive phenotype of clinical hepatocellular carcinomas and to poor outcomes in tumor xenograft assays. Using phospho-mimicking mutants, we showed that ezrin phosphorylation at Thr567 promoted invasion by hepatocarcinoma cells. Phospho-mimicking mutant ezrinT567D, but not the nonphosphorylatable mutant ezrinT567A, stimulated formation of membrane ruffles, suggesting that Thr567 phosphorylation promotes cytoskeletal-membrane remodeling. Importantly, inhibition of Rho kinase, either by Y27632 or RNA interference, resulted in inhibition of Thr567 phosphorylation and a blockade to cell invasion, implicating Rho kinase-ezrin signaling in hepatocellular carcinoma cell invasion. Our findings suggest a strategy to reduce liver tumor metastasis by blocking Rho kinase-mediated phosphorylation of ezrin. Cancer Res; 71(5); 1721–9. ©2011 AACR.

Introduction

Hepatocellular carcinoma presents a major health threat in South-East Asia, especially in China. It ranks third among all malignancies both in incidence and mortality in China and accounts for approximately 42.5% of the total incidence worldwide (1, 2). Despite many years of sustained efforts, the long-term outcome employing current therapies remain dismal as both recurrences of the lesions within the liver and distant metastases continue to increase. Therefore, a better understanding of tumor dissemination and growth is of central importance for optimizing treatment outcome. Cancer metastasis is a multistep process that involves cell detachment from the primary tumor, entry into the vascular or lymphatic system, dispersal through the circulation, and extravasation and proliferation in the target organ (3, 4). Ezrin is an actin-binding protein of the ezrin/radixin/moesin (ERM) family of cytoskeleton-membrane linker proteins involved in a variety of actin-based cellular dynamics (5, 6). Our previous studies showed that ezrin interacts and codistributes with cytoplasmic β-actin at the apical membrane of epithelial cells (7, 8). Recently, we have showed the importance of the phospho-regulation of ezrin in membrane-cytoskeletal remodeling and elucidated how protein phosphorylation regulates the molecular plasticity of ezrin (9, 10). Using mouse genetics, Tamura and colleagues showed the physiology of ezrin in animals by a knock-down approach, resulting in a low-level expression of ezrin in mice (<5% of the wild-type levels). This resulted in severe defects in vesicular membrane trafficking in gastric parietal cells, thus confirming the role of ezrin in orchestrating membrane-cytoskeletal dynamics (11). Aberrant regulation of ezrin activity has been associated with tumor progression in several cancers including HCC, the pediatric solid tumors, rhabdomyosarcoma, and osteosarcoma (12). Yeh et al. (2009; ref. 13) reported 104 cases of HBV-HCC patients in a prospective follow-up study showing the correlation between ezrin expression level and disease outcome. The results suggest that ezrin overexpression may contribute to dedifferentiation and invasion of HBV-HCC cells. HBV-HCC patients with ezrin overexpression were independently associated with smaller size tumor, cirrhotic liver background,
poor differentiation, and vascular invasion. However, it is unclear whether ezrin activity and plasticity is altered in the aforementioned conditions and whether aberrant ezrin expression has a causative role in disease progression.

Recent studies suggest that ezrin is dynamically phosphorylated during osteosarcoma metastasis (14). However, it is unclear whether or not phospho-Thr567 contributes to the malignancy of HCC. To delineate the phospho-regulation of ezrin in liver cancer progression and/or metastasis, we carried out proteomic analyses of paired samples of the primary and metastatic HCC tumor. Our analyses show that ezrin protein phosphorylation at Thr567 is indeed correlated with the invasiveness of HCC in vitro and HCC metastasis in vivo.

Materials and Methods

Cell culture
HepG2 cell line was purchased from ATCC in July 2009, and maintained as per ATCC instruction.

Tumor sample collection & proteomic analyses
HCC and metastasis was diagnosed on the basis of typical clinical and radiological findings, and also confirmed by pathology. The histological definition of HCC was based on the classification proposed by the World Health Organization and the protocol was approved by the Ethics Committee of Xijing Hospital. Matched pairs of primary HCC and samples of intravascular thrombi from the same patient were collected for parallel histological and proteomic studies.

Primary HCC tissue and metastatic embolus samples were dissected and then labeled with Cy3 and Cy5, respectively, using Ettan DIGE Kit (Amersham Biosciences) according to manufacturer’s manual. Following the labeling, the Cy3 and Cy5 labeled proteins were then mixed and precipitated with 85% alcohol and solubilized with lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 2% DTT, 2% IPG buffer [pH 4–8], 1 mmol/L benzamidine, 2 μg/mL pepstatin-A, 20 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L sodium vanadate, 1 μmol/L microcystin-LR) as previously described (9). Excised 2-dimensional protein spots were destained, chopped into small fragments with a razor blade, and subjected to digestion by trypsin according to Fang et al. (15). Detailed description of DIGE and mass spectrometric analyses is listed in Supplemental Methods.

Invasion assay
Matrigel-precoated Transwell chambers with PET membranes containing 8-μm pores (BD Biosciences) were soaked in DMEM and incubated for 60 minutes at 37°C. The invasion assay was carried out according to instruction.

Tumor xenografts
Heptacarcinoma MHCC97-H cells (5×10⁶ in 0.1 mLPBS) infecting with adenovirus carrying CFP, CFP-ezrin, CFP-ezrinT567A, CFP-ezrinT567D were inoculated into the liver of female NOD/SCID mice (6-week old; 10 animals per group). Tumor growth was evaluated by monitoring tumor volume every 2 days for 10 weeks. The MHCC97-H cell line was selected based on its reproducibility of metastasis in NOD/SCID mice and prior proteomic analyses (26). The protocol was approved by the Animal Using Committee of Xijing Hospital.

Immunocytochemistry, Western blotting analyses, and statistical analyses were described in supplemental methods.

Results

Identification of ezrin hyperphosphorylation in HCC metastasis
Metastasis is often the final and fatal stage in the progression of solid tumor malignancies. In order to gain an unbiased understanding of molecular mechanisms underlying HCC metastasis, we initiated a broad search for aberrant posttranslational modification of proteins associated with the metastatic phenotype of HCC. Two-dimensional SDS-PAGE (2D-gel) was used to profile each protein. With different fluorescent reporters, we compared 2 different preparations on the same gel. Together with mass spectrometry, the differentially displayed protein spots were identified (7).

As shown in Figure 1A, proteins from the primary HCC and its paired metastatic tumor embolus were labeled with Cy5 and Cy3, respectively, mixed, and fractionated by IEF followed by SDS-PAGE. Cellular proteins from the metastatic tumor were labeled in Cy5 (green) whereas the proteins from the primary tumor were marked in red (Cy3 labeling). The merged image shows that the overall protein profiles from the 2 distinct histological stages are similar. However, the merged image also exhibits dramatic differences in protein biochemical properties from the aforementioned 2 histological stages. For example, there was a reduction in the relative intensity of the major alkaline spot (0; estimated pI of 6.7) and a shift toward a series of the more acidic isoforms 1, 2, 3, 4, and 5 (Fig. 1B), an estimated pI varying from 6.0 to 6.2, respectively, which suggest that ezrin is hyperphosphorylated in the metastatic sample (arrows) compared with the primary tumor.

To identify the phospho-modification associated with metastasis, the acidic isotype spots of ezrin were removed from the 2D-gel, combined, and subjected to in-gel digestion with trypsin. The corresponding regions of the 2D-gel from primary HCC samples were used as controls. These multiple spots represent the same ezrin protein but with different modifications. The tryptic peptides recovered from mass spectrometric analysis indicate that Thr⁵⁶⁷ is likely phosphorylated in metastatic sample compared with the primary tumor.

To validate if Thr567 is selectively phosphorylated in the metastatic sample, we carried out the Western blotting using a phospho-Thr567-specific antibody. As shown in Figure 1C, phospho-Thr567 antibody strongly reacts with ezrin from the metastatic sample, although there was also very minor reactivity with primary tumor. There was no reactivity with normal liver tissue, suggesting that phosphorThr567 of ezrin correlates with the metastatic phenotype of HCC.

To ascertain the phospho-Thr567 in the specific spots on the 2D-gel, we carried out immunoblotting of phospho-Thr567 on 2D-separated samples as previously described (9). As
shown in Figure 1D, spots #2 and #3 from cancer embolus preparation are most heavily phosphorylated. Quantitative analyses of phospho-Thr567 levels over ezrin protein levels of individual spots from 21 sets of paired samples, shown as graphics, showed that phosphorylation of Thr567 is a dynamic event as spots #4 and #5 bear little phosphorylation on Thr567. The spot #2 often represents the most heavily phosphorylated in embolus samples from 21 patients, suggesting that phosphorylation of Thr567 is an early event of ezrin modification.

Given the heterogeneity of cell types used for proteomic studies, we attempted to validate the Thr567 phosphorylation using immunohistochemical analyses. As shown in Figure 2A, the phospho-Thr567 signal was readily apparent in invasive liver cancer tissue (Fig. 2A; c). Specifically, phospho-Thr567 signal was heavily localized to the cancer embolus of HCC (b; arrows). A survey of 21 sets of tissues samples taken from HCC patients revealed similar pattern shown in Figure 2A. As the ezrin protein levels in normal and HCC samples are similar (Fig. 1C), these results show that the phospho-Thr567 signal correlates with HCC metastasis.

Hyperphosphorylation of Thr567 correlates with invasiveness of HCC

To validate our hypothesis that phospho-Thr567 but not ezrin protein levels play a critical role in HCC progression, we carried out confocal microscopic analyses of double immunofluorescence staining of liver tissues in which ezrin protein levels were visualized by rhodamine-conjugated secondary
antibody (red) and phospho-Thr567 was labeled in FITC-conjugated secondary antibody. As shown Figure 2B (top panel), ezrin was present in normal liver epithelia as primary cytoplasmic staining. However, there is virtually no phospho-Thr567 signal in the green channel, consistent with our early western blotting analysis. The section taken from the primary tumor was then examined. As shown in Figure 2B (middle panel), ezrin was present in the primary tumor in similar profile like that of normal liver epithelia. The signal of phospho-Thr567 was largely negligible, except for a few cells with green fluorescence labeling. As predicted, the signal of phospho-Thr567 was very noticeable in a population of ezrin-positive cells, confirming the link between a dramatically increased level of phospho-Thr567 and HCC invasiveness.

To quantify if phospho-Thr567 levels in invasive HCC cells is significantly higher than those of normal and noninvasive carcinoma cells, we measured the average pixel intensities of phospho-Thr567 staining from 300 cells of each group from 5 different samples. The phospho-Thr567 pixel intensities of each cell were then normalized against ezrin pixel values to account for any variations in staining or image acquisition. As shown in Figure 2C, the phospho-Thr567 level in invasive HCC cells is significantly higher than those of normal and noninvasive carcinoma cells. Further quantification of samples from 21 patients revealed exact same profile. Thus, we conclude that a high phospho-Thr567 level is a potential new marker for invasive HCC.

Phospho-mimicking mutant of ezrin promotes the invasiveness of HCC cells

To investigate the potential role of phospho-Thr567 of ezrin in liver cell invasion and migration, HepG2 cells grown in a filter with recombinant adenoviral constructs were infected with wild-type ezrin, phospho-mimicking ezrin T567D, and nonphosphorylatable ezrin T567A. Western analyses show that all 3 ezrin constructs expressed comparable levels of exogenous ezrin (Fig. 3A, CFP-ezrin). Typically, the exogenously expressed ezrin protein was about 5-times higher than the level of endogenous ezrin in infected HepG2 cells (data not shown).

We next examined if phospho-mimicking ezrin enhances HepG2 cell invasion. To this end, HepG2 cells were infected with adenoviral-CFP-ezrin (wild type, ezrin T567D, and ezrin T567A). Both mutant ezrin proteins have a distribution pattern similar to the wild type (data not shown). As shown in the Figure 3B, HepG2 cells infected with CFP-ezrin began passing through the transwell chamber (arrows). However, examination of HepG2 cells infected with CFP-ezrin T567D
revealed a dramatic increase in the number of cells passing through the transwell chamber (Fig. 3B, a; arrows). In contrast, fewer HepG2 cells infected with CFP-ezrinT567A along passed through the transwell chamber (Fig. 3B, c; arrows). To quantify the effect of ezrin phosphorylation on HepG2 cell invasion ability, we defined the invasive potential of wild-type ezrin expressing HepG2 cells as 100% and related the efficiency of all other testing conditions to that of wild-type ezrin expressing cells. Our statistical analyses indicate that overexpression of phospho-mimicking ezrinT567D promoted cell invasion by 2-folds (P < 0.01; n = 10). Conversely, fewer HepG2 cells infected with CFP or no virus (parental; uninfected; n = 4) passed through the transwell chamber (Fig. 3B, d; arrows). Thus, we conclude that phospho-Thr567 in ezrin promotes the invasiveness of HepG2 cells.

Surprisingly, we noticed that wild-type ezrin overexpression also enhanced HepG2 cells invasive capability whereas non-phosphorylatable mutant of ezrinT567A exerted a reverse effect (Fig. 3B; Fig. 3C). We reasoned that exogenously expressed ezrin became phosphorylated whereas nonphosphorylatable ezrinT567A was not. If phosphorylation of Thr567 on CFP-ezrin is a function of Rho kinase (ROCK), suppression of ROCK would reduce the invasion capability of HepG2 cells overexpressing wild-type ezrin and the level of Thr567 phosphorylation on CFP-ezrin. As shown in Figure 3C, suppression of ROCK activity significantly reduced the invasion capability of HepG2 cells (ROCK siRNA; P < 0.01), which is correlated with the suppression of ezrin phosphorylation at Thr567 (Fig. 3D; Ezrin + ROCK siRNA, P < 0.001). Those analyses suggest that ezrin phosphorylation at Thr567 contributes to HepG2 cell invasion in the chamber assay.

Rho kinase phosphorylates ezrin and promotes the invasiveness of HCC cells

Several studies show that the ROCK signaling pathway is aberrantly upregulated in tumors and plays a key role in...
cancer invasion and metastasis (24). As ezrin Thr567 is a substrate of ROCK (16, 24), we sought to test the role of ROCK in HepG2 cell invasion using pharmacological inhibitor Y27632. To visualize the invasion assay with ease, we have infected HepG2 cells with CFP-ezrin before inhibition of ROCK. As shown in Figure 4A, Y27632-inhibited HepG2 cell invasion in a dose-dependent manner, consistent with an early animal study in which administration of Y27632 blocks intrahepatic metastasis of human HCC in SCID mice (25). The maximal inhibition of HepG2 cell invasion in transwell experiment was achieved with 10⁻⁶ mol/L Y27632.

Next, we correlated the level of phospho-Thr567 with its efficiency in HepG2 cell invasion. As predicted, incubation of HepG2 cells with Y27632 exhibited a dose-dependent inhibition of ezrin phosphorylation judged by western analysis of phospho-Thr567 (Fig. 4B). The phospho-Thr567 as a function of ROCK was also validated by ROCK siRNA treatment in which ROCK knockdown reduced the phospho-Thr567 level. Importantly, mimicking Thr567 phosphorylation desensitizes ROCK inhibition in the invasion assay as overexpression of CFP-ezrinT567D promoted HepG2 cell invasion in ROCK siRNA-treated cells (Fig. 3C). Thus, we conclude that ROCK phosphorylates ezrin at Thr567 and promotes invasion.

To explore the mechanistic insight accounting for the contribution of phospho-Thr567 to HCC metastasis, we have infected HepG2 cells with phospho-mimicking, nonphosphorylatable, wild-type ezrin, and CFP tag only followed by examination of the actin cytoskeletal changes using FITC-conjugated phalloidin. As shown in Figure 4D, expression of phospho-mimicking CFP-ezrinT567D dramatically increased the number of membrane ruffles on HepG2 cells. Neither nonphosphorylatable ezrin mutant nor CFP tag alone induced such changes. As phosphorylation of Thr567 has been observed in dynamic membrane cytoskeletal reorganization (10) and membrane ruffles are readout of membrane dynamics associated with cell migration (15), we reason that phosphorylation of ezrin at Thr567 by ROCK promotes actin-based cytoskeleton dynamics and accelerates membrane-cytoskeletal remodeling for cell motility.

Phosphorylation of Thr567 enhances invasion and metastasis of HCC 

To examine the effect of phosphor-Thr567 on HCC cell invasion and metastasis in vivo, we injected metastatic human HCC cells (MHCC97-H) expressing CFP (C), CFP-ezrin (WT), CFP-ezrinT567A (TA), CFP-ezrinT567D (TD) into the livers of NOD/SCID mice and monitored the tumor growth every other days. As shown in Figure 5A, the CFP-ezrinT567D xenografts exhibit MHCC97-H cell metastases in mouse livers. H&E staining also showed that ezrin-T567D expression led to
massive metastasis in the livers of the mice as compared with ezrin wild type and ezrin-T567A expression (Fig. 5B; 100 x arrows). The number of metastatic nodules in the livers was evident by gross histological analysis (Fig. 5C). The CFP-ezrinT567D xenografts significantly increased number of metastatic nodules compared with those of CFP-ezrin wild type, CFP vector alone, and CFP-ezrinT567A (P < 0.001; n = 10). In addition, expression of ezrin-T567D induced a reduction in mouse body weight (Fig. 5D), consistent with the fact that ezrin phosphorylation at Thr567 enhances HCC metastasis and disease progression. Thus, we concluded that phospho-Thr567 promotes ezrin-mediated metastasis in HCC.

Discussion

HCC is one of the most common and aggressive malignancies in the world. Localized HCC is controlled by surgical resection; however, therapies directed at recurrent and metastatic HCC are less effective. Intrahepatic metastasis of HCC is frequently observed in the advanced disease state and it is postulated to develop through tumor cell dispersal via the portal vein. Our comparative proteomics analysis revealed the involvement of ezrin phosphorylation in intrahepatic metastasis of HCC. Indeed, our functional analysis showed that overexpression of phospho-mimicking ezrin is sufficient to confer metastatic capacity in mouse xenografts.

The identification of ezrin as a key component of pediatric cancer metastasis established an important link to the molecular chain connecting metastasis-associated cell-surface proteins to the signal transduction network and prompted further investigation of protein interactive pathways underlying phospho-ezrin regulated tumor metastasis. It was originally thought that ezrin functions as a simple linker between membrane proteins and actin filaments. The mounting evidence indicates...
that ezrin and ERM family participates in a wide variety of important cellular processes ranging from polarity establishment to dynamic immunological synapse formation and acquisition of the senescent phenotype (27).

Examination of the cellular dynamics in which ezrin is involved reveals that ezrin interacts with several previously identified metastasis-associated molecules, such as CD44, E-cadherin, Rho, HGF receptor, etc. (e.g., ref. #5). Therefore, it is probable that ezrin activity is involved in several interactive but parallel pathways associated with metastasis. Function of ezrin as an organizer of adherent junctions in cell–cell communications could also play an important role in metastasis as the interaction of tumor cells with the normal surrounding stromal cells is important for tumor growth and viability. Ezrin-mediated formation of cell–cell junctions might provide survival advantage of invading tumor cells. Notably, evidence from both canine models and prospective human studies show correlations between ezrin expression levels and poor clinical outcome (12, 13), again emphasizing the crucial role ezrin plays in tumor dissemination.

To metastasize, tumor cells must overcome tissue boundaries, which require increased cell motility driven by remodeling of the cytoskeleton and cell contacts with the extracellular matrix. The Rho family of small GTPases plays key role in both cell adhesion and the cytoskeleton remodeling. Active RhoA can recruit the ROCK family members that phosphorylate a range of cytoskeletal proteins, including ezrin/radixin/moesin-family proteins to facilitate the membrane-cytoskeletal interaction (5). Rho mediates various phenotypes of malignant transformation by Ra and Src through its effectors such as ROCK (25). Early studies have reported that aberrant upregulation of ROCK in liver cancer progression (25), whereas its downstream signaling event has remained elusive. Our present study revealed that phospho-Thr567 is a novel regulatory mechanism by which ezrin plasticity orchestrates HCC invasiveness. The fact that inhibition of Thr567 phosphorylation suppressed HCC cell invasion in vitro and HCC metastasis in mouse Xenografts presented in this study raises the possibility of inhibiting Thr567 phosphorylation in prevention of liver cancer progression. It is worthy noting that our phospho-Thr567 analysis of 2D separated samples indicated that spots #2 and #3 from cancer embolus preparation are most heavily phosphorylated, suggesting that phospho-Thr567 is a dynamic event and spots #4 and #5 bear different phospho-epitope from phospho-Thr567. The dynamic pattern of Thr567 phosphorylation infers that Thr567 phosphorylation might be an early “priming” event to allow ezrin molecule unfolded for other posttranslational modification during the disease progression. Although ezrin protein from primary HCC displays several acidic spots in 2D protein from primary HCC displays several acidic spots in 2D SDS-PAGE, they were not recognized by anti-Thr567 antibody (data not shown). To this end, future effort will be directed to systemic identification of posttranslational modification on ezrin using large scale of stage-defined HCC samples and related temporal dynamics of ezrin phosphorylation to membrane ruffle formation.

Phosphorylation of the membrane-cytoskeleton linker protein, ezrin, has been functionally linked to acid secretion and vesicle recruitment to the apical secretory membrane in gastric epithelial cells (e.g., ref. #6). In other cells, the phospho-Thr567 has been shown to regulate the N-C oligomerization of ezrin thereby promoting the formation of actin-rich surface projections (16). Interestingly, phosphorylation of ezrin on thr567 produces repolarization of the gastric parietal cells (10), suggesting that the phospho-Thr567 alters ezrin molecule plasticity. Our single molecule analyses of ezrin using AFM showed that phospho-Thr567-induced unfolding of ezrin favors the intermolecular association, supporting the notion that stimulus-induced protein conformational change can be used as a signaling mechanism orchestrating cellular dynamics (17). Indeed, expression of phospho-mimicking CFP-ezrin T567D dramatically increased the number of membrane ruffles on HepG2 cells (Fig. 4D), suggesting that extracellular cue may elicit ezrin phosphorylation and molecular conformational changes via ROCK activation that then activates downstream signaling cascade. It would be of great interest to identify the proteins selectively interacting with ezrin in a Thr567 phosphorylation-dependent manner and to elucidate how Thr567 phosphorylation links cytoskeletal dynamics to liver cancer cell motility and invasiveness.

The development and application of DNA microarray technology allows for genome-wide transcriptomic profiling, which has provided great insight into the genetic basis of metastasis. Studies using primary tumor material have identified a gene expression signature for breast cancer metastasis consisting of a set of 70 genes (18). Using in vivo selection of organ-specific metastatic cells from the human breast cancer cell line MDA-MB-231, Massagué and colleagues have recently identified a set of genes that specifically mediate osteolytic bone metastasis (19), lung metastasis (20), and brain (21) in the mouse. Although this transcriptomic approach is very powerful, it may not be sufficient as posttranscriptional regulation may escape detection by genomic screening. Indeed, small GTPase ARF6 protein overexpression by breast cancer cells/tissues was not detected by this genomic analysis as its overexpression was due to the unbalanced protein turnover (22,23). Thus, the proteomic approach could provide an unbiased search for disease stage-specific gene activity. The combination of 2-dimensional separation of hepatic proteins from HCC of different stages with mass spectrometric identification of differentially displayed protein spots established here provides a systemic approach to uncover the proteins and modification underlying tumor metastasis. This strategy would be useful for achieving a deeper understanding of early metastatic events and the development of improved treatments for HCC patients at risk for metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ezrin in Hepatocellular Carcinoma Progression

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