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Protocadherin 9 inhibits epithelial–mesenchymal transition and cell migration through activating GSK-3β in hepatocellular carcinoma

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ABSTRACT

Protocadherin 9 (PCDH9) was found frequently lost in hepatocellular carcinoma (HCC). Here we investigated the role of PCDH9 in the development of HCC. We confirmed that PCDH9 was down-regulated in HCC tissues and cell lines compared with the adjacent non-tumor tissues. PCDH9 downregulation was significantly associated with malignant portal vein invasion of HCC patients. Gain- and loss-of-function studies revealed that downregulation of PCDH9 facilitated tumor cell migration and epithelial–mesenchymal transition (EMT). We identified PCDH9 as a novel regulator of EMT by increasing the activity of GSK-3β and inhibiting Snail1, indicating its potential therapeutic value for reducing metastasis of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and ranks third in global cancer mortality rates [1]. Despite the current use of hepatectomy and liver transplantation to improve the radical cure rates, the long-term outcome for HCC patients after effective treatment still remains generally poor [2]. The main reason for the high early recurrence of HCC is the high potential for vascular invasion and metastasis [3,4]. However, the mechanisms underlying HCC metastasis are still largely unknown. It is thus necessary to discover biological markers that predict early tumor metastasis and identify novel therapeutic targets.

The protocadherins (PCDHs) are a group of calcium-dependent adhesion proteins which make up the largest subfamily (about 80 members) of the cadherin superfamily [5]. In contrast to classical cadherin, which potentiates strong cell–cell adhesion through homophilic interactions, the PCDHs appear to have more varied physiological functions [6,7]. Thus, they act both as a mediator of cell–cell adhesion and as a regulator of other molecules [8]. Based on the genomic structure, the PCDHs family can be divided into two groups, clustered PCDHs and non-clustered PCDHs [9]. Human non-clustered PCDH genes are often located at three chromosomal loci: 4q28–31, 5q31–33 and 13q21 [6]. Recently, PCDHs around 13q21 (PCDH8, PCDH9, PCDH10, PCDH17 and PCDH20) have been reported as candidate tumor suppressor genes, suggested that these PCDHs might be broadly involved in tumor suppression in a variety of tumors [10–14].

The protocadherin 9 (PCDH9) gene has been mapped to 13q21.32 in humans and it encodes a protein which is expressed in a broader variety of tissues [5]. Recent studies have shown that PCDH9 expression was down regulated in non-nodal mantle cell lymphoma (MCL) and glioblastoma, as a result of gene copy number alterations, and that exogenous expression of PCDH9 could inhibits glioma cell invasion [11,15]. Although these results suggest that down-regulation of PCDH9 may be a factor in the carcinogenesis of MCL and glioblastoma, the association between PCDH9 and the pathogenesis of many other cancers remains elusive and

Abbreviations: PCDH9, protocadherin 9; HCC, hepatocellular carcinoma; GSK-3β, glycogen synthesis kinase-3β; Erks, extracellular signal-regulated kinases; EMT, epithelial to mesenchymal transition; LOH, loss of heterozygosity; IHC, Immunohistochemistry.

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more detailed functional analyses are required at cellular and molecular levels.

We have previously performed a 2-Mb array based CGH analysis and identified deletions of PCDH9 gene in 24% (6/25) of HCC tumor specimens tested [16]. This frequent deletion of PCDH9 in HCC has prompted us to explore whether it might act as a potential tumor suppressor gene in HCC. In the present study, we validated the down-regulation of PCDH9 in HCC tumor tissues. We found that PCDH9 could inhibit EMT and cell migration of HCC cells through activating GSK-3β signaling and inhibiting the expression of transcription factor Snail1. Our findings indicated that PCDH9 had a potential function to play a tumor-suppressor role in HCC and re-expression of PCDH9 might serve as a potential therapeutic strategy for HCC.

2. Materials and methods

2.1. Liver cancer cell lines and HCC tissues

Human liver cancer cell lines (SNU-449, SNU-182, Huh-7, SNU-387, SK-HEP-1, SMMC-7721, PLC/PRF/5 and Hep3B) were purchased from ATCC. All cells were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA).

A total of 120 HCC patients who underwent surgery at the Henan Oncology Hospital (Zhengzhou, Henan, China) from 2009 to 2013 were enrolled in this study. The clinico-pathological characteristics of the patients are listed in Supplementary Table S1. Disease-free liver tissues ($n = 12$) were obtained from liver donors in the same hospital. This study was approved by the Ethics Committee of Peking University Health Science Center.

2.2. Quantitative reverse transcription-PCR (qRT-PCR)

Quantitative real-time RT-PCR was performed on a LightCycler 480II Realtime PCR Detection System (Roche, Indianapolis, IN, USA). CTBP1 was included as a house keeping gene control to normalize expression levels [17,18]. The primers used for qRT-PCR assay are listed in Supplementary Table S2.

2.3. Immunohistochemistry and Western blot assay

Immunohistochemical staining was performed using the 2-step plus Poly-HRP Anti-rabbit IgG Detection System (ZSGB-Bio, Beijing, China) according to the manufacturer's recommendations. For direct Western blot analysis was performed as reported previously [19]. The antibodies used in this study were listed in Supplementary Table S3.

2.4. Plasmid constructions

The full-length PCDH9 cDNA with a His-tag at its C-terminal was cloned into the pIRES2-EGFP expression vector (BD Biosciences Clontech, USA). The pAAV-U6 vector encodes two effective shRNA (shRNA1 and shRNA2) against PCDH9. Oligonucleotides of the two shRNAs were showed in Supplementary Table S2.

2.5. Trans-well migration assay and Wound-healing assay

The trans-well migration assay and wound-healing assay was performed as reported previously [19].

2.6. Statistical analysis

All statistical analyses were performed with SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). The Student's t test was used to determine differences between two groups. The comparison of patient's PCDH9 expression was analyzed using the chi-square test, when $n < 5$ Fisher's exact test was used. All tests were 2-sided statistical analyses and a p-value of less than 0.05 was taken as indicative of statistically significant difference.

3. Results

3.1. Down-regulation of PCDH9 expression in HCC tissues

We first detected the PCDH9 mRNA level in 8 HCC derived cell lines, 5 of them (Huh-7, SMMC-7721, SK-HEP-1, SNU-182 and SNU-449) showed down-regulation of PCDH9 as compared to the level in normal liver tissues (Fig. 1A). Then the PCDH9 mRNA levels were compared between paired human HCC samples, compared to that in the corresponding adjacent non-tumor tissue, PCDH9 mRNA was down-regulated in a majority of tumor derived samples (61%, 73/120, $p = 0.0082$) (Fig. 1B). The down regulation of PCDH9 expression was further confirmed at the protein level, by both Western blot analysis (Fig. 1C) and immunohistochemistry (Fig. 1D).

3.2. PCDH9 down-regulation is associated with malignant portal vein invasion in HCC

We further analyzed the correlation between PCDH9 expression and the clinico-pathological characteristics of HCC patients. Stratified statistical analysis revealed that low expression of PCDH9 was significantly correlated with malignant portal vein invasion (34.2% vs. 65.8%, $p = 0.0354$) (Table 1). No association was found between PCDH9 expression and gender, age, cirrhosis background, tumor size or AFP level. Interestingly, the Mantel-Cox test also showed that patients with PCDH9 down-regulation in tumors tissue was more likely had a poor total survival of post curative resection, however such correlation did not reach statistical significance ($p = 0.2532$) (Supplementary Fig. S1).

3.3. PCDH9 suppresses migration of HCC cells

To further explore the role of PCDH9 in HCC, the HCC derived cell line SNU-449 which showed extremely low levels of endogenous PCDH9 expression was transfected with either a PCDH9 expression construct or control pIRES2-EGFP plasmid. Additionally, two HCC cell lines (Hep3B and PLC/PRF/5) that exhibited high levels of endogenous PCDH9 expression were transfected with either PCDH9 knockdown plasmid (shRNA1 and shRNA2) or control pAAV-U6 plasmid. Following G418 selection, SNU-449 cells stably expressing ectopic PCDH9, as well as the Hep3B and PLC/PRF/5 cells in which endogenous PCDH9 expression suppressed were obtained. The alterations of PCDH9 expression in these experimental cell lines were confirmed by real-time RT-PCR and Western blot assays (Fig. 2A).

In order to investigate the effects of PCDH9 expression on HCC cell motility and migration, trans-well assay and wound-healing assay were conducted. By using Trans-well assay, we found that the number of SNU-449 cells migrating through membrane partition into the lower chamber was significantly lower in SNU-449 cells ectopically expressing PCDH9 than in the control cells ($p = 0.0007$, Fig. 3B). By contrast, in both Hep3B and PLC/PRF/5 clones in which PCDH9 expression had been knocked down, the number of cells migrating through the partitioning membrane

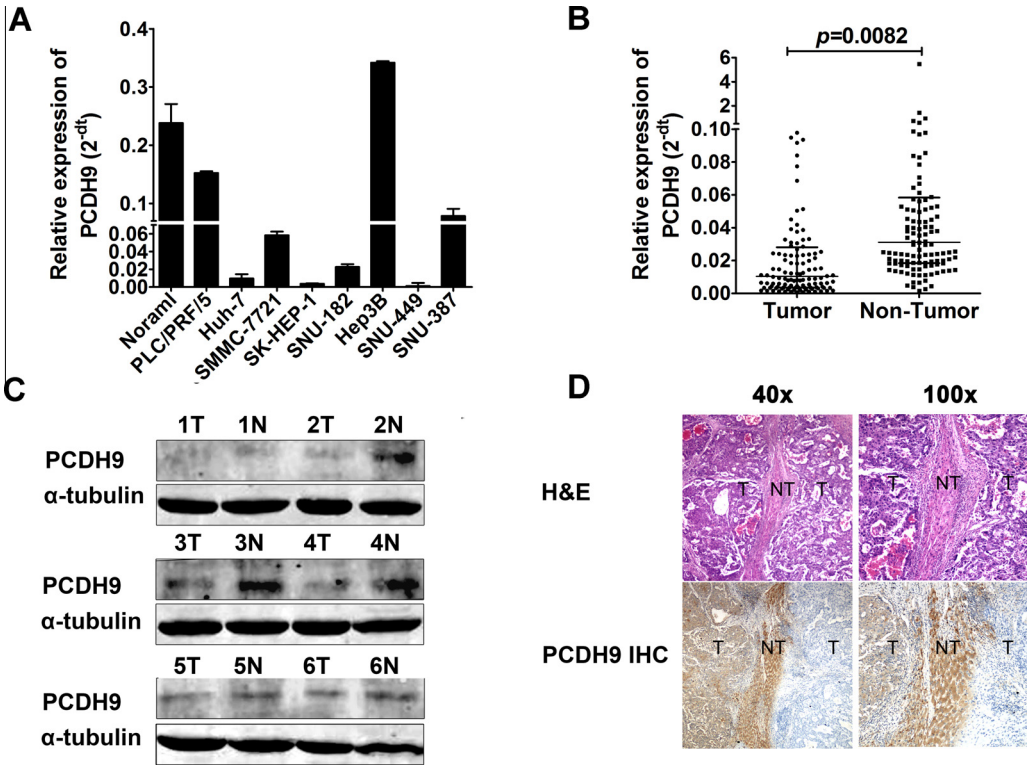

Fig. 1. Down regulation of PCDH9 in HCC cell lines and tumor tissues. (A) Expression of PCDH9 in 8 HCC cell lines and 12 control normal liver tissues (mean ± sd; $n = 3$). (B) Expression of PCDH9 in 120 pairs of HCC and adjacent non-tumor tissues. The lines in the grouped column scatter indicate mean values for tumor and adjacent non-tumor tissues. (C) Expression of PCDH9 in 6 pairs of HCC and adjacent non-tumor tissues as detected by Western blot assay. α-Tubulin was used as a loading control. (D) Representative immunohistochemistry results showing the difference in expression of PCDH9 between HCC tumor tissues (T) and adjacent non-tumor tissues (NT).

Table 1
Association of PCDH9 downregulation with clinical pathology parameters in HCC.

Characteristics		T < N ($n = 73$)	T ⩾ N ($n = 47$)	p value
Gender				0.6483
	Male	46	27	
	Female	27	20	
Age				0.1486
	<50	33	15	
	⩾50	40	32	
Portal vein invasion				0.0354
	Present	25	7	
	Absent	48	39	
	N/A	0	1	
Tumor size				0.6411
	⩾5 cm	53	35	
	<5 cm	17	12	
	N/A	3	0	
Tumor encapsulation				0.4252
	Complete	56	41	
	Incomplete	13	6	
	N/A	4	0	
Intrahepatic dissemination				0.6894
	Present	25	15	
	Absent	47	32	
	N/A	1	0	
AFP level				0.3601
	⩾400	35	17	
	<400	36	30	
	N/A	2	0	
Liver cirrhosis				0.2484
	I–III	47	35	
	IV–VI	26	12	

T: tumor tissues; N: non-tumor tissues; N/A: not available.

was significantly higher than that seen for appropriate controls (Fig. 2B). In consistence with above observation, When a scratch was created in the monolayer cells, restoration of PCDH9 levels by ectopic expression caused SNU-449 to require a longer time to re-fill the gap compared to that seen for control clones ($p = 0.0313$, Fig. 2C). By contrast, the knock-down of PCDH9 expression in Hep3B clones, promote cell migration at 24 h and 36 h post scratching compared with the appropriate controls ($p = 0.0221$ and $p = 0.0401$, Fig. 2C). Similar results were obtained from PLC/PRF/5 clones ($p = 0.0246$ and $p = 0.0251$, Fig. 2C).

3.4. PCDH9 expression inhibits EMT in HCC derived cells

During cell culture of SNU-449 cells ectopically expressing PCDH9, we noticed a loss of mesenchymal morphologic feature and re-emergence of epithelial features (Fig. 3A). These morphological changes prompted us to test the expression of a series of key EMT markers. Quantitative RT-PCR assays revealed that levels of a number of mesenchymal markers including N-cadherin (CDH2), Vimentin (VIM) and Fibronectin (FN) were significantly decreased. By contrast, the expression of epithelial markers, such as E-cadherin (CDH1, 13.5-fold up), Occludin (OCLN, 6.5-fold up), Desmoplakin (DSP, 3.7-fold up) and Keratin 18 (KRT18, 2.2-fold up) were all markedly increased (Fig. 3B). It is also noteworthy that knocking down PCDH9 expression in Hep3B or PLC/PRF/5 cells resulted in increased expression of mesenchymal markers and suppression of epithelial marker (Fig. 3C). The altered expression of the mesenchymal and epithelial markers E-cadherin, Snail1 and N-cadherin were independently confirmed using Western blot analysis (Fig. 3D).

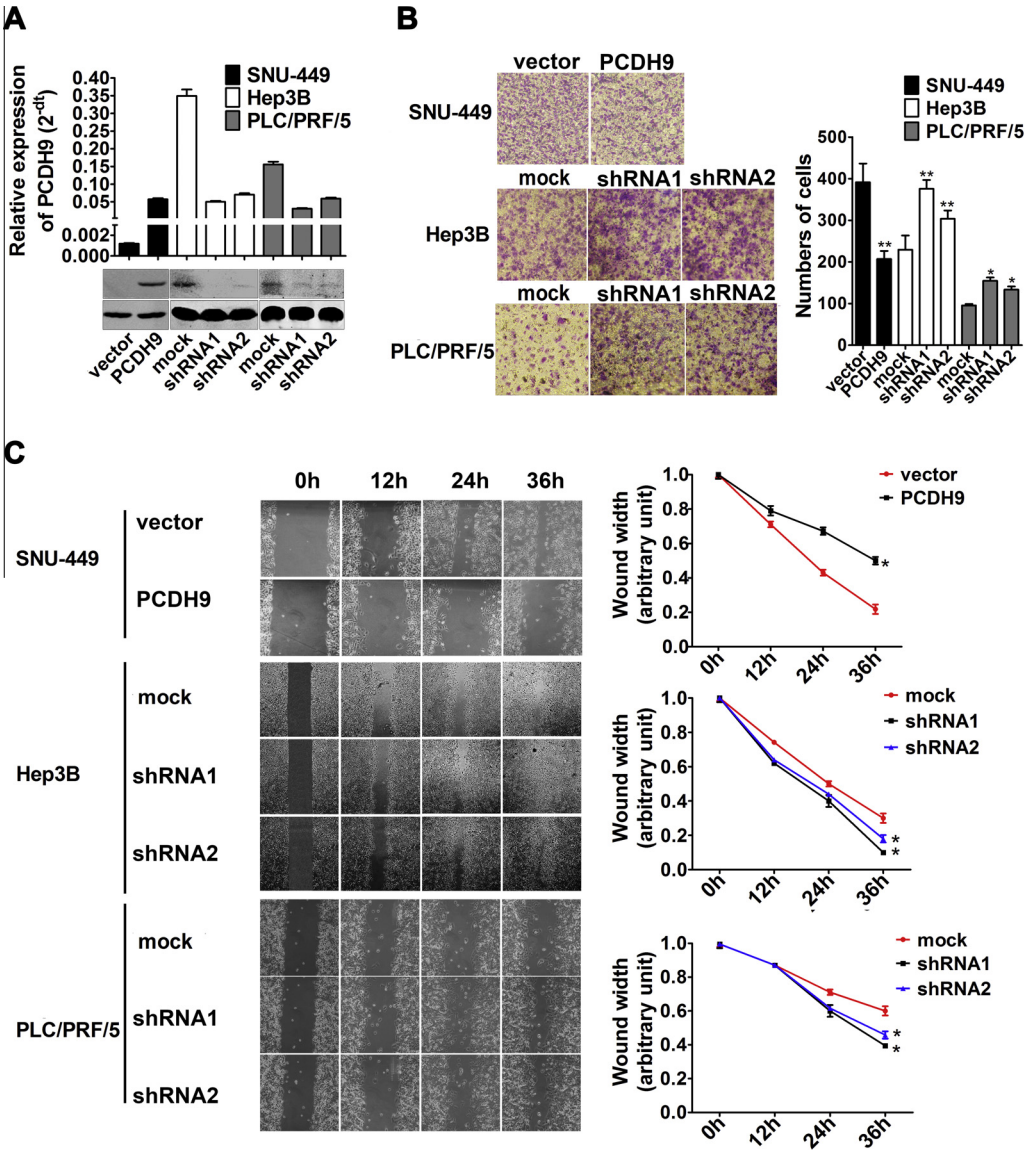

Fig. 2. PCDH9 expression can suppress cell migration in HCC derived cell lines. (A) The expression of PCDH9 in stable cell clones overexpressing and having had expression knocked down was measured by quantitative RT-PCR (mean ± sd; $n = 3$) and Western blot analysis, respectively. (B) Shows cell motility as determined in a wound-healing assay. All experiments were performed in triplicate, with the scratch area being arbitrarily assigned a value of 1.0 at time 0. (C) Shows are presentative trans-well migration assay and histograms of the number of cells migrating through the partitioning membrane. The columns show the mean for three separate experiments, each of which was done in triplicate; bars, SD. $^{*}p < 0.05$, $^{**}p < 0.005$ versus appropriate controls (Student's t test).

3.5. Activation of GSK-3β is required for PCDH9 to inhibit EMT

Snail1 plays a key role in the complex signaling network orchestrated during epithelial to mesenchymal transitions, and it is rapid turnover dependent on the GSK-3β mediated phosphorylation on it [20,21]. To determine whether GSK-3β was involved in the Snail1 down regulation induced by ectopic PCDH9, the level of phospho-GSK-3β (Ser9), the inactive form of GSK-3β, was measured by western blot assay. As shown in Fig. 4A, the level of phospho-GSK-3β (Ser9) was decreased in SNU-449 cells ectopically expressing PCDH9, whereas knockdown of PCDH9 expression in Hep3B and PLC/PRF/5 cells resulted in its level being increased. These results suggest that PCDH9 expression can modulate GSK-3β kinase activity.

To verify whether the activation of GSK-3β is required for PCDH9 to inhibit EMT, SNU-449 cells were treated with the GSK-3β inhibitor lithium chloride (LiCl) and the expression of EMT markers were analyzed. As showed in Fig. 4B, western blot assay revealed that treatment of SNU-449 cells with LiCl could reverse PCDH9 mediated Snail1 down-regulation. LiCl treatment also increased N-cadherin and decreased E-cadherin levels. Meanwhile, LiCl treatment eliminated the PCDH9-induced expression change of other EMT markers (Fig. 4C).

Both Akt and Erks can phosphorylate GSK-3β at the crucial Ser9 residue. Therefore, western blot analysis was carried out and the results demonstrated the down-regulation of phospho-Akt (Ser473) and phospho-Erk (Thr202/Tyr204) in SNU-449 cells ectopically expressing PCDH9. By contrast, knockdown of endogenous PCDH9 expression resulted in the up-regulation of phospho-Akt and Erk in Hep3B and PLC/PRF/5 cells (Fig. 4A), suggesting strongly that PCDH9 might activate the GSK-3β via modulating Erks and Akt activation.

4. Discussion

HCC development is clearly a multistep process involving both the activation of oncogenes and the silencing of tumor suppressor

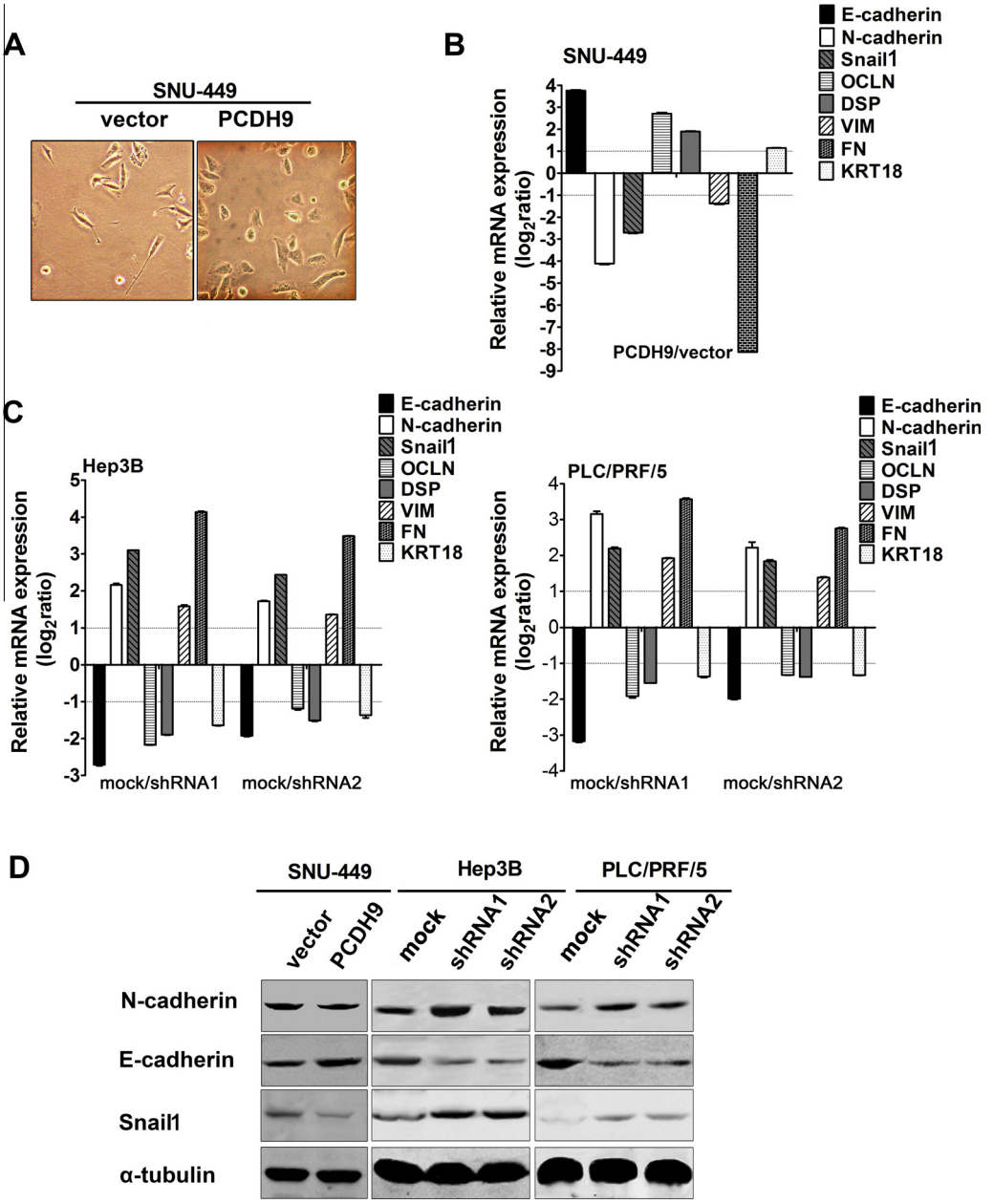

Fig. 3. Regulation of PCDH9 expression on EMT in HCC derived cell lines. (A) Shows morphological changes in SNU-449 cells ectopically expressing PCDH9. (B) Shows quantitative real time RT-PCR analysis of indicated EMT biomarkers in SNU-449 cells ectopically expressing PCDH9. The $\log_2$ratios of EMT markers, which reflect the change of expression levels, were muffled to less than 1 or −1. The PCDH9/vector ratio shown is a $\log_2$ratio scale where 0 means equal, +1 means double and −1 means 50% (mean ± sd; $n = 3$). (C) Shows quantitative real time RT-PCR analysis of indicated EMT biomarkers in Hep3B or PLC/PRF/5 cells in which PCDH9 expression has been suppressed. The $\log_2$ratio of shRNA1/mock or shRNA2/mock ratio is calculated. (D) Shows Western blot analysis of the EMT-related molecules E-cadherin, N-cadherin and Snail1.

genes. Published studies on HCC have shown that chromosome region 13q is one of the most frequently affected chromosome arms in a number of cancers including HCC [22,23]. A recently published study has reported that allelic losses on 13q can result in more aggressive tumor behavior, suggesting that this chromosomal region is likely to contain TSGs [24,25]. In this study, we provide direct evidence for the tumor-suppressing role of PCDH9, which is located on chromosome 13q21.32 and frequently deleted in HCC.

PCDH8, PCDH10, PCDH17 and PCDH20 are co-localized with PCDH9 on chromosome 13q21 [6]. The genetic and/or the epigenetic aberration of these PCDHs have been found in many kinds of tumors [4,6,10,12,14]. However, the expression and molecular function of PCDH9 in HCC has not previously been analyzed in any detail. In this study, we detected the PCDH9 mRNA level in 120-paired human HCC tissues and 8 HCC derived cell lines. The results showed that expression of PCDH20 is frequently decreased in HCC tumor tissues (73 of 120, 61%) and cell lines (5 of 8, 62.5%). Further analysis revealed that reduced expression of PCDH9 was significantly associated with the malignant portal vein invasion of HCC patients, suggested that down-regulation of PCDH9 plays a critical role in establishment of HCC metastasis. In consistent with the above-suggested function, overexpression of PCDH9 in HCC derived cell lines inhibited cell migration, whereas loss of PCDH9 expression enabled HCC cells to gain higher invasive capability. These results further confirmed the migration-suppressive

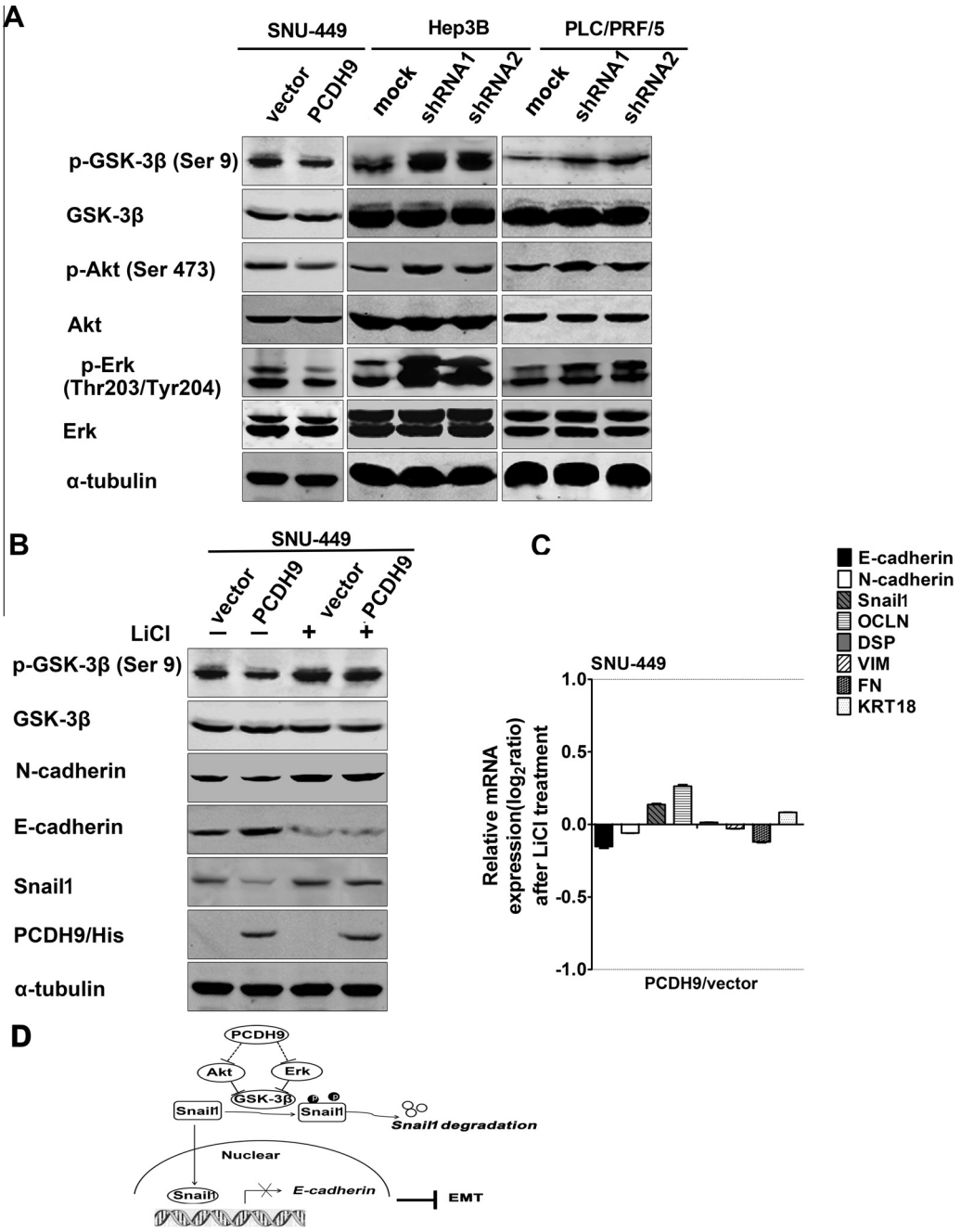

Fig. 4. Activation of GSK-3β is required for PCDH9-induced modulation of EMT. (A) Shows Western blot of phosphorylated GSK-3β, Erks, and Akt in SNU-449 cells ectopically expressing PCDH9 or Hep3B or PLC/PRF/5 cells in which PCDH9 expression has been suppressed. (B and C) Shows the expression of EMT molecular biomarkers determined in SNU-449 cells after LiCl treatment by Western blot analysis (B) and real-time RT-PCR (C). (D) The Intracellular signaling cascades of EMT inhibition triggered by PCDH9. '→' means positive regulation, '–|' means negative regulation, an un-broken line means direct regulation and a dotted line means indirect regulation.

function of PCDH9 in HCC. Notably, the frequency of the decreased expression of PCDH9 mRNA expression in primary HCC tissues (61%) was higher than that expected from the gene loss (24%), suggesting that other mechanisms such as epigenetic regulation and post-translational modification, which also contribute to the down-regulation of PCDH9 in HCC.

EMT is a key event in the tumor invasion and metastasis process by epithelial cell layers lose polarity and cell–cell contacts and remodel the cytoskeleton [26,27]. The decreased invasive capability and morphological changes of SUN-449 cell line induced upon ectopic PCDH9 expression prompted the investigation of PCDH9's involvement in the regulation of the epithelial–mesenchymal transition in this study. As expected, ectopic expression of PCDH9 could up-regulate epithelial markers, such as E-cadherin, Occludin and KRT18, and down-regulate mesenchymal markers such as N-cadherin, Vimentin and Fibronectin, and particularly the EMT-associated transcription regulator Snail1. Consistent with these results, knockdown of PCDH9 expression caused a decrease in the expression of these epithelial markers and an increase in expression of mesenchymal markers. The expression pattern of these EMT markers are consistent with the alteration of migratory properties induced by PCDH9 in HCC cells, supporting that PCDH9

may be a key regulator of invasion and metastasis by inhibiting EMT of HCC cells. This involvement of PCDH9 in regulating EMT also provided an explanation for the association of the down-regulation of PCDH9 expression with the presence of portal vein invasion in HCC patients.

Amongst the various biomarkers of EMT, Snail1 is generally considered to be a critical transcriptional regulator because of its direct suppressive effect on CDH1 (encoding E-cadherin) promoter [20,28]. In addition, GSK-3β has been recognized as the primary kinase involved in regulating both the sub-cellular location and stability of the Snail1 protein [21]. It was therefore plausible to hypothesize that PCDH9 may down-regulate Snail1 expression through activating the GSK-3β kinase. As expected, our data demonstrated that PCDH9 could activate the GSK-3β kinase and reduce the expression of Snail1. Treatment of cells with LiCl, a known inhibitor of GSK-3β abolished down-regulation Snail1 induced by ectopically expressing of PCDH9, meanwhile the changes of EMT biomarkers disappeared. These results allow the construction of a putative pathway for PCDH9 conducting its biological action (Fig. 4D). This proposes that, acting through an undefined mechanism, PCDH9 inhibits both Akt and Erks kinase activities and this leads to the activation of the GSK-3β kinase, which in turn suppress the Snail1 expression. Reduced expression of Snail1 emancipates the transcription of E-cadherin and results in an up-regulation of its expression, which inhibits the EMT process and cell migration of HCC cells.

Several recent studies have indicated that PCDH19, a member of PCDHs subfamily might act synergistically with N-cadherin to regulate cell adhesion during anterior neurulation in zebrafish [29,30]. However, PCDH9 showed neither homophilic interaction with itself nor heterophilic adhesion with N-cadherin in the present study (Supplementary Fig. S2), this is consistent with another PCDH member PCDH20 which we recently demonstrated it as a tumor suppression gene in HCC [19]. We found that both PCDH20 and PCDH9 could conduct their anti-tumor property through suppressing activation of Akt and Erks and promoting GSK-3β kinase activities [19]. Unlike PCDH20's capability to inhibit Wnt/β-catenin signaling via down-regulating β-catenin, PCDH9 could not suppress Wnt/β-catenin signaling pathway in HEK 293T or any of 5 HCC derived cell lines tested (Huh-7, SMMC-7721, SK-HEP-1, SNU-182 and SNU-449) (data not shown). Instead, PCDH9 exhibits its tumor suppressing function by down-regulating Snail1 and inhibiting epithelial to mesenchymal transition. Though Snail1 and β-catenin are both downstream molecular of GSK-3β, the different modulation on GSK-3β signaling by PCDH9 and PCDH20 suggested that different members of PCDH could play diverse roles as intracellular signaling regulators. Further study will be conducted to determine the precise intracellular signaling and explore other potential mechanisms of PCDH9 in the carcinogenesis of HCC.

In summary, this study has shown for the first time that PCDH9 acts as a potential tumor suppressor gene in HCC, and the loss of PCDH9 expression appears to be a driver in a significant fraction of hepatocarcinogenesis.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

We thank Professor Quanjun Lv for his enthusiastic help to establish the HCC tissue specimen database.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.101.

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