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Identification of Dermcidin as a novel binding protein of Nck1 and characterization of its role in promoting cell migration

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ABSTRACT

A distinct feature of hepatocellular carcinoma (HCC) is the tendency of tumor cells to disperse throughout the liver. Nck family adaptor proteins function to couple tyrosine phosphorylation signals to regulate actin cytoskeletal reorganization that leads to cell motility. In order to explore the role of Nck in HCC development, we performed GST pull-down assay using the SH2 domain of Nck1 as bait. The resulting precipitates were separated by 2-DE. Mass spectrometry analysis revealed a group of Nck1 SH2 domain-binding proteins that were differentially expressed in HCC. One of these proteins, dermcidin (DCD), and its interaction with Nck1, was further validated in vitro. GST pull-down assay revealed that Nck1 SH2 domain binds to the phosphotyrosine residue at position 20 (Y20) of the DCD. Pervandate treatment significantly enhanced the interaction between DCD and Nck1. Moreover, we demonstrated that forced expression of DCD could activate Rac1 and Cdc42 and promoted cell migration. Taken together, these data suggest a role of DCD in tumor metastasis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive human malignancies [1]. High metastasis and recurrence rate are major obstacles to further improve the long-term survival rate of HCC patients [2]. Accumulating evidence suggests that cell migration plays a critical role in tumor metastasis and invasion. Cell migration is governed by remodeling of the actin cytoskeleton, i.e., the dynamic assembly and disassembly of filamentous actin. Malignant tumor cells often show excessive cell protrusive activity due to aberrant activation of signaling pathways that regulate actin cytoskeleton rearrangement [3,4].

The Nck adaptor proteins consists three SH3 domains, followed by a C-terminal SH2 domain. Both *Caenorhabditis elegans* and *Drosophila melanogaster* have a single Nck gene, whereas mammals have two Nck homologues, Nck1 and Nck2 (also named Grb4). The two murine Nck gene products exhibit 68% amino acid identity to one another. Nck can bind, via its SH2 domain, to a number of receptor tyrosine kinases such as the platelet derived growth factor receptor (PDGFR), the epidermal growth factor receptor (EGFR), and the ephrin receptor (Eph), as well as tyrosine phosphorylated docking proteins such as p62Dok-1 and p130Cas [5–7]. On the other hand, the Nck SH3 domains are capable of binding to proline-rich sequences on a host of effecter proteins implicated in cytoskeleton regulation. For instance, binding of Nck to Pak/PIX complex stimulates Rho GTPases Rac1 and Cdc42 activation and regulates cell adhesion, migration as well as gene transcription [8,9].

Accumulating evidence in both invertebrates and mammals indicates that one of the major cellular functions of Nck is to link phosphotyrosine signals to regulation of the actin cytoskeleton, which is the prerequisite for various biological responses such as axon path finding, migration, chemotaxis and endocytosis [10]. Because tumor cells often possess aberrant kinase activation [11], it is possible that the binding partners of the Nck SH2 domain in tumors are different from those of normal tissues. In order to identify Nck SH2 domain binding proteins associated with HCC development, we used the GST fused Nck1 SH2 domain to pull down associated proteins from either

Abbreviations: HCC, hepatocellular carcinoma; DCD, dermcidin; PDGFR, platelet derived growth factor receptor; EGFR, epidermal growth factor receptor; Eph, ephrin receptor; DMEM, Dulbecco's modified Eagle's media; PCR, polymerase chain reaction; siRNA, small interference RNA; IPTG, isopropyl-β-D-1-thiogalactopyranoside; EGTA, ethylene glycol tetraacetic acid; Rac1, Ras-related C3 botulinum toxin substrate 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ACN, acetonitrile; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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normal or HCC tumor samples. Then 2-DE gels were used to resolve the resulting complexes and differentially expressed protein spots were analyzed by mass spectrometry. Our study identified dermcidin (DCD), a protein that is upregulated in HCC, as a novel binding protein of Nck1. We further demonstrated that DCD overexpression could promote cell migration.

2. Materials and methods

2.1. HCC patient samples and cell lines

HCC tissues were collected from 21 HCC patients who underwent hepatectomy at the First Affiliated Hospital of Sun-Yat Sen University. None of these patients received preoperative chemotherapy or radiotherapy. Normal liver tissues were obtained from 8 patients diagnosed with liver hemangioma or cholelithiasis. Specimens were obtained with written informed consent from all patients. The study was carried out with prior approval of the Committees for Ethical Review of Research involving Human Subjects of the First Affiliated Hospital of Sun-Yat Sen University (Guangzhou, China).

The normal human liver cell line, Chang liver, the hepatoma cell line HepG2, Hep3B, SMMC-7721 and BEL-7402, and an endothelial cell line of hepatic origin SK-HEP-1 were obtained from the Cell Bank, Chinese Academy of Medical Sciences (Shanghai, China). The cells were maintained in high-glucose Dulbecco's modified Eagle's media (DMEM) (Gibco, Australia) supplemented with 10% fetal bovine serum (Gibco, Australia).

2.2. Plasmid constructs and transfection

The full-length dermcidin cDNA was amplified and cloned into the pReciever M06 expression vector (FulenGen, Guangzhou, China). GST tagged SH2 domain of Nck1 was generated by PCR amplification of human Nck1 template and ligated into pGEX-4T-3 expression vector. The Y20F DCD mutant and the R308K Nck1 mutant were generated by site-directed mutagenesis kit according to the manufacturer's instructions. The expression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. GST fusion protein purification and GST pull-down assay

Escherichia coli (BL21) was transformed with pGEX-4T-3 or pGEX-Nck1-SH2, and incubated with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 4 h. The GST fusion proteins were purified from bacterial lysates with GSH-Sepharose 4B beads according to the manufacturer's instruction (Amersham Biosciences, NJ, USA).

Tissue/Cell lysates were prepared and spun at 15,000×*g* for 15 min and the supernatants were incubated with GST or GST-Nck1-SH2 conjugated Sepharose beads for 2 h at 4 °C. After extensive washing, fusion proteins were eluted with glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) and desalted with 2-DE cleaning up kit (Amersham Biosciences, NJ, USA).

2.4. 2-DE and image analysis

Protein samples (250 μg) were diluted to 450 μl with rehydration solution (7 M urea, 4% CHAPS, 0.5% IPG ampholyte, 65 mM DTE, 2 M thiourea, and 0.0002% bromophenol blue), and then loaded onto IPG gel strips (pH 3–10 linear, 24 cm long, Amersham Biosciences, NJ, USA). The first dimensional separation, IEF, was carried out using the IPGphor system (Amersham Biosciences, NJ, USA) at 18 °C with 8000 V for a total of 90 k VHS. After IEF, the IPG strips were subjected to reduction with 2% DTE in equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol), followed by alkylation with 2.5% iodoacetamide in the same buffer. Gels were stained with Silver staining kit (Amersham Biosciences, NJ, USA) according to manufacturer's instruction. The developed gels were scanned as 2-DE images using an Image scanner (Amersham Biosciences, NJ, USA), and then analyzed using ImageMaster software (Amersham Biosciences, NJ, USA).

2.5. In-gel digestion and protein identification

2-DE gels of interest were washed in water/acetonitrile (ACN) 1:1 and then dehydrated in ACN. The gel pieces were air-dried and rehydrated in 20 μl of 10 mM DTT, 0.1 M NH_4HCO_3. Reduction of disulfide bonds was performed at 56 °C for 45 min. The supernatant was discarded and cysteine residues were modified to *S*-carboxyamidomethylcysteine in 55 mM iodoacetamide, 0.1 M NH_4HCO_3. After washing with 0.1 M NH_4HCO_3/ACN (1:1) for 15 min followed by ACN, the gel pieces were air-dried, rehydrated in chilled 50 mM NH_4HCO_3 and 12.5 ng/μl trypsin, and incubated at 37 °C overnight. The supernatant was collected and peptides were extracted from the gel twice with 50 mM NH_4HCO_3/ACN (1:1) followed by 5% formic acid/ACN (1:1). The combined extracts were evaporated to dryness in a vacuum centrifuge. Prior to mass spectrometric analysis, peptides were re-dissolved in 10 μl of 0.1% formic acid. Online peptide separation was performed after trapping each sample on 180 μm × 20 mm Symmetry® C18 Nano Acquity™ UPLC™ column with 1% ACN and 0.1% formic acid at 15 ml/min flow rate following the separation on a 75 μm × 250 mm BEH130 column (Nano Aquity™ UPLC™) with a 50 min gradient from 5 to 95% ACN and 0.1% formic acid at a flow rate of 300 nl/min. A tapered fused silica was used as an emitter. Mass analyses were performed with a Quadruple Time of Flight mass spectrometer (Q Tof, Waters, USA). The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra (from *m/z* 400 to 1800) were acquired in the Q Tof, and the four most intense ions in each survey scan were fragmented and analyzed. Proteins were identified by automated database searching (Spectrum Mill, Agilent technologies and MASCOT, matrix-science) of all MS and MS/MS spectra using the IPI Human, Swiss-Prot and NCBinr databases. Raw data files were converted to .pkl files by the ProteinLynx Global Server (PLGS), Waters Corp software utility. Search parameters were set as follows: MS accuracy, 0.15 Da; MS/MS accuracy, 0.15 Da; two missed cleavage allowed; variable carbamidomethyl modification of cystine and variable oxidation of methionine and all entries of the databases were searched.

2.6. Western blot and immunoprecipitation

Western blot and immunoprecipitation were performed as described previously [12]. Dermcidin antibody and HA tag antibody were from Abcam (Cambridge, MA). Anti-Nck1 was from Santa Cruz (CA, USA). GAPDH was from Sigma-Aldrich (St. Louis, MO, USA).

2.7. Rho family small GTPase activation assay

The intracellular activities of Rho family GTPases Rac1 and Cdc42 were examined using Rac1 and Cdc42 activation assay kits, respectively (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocols. In brief, cells were rinsed with ice-cold PBS and lysed with Mg^{2+} lysis/wash buffer. After clarifying the cell lysates with glutathione agarose and quantifying the protein concentrations, aliquots with equal amounts of proteins were incubated with Rac/Cdc42 assay reagent (PAK-1 PBD, agarose) at 4 °C for 1 h, using the GTPgS-pretreated lysates as positive controls. The precipitated GTP-bound Rac1 and Cdc42 were then eluted in Laemmli reducing sample buffer, resolved in a 12% SDS-PAGE, and immunoblotted with monoclonal antibodies specific for Rac1 and Cdc42. Five percent of the cell lysate were also resolved in a 10% SDS-PAGE and

immunoblotted with indicated antibodies to measure the total amount of Rac1 and Cdc42.

2.8. Migration assay

Migration assay was performed as described previously [13].

2.9. Statistic analysis

Statistical differences between two groups were determined by the Student's *t* test. A $P<0.05$ was considered statistically significant. The results were expressed as mean ± SD from at least three experiments.

3. Results

3.1. Proteomic analyses identified differentially expressed Nck1-SH2 binding proteins between HCC and normal liver tissue

The proteins extracted from HCC tissues and normal tissues (Table 1) were pooled separately and incubated with purified GST-Nck1-SH2. The protein complex binding to Nck1-SH2 was eluted from glutathione beads and separated by 2-DE. Representative gel images of the protein complex binding to Nck1-SH2 in HCC and normal liver tissue were shown in Fig. 1. A comparison of the 2-DE images revealed that 28 protein spots were up-regulated in HCC more than twofold compared to normal liver tissues ($T/N>2$), with 15 of them detected only in HCC samples. Finally, 13 proteins were identified from 14 spots by mass spectrometry (Table 2), most of which have not previously been reported to be associated with Nck1, nor with HCC. Because DCD was previously reported as a candidate oncogene in breast cancer [14], we selected it for further study.

Table 1
Clinical features of patients analyzed by 2DE.

		Gender	Age	HBV	AFP	Diagnosis	Edmonson
Normal	1	F	68	(−)	(−)	Hepatolithiasis	
	2	M	59	(−)	(−)	Hepatolithiasis	
	3	F	26	(−)	(−)	Hepatolithiasis	
	4	M	51	(−)	(−)	Hepatolithiasis	
	5	M	56	(−)	(−)	Hemangioma	
	6	F	40	(−)	(−)	Hepatolithiasis	
	7	F	54	(−)	(−)	Hepatolithiasis	
	8	F	55	(−)	(−)	Hepatolithiasis	
HCC	1	M	41	HBsAg	(+)	HCC	II
	2	M	57	HBsAg	(+)	HCC	II
	3	M	40	HBsAg	(+)	HCC	III
	4	M	54	HBsAg	(+)	HCC	II
	5	M	79	HBeAb+HBcAb	(+)	HCC	III
	6	M	49	HBsAg	(+)	HCC	II
	7	M	55	HBsAg	(+)	HCC	III
	8	M	53	HBsAg	(+)	HCC	II–III

3.2. Expression of DCD is significantly increased in HCC tissues

Since the interaction between DCD and Nck-SH2 was only detected in HCC tissues but not in normal liver tissues, we then examined DCD expression in 21 HCC and adjacent non-cancerous liver tissues by Western blot. As shown in Fig. 2A–B, DCD was detected in all tumor tissues but not in the adjacent non-tumor tissues. We further examined the expression of DCD in normal and HCC cells. As shown in Fig. 2C, the protein level of DCD in a normal liver cell line (Chang liver cell) is relatively lower than that in HCC cells such as HepG2, Hep3B, SMMC-7721 and BEL-7402. It is interesting to note that the level of DCD is extremely high in SK-HEP-1 cell which is a high invasive endothelial cell line of hepatic origin (Fig. 2C), suggesting that increased DCD expression might be associated with tumor metastasis.

3.3. Interaction between DCD and Nck1 is tyrosine phosphorylation-dependent

Since DCD was identified as a binding protein of the Nck1-SH2 domain in HCC tissues, we further confirmed this interaction in vitro. HA-tagged DCD was transiently transfected into SK-HEP-1 cells and the interaction between DCD and Nck1-SH2 was examined by GST pull-down assay. As shown in Fig. 3A, DCD bound to Nck1-SH2 but not GST. Since DCD is highly expressed in SK-HEP-1 cells, we therefore performed co-immunoprecipitation experiment to analyze the association of endogenous DCD with Nck1 in SK-HEP-1 cells. As shown in Fig. 3B, DCD was detected by anti-DCD antibody in the anti-Nck1 immunoprecipitate, but not in the precipitate obtained using a control IgG.

Since SH2 domains are known to bind to phosphotyrosine residues in proteins, Nck1-DCD interaction should be in a phosphorylation-dependent manner. To address this issue, we treated HA-tagged DCD transfected cells with phosphatase inhibitor pervanadate. As shown in Fig. 3C, the interaction between GST-Nck1 and DCD was enhanced by pervanadate treatment. Analysis of DCD sequence revealed one tyrosine residue, Tyr^{20}, in DCD. Thus, site-directed mutagenesis was employed to substitute Tyr^{20} to phenylalanine (Y20F). We next transfected either wild type DCD or DCD mutant Y20F into SK-HEP-1 cells, respectively. Forty-eight hours after transfection, the interaction between DCD and Nck1-SH2 was analyzed by GST pull down. As shown in Fig. 3D, the interaction between Y20F and Nck1-SH2 was dramatically attenuated compared to wild type DCD. Collectively, these data indicate that the interaction between DCD and Nck1 is tyrosine phosphorylation-dependent.

3.4. DCD expression activated Rho GTPases and promoted cell migration via interaction with Nck1

It has been demonstrated that, upon receptor tyrosine kinases activation, Nck binds to Pak/PIX complex and then leads to Rac1 and Cdc42 activation. Therefore, to test the functionality of DCD–Nck1 interaction, SK-HEP-1 cells were transfected with wild type DCD or Y20F mutant DCD, respectively. As shown in Fig. 4A and B, overexpression of wild type DCD resulted in a remarkable increase in the activation of both Rac1 and Cdc42 compared with control cells. However, both Rac1 and Cdc42 activities in Y20F mutant transfected cells were dramatically lower than that of wild type DCD transfected cells.

Given the important role of Rac1 and Cdc42 activation on cell migration, we next examined the effect of overexpressing DCD on cell migration. Boyden chamber cell motility assay revealed that DCD-transfected cells displayed a greater ability to migrate across the filter than the vector controls (Fig. 4C). Quantification of the data indicated an almost 100-fold increase in the number of cells migrating across the transwell filters for DCD-overexpressing cells over control cells (Fig. 4D), whereas, the motility of Y20F mutant transfected cells drastically reduced compared to wild type DCD.

It has been reported previously that Nck1-R308K mutant disrupts the ability of the SH2 domain to bind to phosphotyrosine residues [15]. To confirm the role of Nck1-SH2 domain in DCD-mediated cell migration, we cotransfected HA-tagged DCD with Nck1-WT or Nck1-R308K into SK-HEP-1 cells. Forty-eight hours after transfection, the immunoprecipitated proteins by Nck1 antibody were analyzed by Western blot and significant amount of DCD was found to be coprecipitated with Nck1-WT but not with Nck1-R308K (Fig. 5A). Moreover, Nck1-R308K dramatically inhibited DCD-augmented cell migration (Fig. 5B–C) compared with wild type Nck1. Collectively, these findings indicated that DCD overexpression, via interaction with Nck1-SH2, activates Rho GTPases and promotes cell migration.

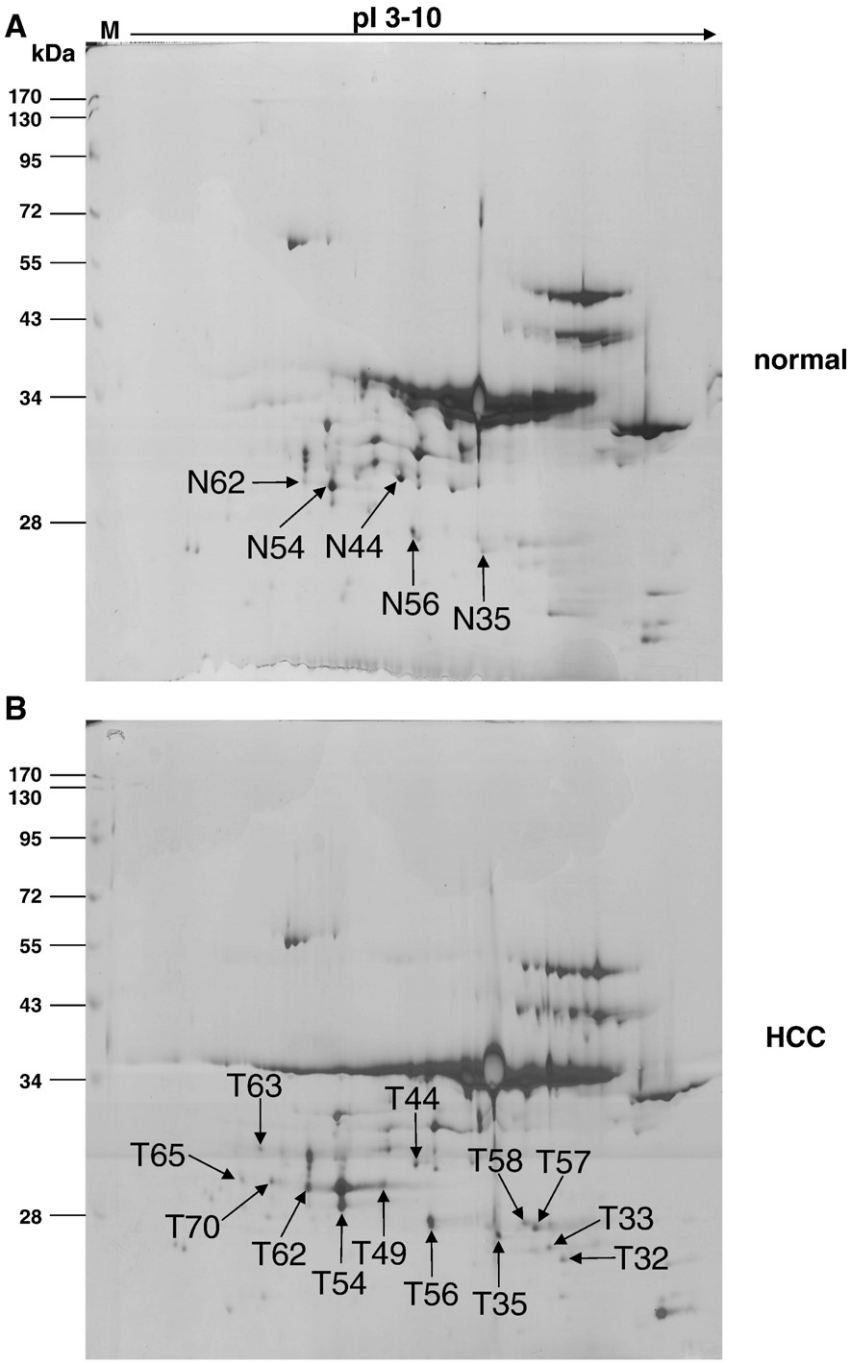

Fig. 1. Differentially expressed Nck-SH2 binding proteins in normal versus HCC samples. Representative 2-DE images of GST-Nck1-SH2 binding proteins isolated from normal liver tissue (A) and HCC samples (B). The significantly up-regulated spots ($T/N>2$) in HCC are marked with numbers. T, HCC sample; *N*, normal liver tissue.

4. Discussion

Using 2 DE combined with MS analysis, we successfully identified DCD as a novel binding protein of SH2 domain of Nck1 in HCC tissues. Western blot demonstrated the increased expression of DCD in HCC samples compared with adjacent non-tumor tissues. Consistent with our data, DCD has been previously shown as a candidate oncogene overexpressed in 10% of invasive breast carcinomas [14]. In addition, a small proteolytic peptide, proteolysis-inducing factor (PIF), derived from the protein encoded by the dermcidin gene was identified as a cachectic factor and was purified from a cachexia- inducing murine tumor and in the urine of weight-losing patients with pancreatic cancer [16,17].

The oncogenic function of DCD seems to be versatile in different tumors. It has been shown that DCD enhanced cell growth and survival by reducing serum dependency in breast cancer cells [14]. In hepatoma HuH7 cells, overexpression of DCD improved cell survival under oxidative stress by decreasing necrosis and increasing the number of cells undergoing apoptosis. The growth and survival-promoting effects of DCD in HuH7 cells have been shown to involve different pathways [18]. Moreover, it has been reported that the induction of skeletal muscle proteolysis by PIF is mediated through increasing NF-κB accumulation in the nucleus [19]. In the present study, we provided evidence that the expression of DCD was upregulated in hepatoma cells than that of normal liver cells. It is noteworthy that the expression of DCD is particularly high in SK-HEP-

Table 2
Differentially expressed Nck-SH2 binding proteins in HCC tissues.

Spot number	SwissProt ID	Protein name	MW (KDa)	No. of matched peptide	*T/N* ratio
T14	Q9NTJ3	Structural maintenance of chromosomes protein 4	147.18	4	>2
T35	P16152	Carbonyl reductase [NADPH] 1	30.37	4	>2
T44	Q86YZ3	Hornerin	282.39	5	>2
T52	P60660	Myosin light polypeptide 6	16.93	1	>2
T54	Q969J3	Loss of heterozygosity 12 chromosomal region 1	22.22	3	>2
T56	P62736	Actin, aortic smooth muscle	42	2	>2
T25	Q99943	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	31.71	1	nd *N*
T32	Q6T4R5	Nance-Horan syndrome protein	176.7	2	nd *N*
T33	Q86YZ3	Hornerin	282.39	12	nd *N*
T42	Q99583	Max-binding protein MNT	62.3	2	nd *N*
T57	P60709	Actin, cytoplasmic 1	41.74	10	nd *N*
T58	P81605	Dermcidin [Precursor]	11.28	2	nd *N*
T63	Q92556	Engulfment and cell motility protein 1	83.83	1	nd *N*
T70	P60059	Protein transport protein Sec61 subunit gamma	7.74	4	nd *N*

nd *N*, not detected in normal tissue.

1 cells, a high invasive endothelial cell line of hepatic origin, suggesting a role of DCD in tumor metastasis. Therefore, we think this cell line is suitable for analyzing the role of DCD in promoting cell migration and the underlying mechanism. Further analysis revealed that overexpression of DCD could promote SK-HEP-1 cell migration and the mechanism is by activating Rho GTPase Rac1 and Cdc42. Taken together, these data supported a role of DCD in tumor metastasis.

Previous studies showed that the function of DCD is mediated via the action of the core peptide of PIF [18,20]. In the present study, we demonstrated that Tyr^{20} of DCD is essential for its interaction with Nck1 and mutation of Tyr^{20} to phenylalanine abolished its binding to Nck1 and attenuated cell migration. Since the Tyr residue is located within the core peptide of PIF, it is plausible to postulate that the core peptide of PIF is essential for the function of DCD in tumor metastasis.

In the present study, we identified DCD as a novel binding protein of SH2 domain of Nck1. The Nck SH2 domain belongs to the group I family, containing an aromatic amino acid (phenylalanine) at the βD5 position. Based on studies using degenerated phosphopeptide libraries, the SH2 domain of Nck1 prefers a YDXV consensus motif in tyrosine- phosphorylated proteins [21]. We showed that Nck1 binds to the phosphorylated Y20 of DCD. Interestingly, YDPE is not a perfect fit to the consensus binding motif of Nck. Consistent with our finding, the binding motif for Nck1 in the PDGF receptor is YVPM [22,23]. These studies suggest that some flexibility exists in the interaction between the SH2 domain and the phosphotyrosine motif. Therefore, it is necessary to identify in vivo binding partners for certain SH2 domain in order to fully understand the specificity of the domain.

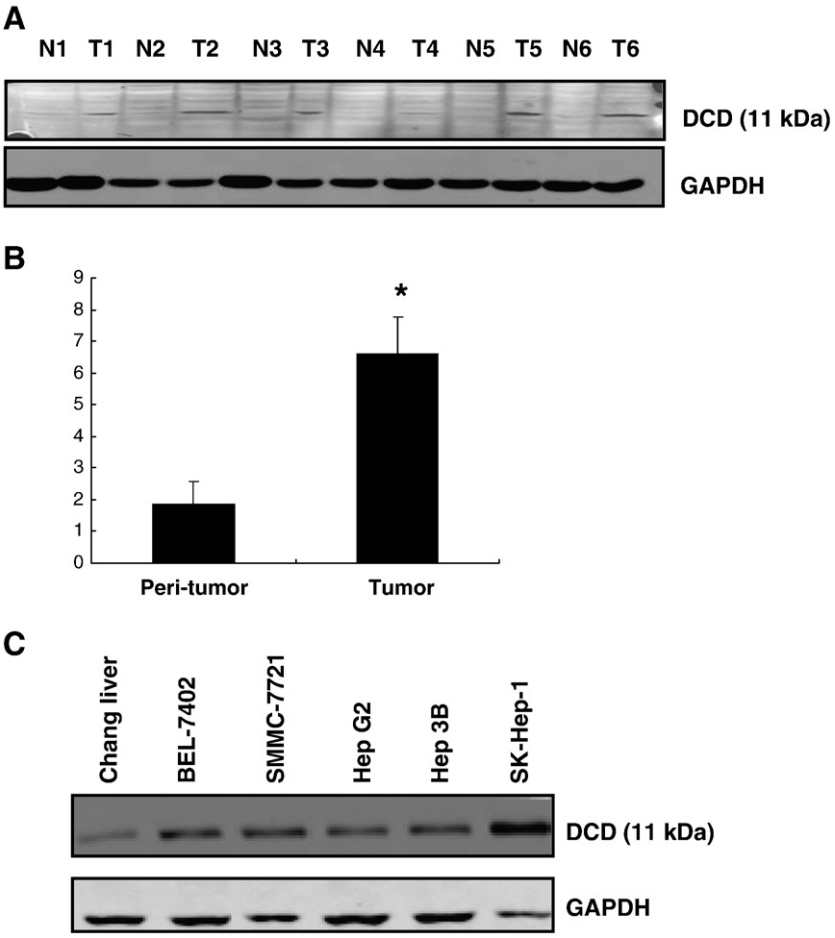

Fig. 2. (A) DCD is overexpressed in HCC tissues. Total protein extracted from tumor tissue (T) or adjacent non-tumor tissue (*N*) was immunoblotted with antibody against DCD. The same membrane was re-blotted with an anti-GAPDH antibody to verify equal loading. (B) Quantification of (A) to show the relative abundance of DCD in tumor versus peri-tumor tissues. The DCD signal was normalized to that of GAPDH for comparison. Data are expressed as mean ± SE for 21 pairs of samples. $^{*}P<0.05$ compared with adjacent non-tumor tissue. (C) Expression of DCD in normal and tumor cell lines was detected by Western blot. Equal amount of whole cell lysates extracted from indicated cell lines was immunoblotted with antibody against DCD. The same membrane was re-blotted with an anti-GAPDH antibody to verify equal loading.

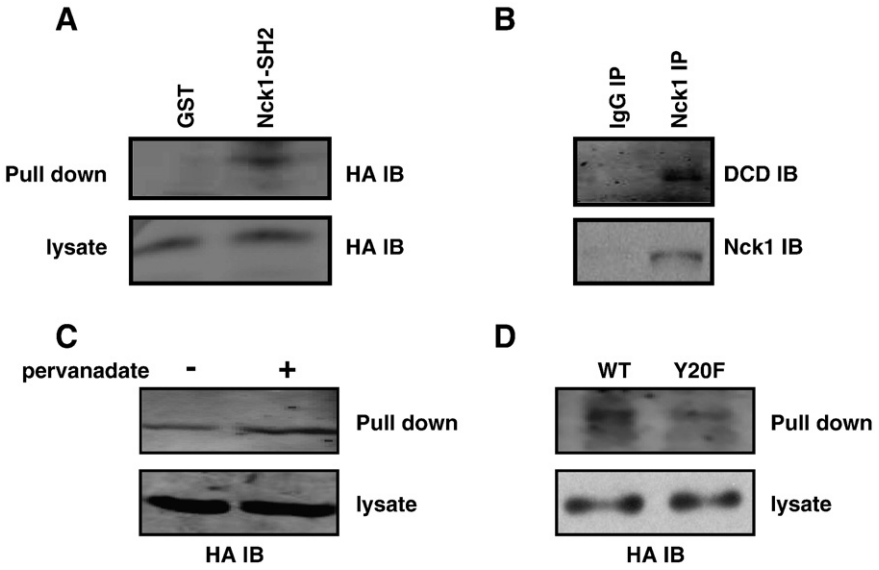

Fig. 3. DCD interacts with Nck1 in a tyrosine phosphorylation-dependent manner. (A) GST-Nck1-SH2 was incubated with cell lysates from SK-HEP-1 cells transfected with either empty vector or HA-tagged DCD. The bound protein was detected by immunoblot with anti-HA antibody. 10% of the SK-HEP-1 cell lysates used in GST pull-down assay was loaded to verify equal loading. (B) SK-HEP-1 cell lysates were immunoprecipitated with an anti-Nck1 antibody or IgG as control, followed by anti-DCD or anti-Nck1 immunoblot. (C) SK-HEP-1 cells were transfected with HA-tagged DCD. 48 h after transfection, cells were treated with 100 μM pervanadate for 10 min and then cell lysates were harvested. The corresponding cell lysate was incubated with purified GST-Nck1-SH2. Bound protein was detected by immunoblot using anti-HA antibody. 10% of the cell lysates used in GST pull-down assay was loaded to verify equivalent loading. (D) SK-HEP-1 cells were transfected with HA-tagged wild type DCD or Y20F mutant. 48 h after transfection, cell lysates were harvested and the corresponding cell lysate was incubated with purified GST-Nck1-SH2. Bound protein was detected by Western blot using anti-HA antibody. 10% of the cell lysates used in GST pull-down assay was loaded to verify equivalent loading.

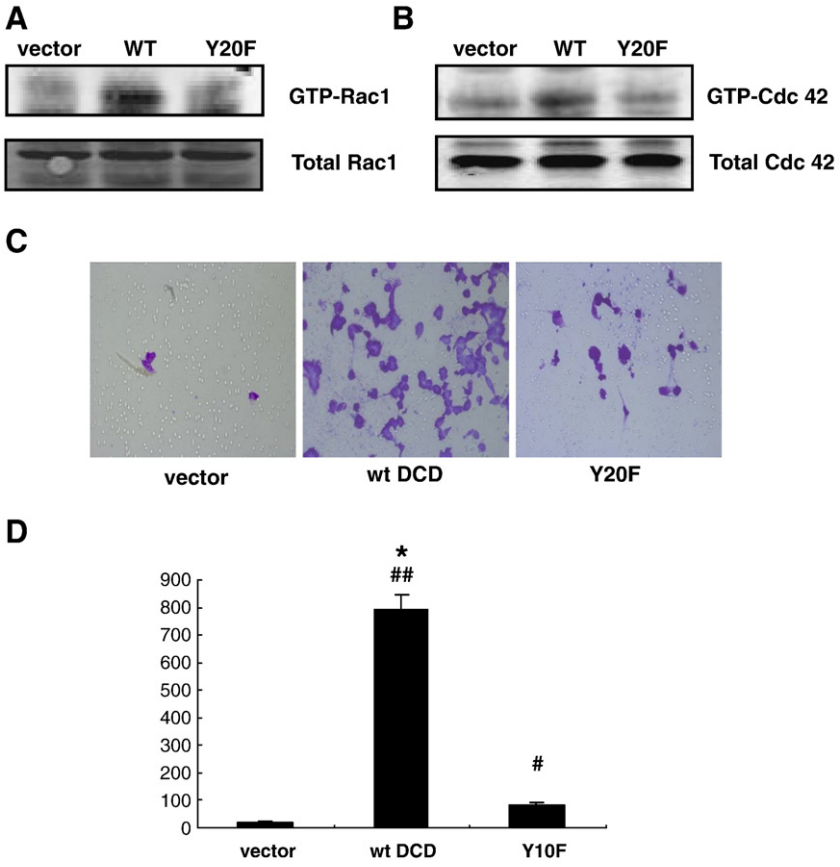

Fig. 4. Overexpression of DCD activated Rac1 and Cdc42 and promoted cell migration. SK-HEP-1 cells were transfected with empty vector, HA-tagged wild type DCD or Y20F mutant, respectively. 48 h after transfection, the amount of active GTP-bound Rac1 (A) and Cdc42 (B) was determined by antibodies specific for Rac1 and Cdc42. (C) For Boyden chamber motility assay, cells were seeded onto the filter in the upper compartment of the chamber and incubated for 18 h. Cells and cell extensions that migrated through the pores of transwell plates were counted and reported in (D). Photographs were taken using a Nikon microscope (phase contrast). Magnification, 200×. $^{\#\#}P<0.01$ vs. vector transfected cells; $^{\#}P<0.05$ vs. vector transfected cells; $^{*}P<0.05$ vs. Y20F mutant DCD transfected cells.

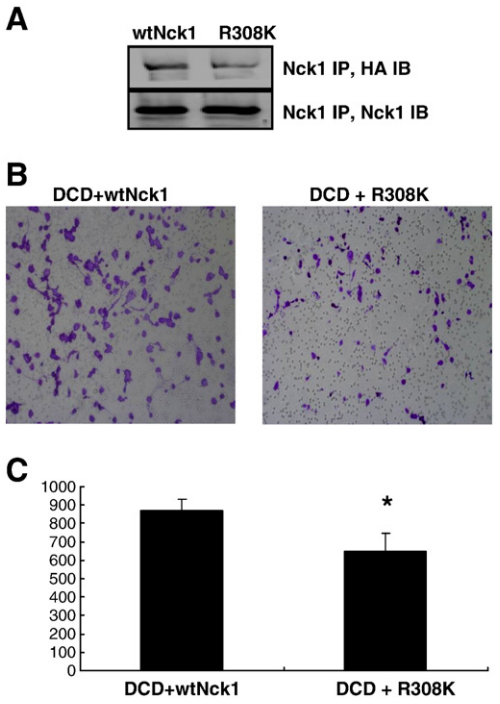

Fig. 5. Intact SH2 domain of Nck1 is important for DCD–Nck1 interaction and DCD-induced cell migration. (A) SK-HEP-1 cells were co-transfected HA-tagged DCD with either Nck1-WT or Nck1-R308K, respectively. 48 h after transfection, cell lysates were immunoprecipitated with antibody against Nck followed by immunoblot with antibody against HA or Nck respectively. (B) Boyden chamber motility assay was performed according to the method described in Fig. 4. Magnification, 200×. *P<0.05 vs. Nck-WT transfected cells.

Protein tyrosine kinases and protein tyrosine phosphatases play pivotal role in a variety of important signaling pathways in multicellular organisms and aberrant kinase activities are often associated with malignant transformation [24,25]. Thus, profiling the global state tyrosine phoshporylation state, especially comparing the tyrosine phoshporylation state between normal and tumor tissues, will provide a useful means for novel molecular diagnostic approaches. The SH2 domain is a small modular protein domain that binds specifically to tyrosine-phosphorylated peptide ligands [26]. Therefore, characterizing the SH2 domain binding protein complex can enrich low-abundance tyrosine phosphorylated proteins and efficiently capture information relevant to the activation state of signaling pathways in cells and tissues. Machita et al. developed a SH2 profiling method based on far-Western blotting by using GST-SH2 fusion proteins as probes and proposed that SH2 binding profiles may be useful as a molecular diagnostic tool for classifying tumor cells and predicting clinical outcomes [27]. By combining affinity purification of Grb2-SH2 domain binding proteins with SILAC, Blagoev et al. identified 28 proteins, including EGFR and Shc, that were selectively enriched after EGF stimulation in cells, suggesting this strategy is effective in identifying functional protein complexes [28]. However, all the studies were performed in cells lines. In the present study, we applied this strategy to investigate proteins associated with a disease and identified DCD as a novel Nck1-SH2 domain binding protein that differentially expressed in HCC tissues.

It is noteworthy that the advantage of affinity purification of SH2 domain binding proteins is not limited to enriching low-abundance tyrosine phosphorylated proteins. We were able not only to identify DCD as a novel tyrosine phosphorylated protein but also to identify Tyr^{20} as the phosphorylation site for SH2-binding. Moreover, based on the role of Nck1 on actin polymerization, we demonstrated that the functional consequence of highly expressed DCD is to activate Rho GTPases, suggesting that this strategy is effective in identifying novel SH2 domains associated phosphorylated proteins and the correlated signaling pathways.

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