PCDH9 acts as a tumor suppressor inducing tumor cell arrest at G_0/G_1 phase and is frequently methylated in hepatocellular carcinoma

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Abstract. Tumor suppressor genes (TSGs) are frequently involved in the pathogenesis of hepatocellular carcinoma (HCC). The epigenetic and genetic alterations of a novel TSG-protocadherin 9 (PCDH9) and its functions in the pathogenesis of HCC were investigated. The methylation status of the PCDH9 promoter was quantitatively analyzed, and the PCDH9 expression was analyzed in HCC cell lines treated with 5-azacytidine. The effects of PCDH9 re-expression and knockdown on growth, proliferation and tumorigenic potential were determined. The results indicated that expression of PCDH9 mRNA was restored in hypermethylation HCC cells following treatment with the DNA de-methylation reagent 5'-Aza. Methylation of the PCDH9 promoter was observed in 22% primary HCC tissues (24/111 tumors). Among the primary HCC cases, the methylated PCDH9 appeared to be associated with a larger tumor size (≥5 cm; P=0.0139) and a more pronounced intrahepatic dissemination (P=0.0312). In addition, it was observed that restored PCDH9 expression could inhibit tumor cell proliferation and xenograft tumor formation. Furthermore, restored PCDH9 expression could inhibit cell proliferation of HCC cell lines via inducing cell cycle arrest at G_0/G_1 phase. Thus, it is suggested that PCDH9 may act as a novel tumor suppressor candidate gene in HCC pathogenesis.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and ranks the third in global cancer mortality rates (1). HCC mortality increases with tumor invasiveness, despite advances in detection, treatment and surveillance strategies. Novel molecular biomarkers are required in order to assist in outcome prediction and in the identification of high-risk patients who would benefit from aggressive postoperative therapy.

Genetic alterations including point mutations, chromosomal deletion/amplification, or epigenetic changes including promoter hypermethylation and genome wide hypomethylation may be implicated in this process (2,3). At present, published studies have reported the loss of heterozygosity (LOH) are frequently detected in HCC and potential tumor-suppressor genes on chromosomes 1p, 4q, 5q, 8p, 8q, 10q, 11p, 13q, 16q and 22q have been suggested (4-8). A previous study implies that allelic losses on 13q may bring about a more aggressive tumor behavior, and inactivation of these genes via allelic losses likely enhances tumor progression in HCC (9).

The cadherin superfamily, a large family of transmembrane or membrane-associated glycoprotein, serve an essential role in regulation of organ and tissue development during embryogenesis and formation of stable cell-cell junctions and maintenance of normal tissue structure in adult organisms (10,11). Certain cadherins have been identified to act as tumor suppressor genes (TSGs), including E-cadherin, a classic cadherin member, which was first described among the cadherin protein family as L-CAM in chicken and then defined as a potential suppressor of invasion and metastasis

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Abbreviations: PCDH9, protocadherin 9; HCC, hepatocellular carcinoma; LOH, loss of heterozygosity

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in lobular breast carcinoma, and pancreatic and epithelial ovarian cancer (12-14).

The protocadherins (PCDHs) are a group of calcium-dependent adhesion proteins that make up a major subfamily of the cadherin superfamily (15). They are abundantly expressed in the central nervous system during embryonic development and during adulthood (16). In contrast to classical cadherins that potentiate strong cell-cell adhesion through homophilic interactions, the PCDHs appear to have more varied physiological functions (17). Previous studies have suggested that PCDHs (PCDH8, PCDH10, PCDH17 and PCDH20) can function as candidate TSGs in a variety of tumor types (18-21). Epigenetic modifications, particularly DNA CpG methylation, serve a key role in the silencing/inactivation of these TSGs (22,23). The PCDH9 gene has been mapped to 13q21.32 in humans and it encodes a protein that is expressed in a broader variety of tissue types (15). Previous studies have demonstrated that PCDH9 expression was downregulated in non-nodal mantle cell lymphoma and glioblastoma (24,25) as a result of gene copy number alterations, and that exogenous expression of PCDH9 could inhibits tumor cell migration. However, whether this gene contributes to HCC tumorigenesis and metastasis remains unknown.

A 2-Mb array-based comparative genomic hybridization (aCGH) analysis was performed, which identified deletions in chromosome 13q21 (the region to which the PCDH9 gene maps) in 24% (6/25) of tumor specimens tested (26). This frequent deletion of PCDH9 in HCC has prompted the investigation of whether it may act as a potential TSG in HCC pathogenesis using *in vitro* and *in vivo* based assay protocols. In the current study, the inactivation of PCDH9 in HCC and its functions in the development of HCC were investigated.

Materials and methods

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 obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Shanghai, China).

A total of 111 patients with HCC who underwent surgery at the Henan Oncology Hospital (Zhengzhou, China) between 2009 and 2013 were enrolled in the present study. Disease-free liver tissues (n=12) were obtained from liver donors in the same hospital. The present study was approved by the Ethics Committee of Peking University Health Science Center (Beijing, China). Written informed consent was obtained from all patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and quantificational methylation assay. RT-qPCR and quantificational methylation assay were performed as described previously (27). The primers used for this methylation status assay are listed in Table I.

Plasmid constructions. The full-length PCDH9 cDNA with a His-tag at its C-terminal was cloned into the pIRES2-EGFP expression vector (Clontech Laboratories, Inc., Mountainview, CA, USA). The pAAV-U6 vector (Cell Biolabs, Inc. San Diego, CA, USA) encodes two effective shRNA (shRNA1 and shRNA2) against PCDH9. Oligonucleotides of the two shRNAs are presented in Table I.

MTT assay. Cell viability was measured using the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay as described previously (28).

Soft agar growth and in-vivo tumorigenicity assay in nude mice. The anchorage-independent growth capability of cells was measured as described previously (28). The *in vivo* tumorigenic potential of cells was investigated in 6-week-old male nude mice (weight, ~20 g; n=6). Nude mice were purchased from the Department of Laboratory Animal Science in Peking University Health Science Center (Haidian, Beijing) and housed in the same place. Mice were maintained in specific pathogen-free rooms, using a microisolator (filter bonneted) or pressurized, individually ventilated cages. The animals were kept in an animal room under SPF conditions at a room temperature of 22±1˚C, with 55±10% relative humidity. Food and water made freely available to the mice. Sterilized or disinfected bedding and cages were used, as was anything that came into contact with the mice. 6-week-old male nude mice received subcutaneous injections of $5x10^6$ HCC cells (SNU449, Hep3B or PLC/PRF/5) in either side of the posterior flank in a volume of 100 μl. Tumor formation was monitored every week over a 6 week period (SNU449) or 5 weeks period (Hep3B or PLC/PRF/5), depended on the size of the tumor. The tumor volume was calculated by the formula: volume=0.5 x length x width^2 (in mm), the maximum tumor size was 616.1 mm^3 (29).

Western blot assay. Western blot analysis was performed as described previously (28). Cells were lysed in RIPA buffer (R0278; Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) and the total cellular protein was resolved on denaturing polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and blotted with primary antibodies. The dilution and catalog no. of primary antibodies used in the present study are listed in Table II. Protein-antibody complexes were visualized using secondary antibodies conjugated with Cy5.5 (catalog. no. RPN998; 1:10,000 dilution; GE Healthcare Life Sciences) and visualized by LI-COR Odyssey IR Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. All statistical analyses were performed with SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). The comparison of the patient genome methylation status was analyzed using the Chi-square test, when n<5 Fisher's exact test was used. The significant differences in the cell growth of clones stably overexpressing PCDH9 and clones transfected with control plasmid were determined using paired t-tests. Differences in tumor growth rate were determined as determined by an analysis of variance followed by Tukey's post hoc test. All tests were 2-sided statistical analyses and $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used in the current study.

Primer	Sequence (5'-3')
PCDH9-methy-F1	CTCCAGGCGCACACTTCGCA
PCDH9- methy-R1	GCTGACCGAGGGGACCCAGA
PCDH9-LOH-F1	TACCGTGTGCGTCAGAGCTT
PCDH9-LOH-R1	GTCCACTAACGTTGTTAACAGC ACA
PCDH9-LOH-F2	TGTGGCAAAGTCCTACAACAGGCA
PCDH9-LOH-R2	TCTCTTGGCTGCAATTTTCTCTCCT
PCDH9-LOH-F3	TGGTAACATCAGCTCACAACATC TG
PCDH9-LOH-R3	CCACACATGGGCATAAGAATG CTCT
PCDH9-LOH-F4	CAGGACTGATGGCAAGGAGC
PCDH9-LOH-R4	TGGCTCCACTAATGTAGAAATCAC
PCDH9-LOH-F5	CAGCATTGCAGATGGAGTCAAG AGA
PCDH9-LOH-R5	AGGGTGGGGTCATGATGATGAA GAT
PCDH9-F	TCCCAACTCTGATGGGCCTTTGGG
PCDH9-R	GGCTCTGGTCAGGGTGTGCC
shRNA1-1	GATCCCCGCGGTATATGACAA CCAATATTTCAAGAGA
shRNA1-2	ATATTGGTTGTCATATACCGCTT TTTA
shRNA1-3	ATATTGGTTGTCATATACCGCGGG
shRNA1-4	AGCTTAAAAAGCGGTATATGACAA CCAATATTCTCTTGAA
shRNA2-1	GATCCCCCCCCAAGTTTACTCATAA TCATTTCAAGAGA
shRNA2-2	ATGATTATGAGTAAACTTGGGTT TTTA
shRNA2-3	ATGATTATGAGTAAACTTGGGGGG
shRNA2-4	AGCTTAAAAACCCAAGTTTACTC ATAATCATTCTCTTGAA

PCDH9, protocadherin 9; F, forward; R, reverse; LOH, loss of heterozygosity; sh, short hairpin.

Table II. Antibodies used in western blotting.

Antibody	Company (catalog number)	Dilution
Anti-α-tubulin	MBL (JM-3708-100)	1:3,000
Anti-GAPDH	MBL (M171-3)	1:3,000
Anti-PCDH9	Santa cruz (sc-84564)	1:200
Anti-his	MBL (D291-3)	1:2,000
Anti-$p21^{Waf1/Cip1}$	MBL (K0081-3)	1:500
Anti-$p27^{Kip1}$	Cell signaling (#3698)	1:500
Anti-cyclin D1	MBL (K0062-3)	1:500
Anti-cyclin E	MBL (K0172-3)	1:500

MBL, MBL International, Woburn, MA, USA; PCDH9, protocadherin 9; Santa Cruz, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; His, histidine; Cell Signaling, Cell Signaling Technology, Inc., Danvers, MA, USA.

Results

Downregulation of PCDH9 expression in HCC tissues. Downregulation of PCDH9 mRNA was detected in 5 (Huh-7, SMMC-7721, SK-HEP-1, SNU-182 and SNU-449) of the 8 HCC-derived cell lines, compared with the level observed in controls obtained from normal liver tissues (Fig. 1A).

DNA methylation and histone deacetylation contributes to the downregulation of PCDH9 expression seen in HCC. In the aCGH assay performed on a subset of the HCC samples, deletions of the chromosomal region (13q21.32) where PCDH9 located was detected in only certain 25% (6 of 25) tumor tissues analyzed (data not shown). Therefore, to investigate if hypermethylation also contributes to PCDH9 downregulation in HCC, the methylation status of the PCDH9 promoter was examined in HCC cell lines and tumor-derived tissue samples. Subsequently, the CpG island in the promoter of PCDH9 was analyzed and the primer was designed to detect methylation status of PCDH9 as presented in Fig. 1B. Amongst the 8 HCC cell lines examined, SMMC-7721 alone exhibited significant PCDH9 promoter hypermethylation and in this case, treatment of cells with the DNA demethylation reagent 5-Aza (5-aza-2'-deoxycytidine) restored PCDH9 gene expression, however similar treatment of HCC-derived cell lines that did not show hypermethylation of the PCDH9 promoter had no effect on PCDH9 expression (Fig. 1C). In addition, it was identified that treatment of cells with the specific histone deacetylase inhibitor trichostatin (TSA), restored PCDH9 gene expression in Huh-7 and SNU-182 cells. While combined treatment of SMMC-7721 cells with TSA and 5-Aza indicated that the expression of PCDH9 was higher than 5-Aza treatment alone (Fig. 1D), these results indicated that methylation and histone deacetylation of the PCDH9 had effects on PCDH9 expression. In addition, 22% of the HCC tumor-derived tissues tested (24/111 paired samples successfully analyzed) indicated higher levels of methylation in the tumor tissue sample compared with that observed in the corresponding adjacent non-tumor tissues and normal liver tissues, however such differences did not reach statistical significance ($P>0.05$; Fig. 1E). Nevertheless, the level of PCDH9 mRNA in tumor tissues that exhibited hypermethylation was also identified to be significantly lower than that observed in the un-methylation group ($P=0.032$; Fig. 1F). These data suggest that DNA hypermethylation can also mediate the downregulation of PCDH9 expression observed in HCC. A further notable result was the significant association of PCDH9 hypermethylation with larger tumor size observed (≥5 cm; $P=0.0139$) and the more pronounced intrahepatic dissemination ($P=0.0312$) were identified (Table I).

PCDH9 inhibits tumor cell proliferation and xenograft tumor formation. To further explore the role of PCDH9 in HCC, the HCC-derived cell line SNU-449 that exhibited low levels of endogenous PCDH9 expression was transfected with either a PCDH9 expression constructor or control pIRES2-EGFP

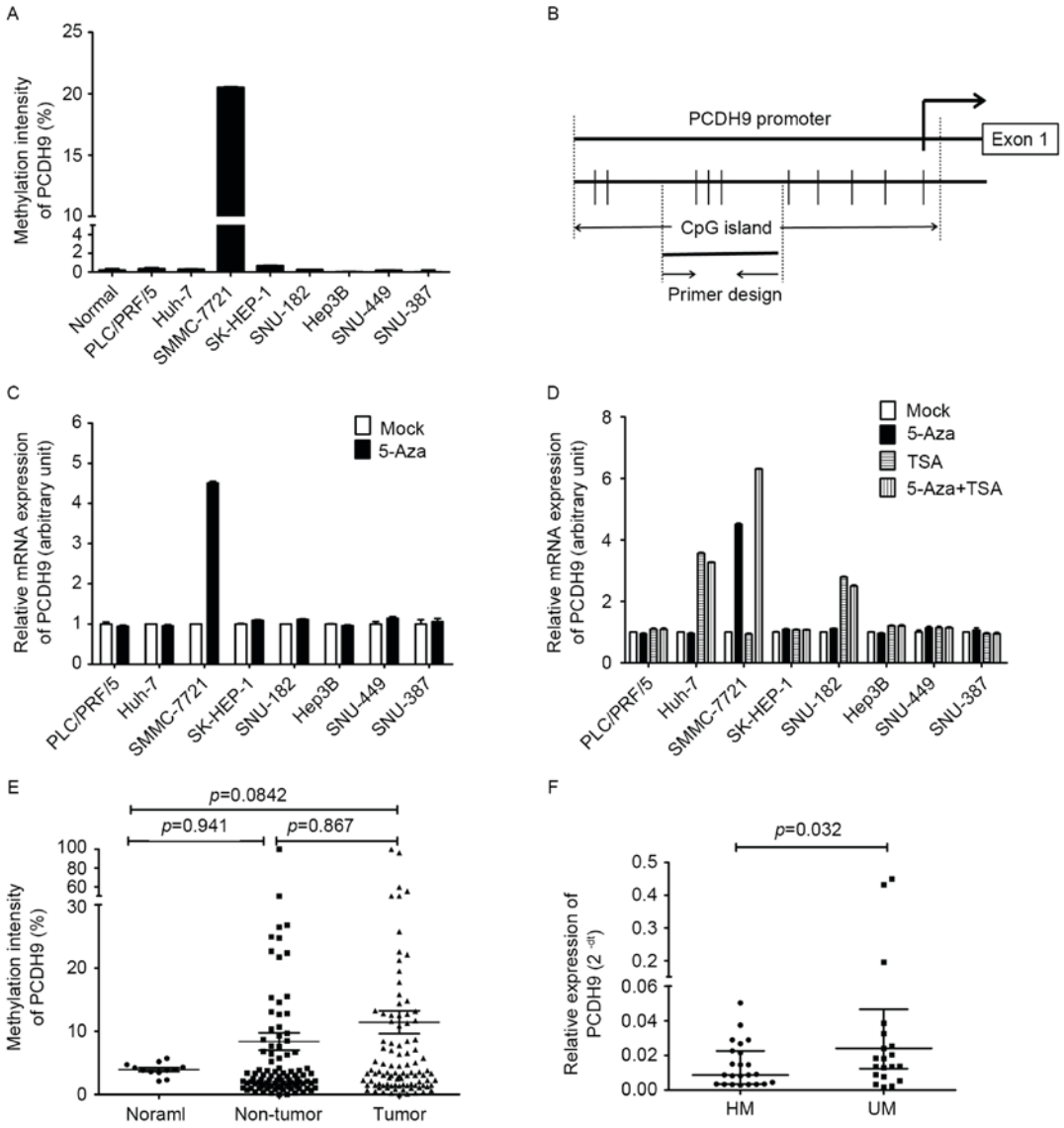

Figure 1. Downregulation of PCDH9 in HCC cell lines and tumor tissues. (A) Expression of PCDH9 in 8 HCC cell lines and 12 control normal liver tissues (mean ± standard deviation; n=3). (B) CpG island in the promoter of PCDH9 and methylation primer design of PCDH9. (C) The expression of PCDH9 in 8 HCC cells treated by 5-Aza (mean ± standard deviation; n=3). The PCDH9 expression level in mock treated cells was set at an arbitrary value of 1 to facilitate comparison. (D) The expression of PCDH9 in 8 HCC cells treated by TSA (mean ± standard deviation; n=3). The PCDH9 expression level in mock treated cells was set at an arbitrary value of 1 to facilitate comparison. (E) The methylation intensity of PCDH9 in normal liver, non-tumor and tumor tissues. Significant differences were determined using Student's t-test. (F) The expression of PCDH9 in hypermethylation group and unmethylation group. The lines in the grouped column scatter indicate the mean ± standard deviation. 'MI'≥10%': Hypermethylation group; 'MI<10%': Unmethylation group. Significant differences were determined using Student's t-test. PCDH9, protocadherin 9; HCC, hepatocellular carcinoma; 5-Aza, 5-aza-2'-deoxycytidine; TSA, trichostatin; MI, methylation intensity.

plasmid. Separately, two HCC cell lines (Hep3B and PLC/PRF/5) that exhibited high levels of endogenous PCDH9 expression were transfected with either PCDH9 knockdown (shRNA1 and shRNA2) or control pAAV-U6 plasmids. Following G418 selection, stable clones of SNU-449 cells in which the expression of PCDH9 had been increased were obtained, in addition to stable clones of Hep3B and PLC/PRF/5 in which endogenous PCDH9 expression had been suppressed. The overexpression of PCDH9 and suppression of endogenous PCDH9 expression was confirmed by western blot analysis (Fig. 2A).

The ability of PCDH9 expression to affect cell proliferation was analyzed in these over- and underexpressing cell clones using an MTT assay. This indicated that the growth of SNU-449 cells overexpressing ectopic PCDH9 was significantly suppressed compared with that observed in clones transfected with control plasmid (P=0.0125; Fig. 2B). By contrast, the use of shRNA1 and shRNA2 to efficiently knockdown endogenous PCDH9 expression significantly enhanced cellular proliferation in Hep3B-(P=0.0107 and P=0.0110; Fig. 2B) and PLC/PRF/5 (P=0.0118 and P=0.0120; Fig. 2B)-derived clones.

A second *in vitro* assay measuring the ability of these clones to form colonies in soft agar was also undertaken as an indicator of their tumorigenic potential. This indicated that the SNU-449-derived clones had decreased colony-forming efficiency compared with mock-transfected controls (P=0.0101; Fig. 2C). By contrast the PCDH9 knockdown in the Hep3B derived clones induced the cells to form much larger colonies

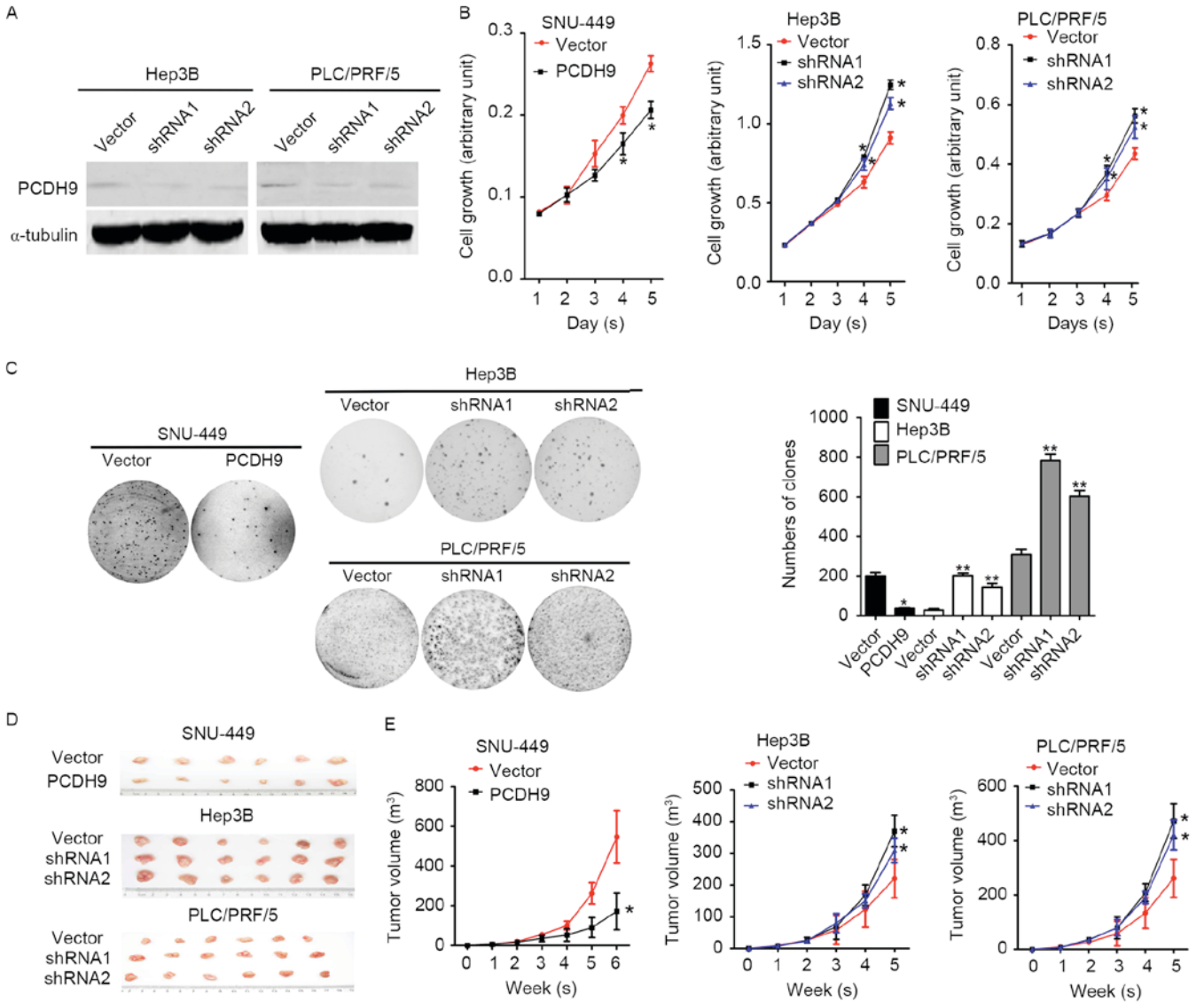

Figure 2. Ectopic expression of PCDH9 can inhibit the tumorigenic properties of HCC cell lines. (A) The expression of PCDH9 in stable cell clones overexpressing and having had expression knocked down was measured by western blot analysis, respectively. (B) The growth of SNU-449, Hep3B and PLC/PRF/5 cell clones stably overexpressing PCDH9, or in which expression of the protein had been knocked down were compared with clones transfected with control plasmid using the MTT assay subsequent to being cultured for varying numbers of days. Values are presented the mean ± standard deviation of three independent experiments and error bars represent standard error of the mean with 3 replicates for each day. Significant differences in the cell growth of clones stably overexpressing PCDH9 and clones transfected with control plasmid were determined using paired t-tests. Asterisks indicate a P-value of <0.05 (statistically significant). (C) Colony formation was analyzed using an anchorage-independent growth assay in softagar. The assay was repeated three times and the histograms indicate the numbers of cell clones detected in a representative assay. Significant differences in the numbers of clones detected were determined using Student t-tests. PCDH9 tumorigenicity was measured in the nude mice xenograft model. (D) Tumor volumes were measured and then were plotted as the mean ± standard deviation. (E) Graphs showing the rate of tumor growth are presented (n=6/group). PCDH9, protocadherin 9; HCC, hepatocellular carcinoma; sh, short hairpin.

in soft agar than that observed in controls (P=0.0025 and P=0.0086, respectively; Fig. 2C). Similar results were also obtained with the PLC/PRF/5-derived knockdown clones (P=0.0004 and P=0.0011, respectively; Fig. 2C).

To investigate tumorigenic potential *in vivo*, a tumor formation assay was conducted in nude mice. A total of 5 weeks after injection, significant differences in average tumor size were observed; 545.8±131.9 mm^3 in mice injected with control SNU-449 cells and only 171.5±92.1 mm^3 in mice injected with SNU-449 expressing ectopic PCDH9 (P=0.0151; Fig. 2D and E). This is contrasted with the five week post-injection image observed for Hep3B clones in which PCDH9 expression had been knocked-down following transfection with plasmids expressing either shRNA1 or shRNA2, an average 5 week post injection tumor size of 220.3±60.8 mm^3 in mice injected with control clones and 372.7±49.8 mm^3 for clones expressing shRNA1 (P=0.0364), and 309.2±38.6 mm^3 for clones expressing shRNA2 (P=0.0401; Fig. 2D and E). PCDH9-knockdown with either shRNA1 or shRNA2 also resulted in PLC/PRF/5 clones which had significantly increased tumor formation when injected into nude mice compared with that produced following injection of the control counterpart (P=0.0401 and P=0.0456; Fig. 2D and E).

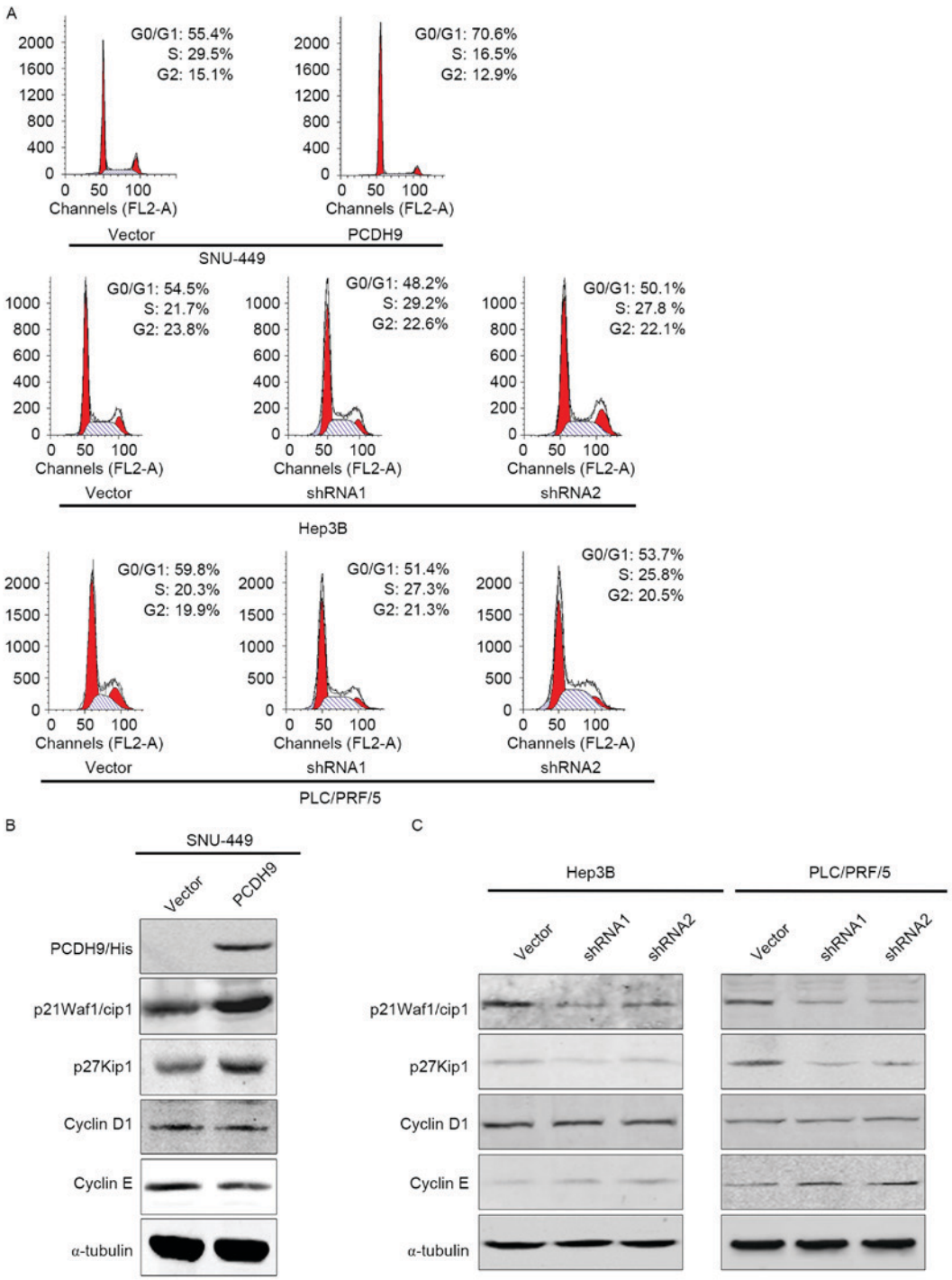

Figure 3. PCDH9 can inhibit tumor cell arrest in G_0/G_1 phase. (A) Ectopic expression of PCDH9 could increase the percentage of G_0/G_1 phase from 55.4 to 70.6% in SNU449 cell lines, while the percentage of S phase was reduced from 29.5 to 16.5%. Knockdown of PCDH9 decreased the percentage of G_0/G_1 phase from 54.5 to 48.2 and 50.1% in the Hep3B cell lines, while it increased the percentage of S phase from 21.7 to 29.2 and 27.8%. Knockdown of PCDH9 decreased the percentage of G_0/G_1 phase from 59.8 to 51.4 and 53.7% in the PLC/PRF/5 cell lines, while it increased the percentage of S phase from 20.3 to 27.3 and 25.8%. (B) Ectopic expression of PCDH9 could increase the expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ while it reduced cyclin D1 and cyclin E. (C) Knockdown of PCDH9 decreased the expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ while it increased cyclin D1 and cyclin E. PCDH9, protocadherin 9; sh, short hairpin.

PCDH9 inhibits cell proliferation of HCC cell lines via inducing cell cycle arrest at G_0/G_1 phase. PCDH9 was also identified to have effects on HCC cells in which there was ectopic overexpression or knockdown of the protein PCDH9. Flow cytometry indicated that in SNU-449 cells, PCDH9 overexpression blocked G_1/S transmission with the percentage of cells in G_1 phase increasing from >55% to ~71%, whilst the percentage in S phase decreased from 29 to 16% (Fig. 3A). By contrast, in Hep3B cells, PCDH9 shRNA1 or shRNA2 decreased G_0/G_1 phase from ~55 to 48% or 50%, respectively, whilst the percentage in S phase increased from ~22 to 29% or 28%, respectively (Fig. 3A). Similarly, in PLC/PRF/5 cells, PCDH9 shRNA1 or shRNA2 decreased G_0/G_1 phase from ~60 to 51% or 54%, respectively, whilst the

percentage in S phase increased from ~20 to 27% or 26%, respectively (Fig. 3A).

The expression of the cell cycle progression regulators cyclin D1, cyclin E, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ were also monitored using a western blot assay. Ectopic expression of PCDH9 in SNU-449 cells significantly increased the levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, however suppressed cyclin E expression and the protein level of cyclin D1 remained unchanged (Fig. 3B). In addition, in Hep3B and PLC/PRF/5 cells, PCDH9 shRNA1 or shRNA2 decreased the protein levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, while increasing the cyclin E expression, and the protein level of cyclin D1 was unchanged (Fig. 3C). These results suggested that it may induce G_1 phase arrest by regulating the expression of cell cycle regulators.

Discussion

Previously, the downregulation of PCDH9 was observed in primary HCC, and PCDH9 was identified to inhibit epithelial-mesenchymal transition and cell migration through activating GSK-3β (30). However, the mechanism remains unclear. The current study aimed to investigate the mechanism and the function of the PCDH9 in HCC cells.

The pathogenesis and development of HCC is a multi-step process involving genetic and epigenetic alterations. Epigenetic alterations (such as DNA methylation and histone deacetylation) are clinically notable because they may be able to reverse such changes and restore gene function. Therefore, epigenetic markers could be clinical indicators for the detection, outcome prediction and treatment of HCC. The frequent LOH of PCDH9 gene identified in previous aCGH data implies that chromosomal deletion accounts for the downregulation of PCDH9 in a significant fraction of HCC cases (26). Epigenetic inactivation of PCDH8, PCDH17 and PCDH20 located on region of chromosome 13 (13q) surrounding the PCDH9 gene has been previously reported in several types of human cancer (18,20,21), thus prompting the investigation of the role of hypermethylation of the PCDH9 promoter in the downregulation of PCDH9 in HCC. As predicted, hypermethylation of the PCDH9 promoter was detected in approximately one fifth of tumor tissues tested, and it was identified to correlate with low-expression of PCDH9 in tumor-derived tissue. Notably, statistical analysis indicated that hypermethylation of the PCDH9 promoter was strongly associated with large tumor size and increased intrahepatic dissemination. This strongly suggests that epigenetic repression of PCDH9 expression may permit tumor growth, and tumor cell migration, resulting in accelerated liver cancer development. In addition, restoration of PCDH9 expression through ectopic expression of the protein was observed to markedly slow down the proliferation of HCC-derived cell lines, to arrest cells in the G_1 phase, to inhibit cell capacity for anchorage-independent growth and notably to increase tumorigenicity in nude mice. In addition, decreased expression of cyclin E, alongside increased expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ proteins, two major negative regulators in cell cycle control, provided experimental evidence supporting G_1 phase arrest in HCC-derived cells in which PCDH9 expression had been restored by ectopic expression. By contrast, knockdown of PCDH9 through shRNA could increase the proliferation of HCC-derived cell lines, it increased the cell capacity for anchorage-independent growth and notably increased tumorigenicity in nude mice. In addition, knockdown of PCDH9 could increase expression of cyclin E, while decreasing expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ proteins. These data supported the hypothesis that PCDH9 arrests cells in the G_1 phase.

Furthermore, in the experiments involving the restored expression of PCDH9 and knockdown of PCDH9, there were no significant impacts on the expression of cyclin D1, a novel protein involved in the regulation of cell cycle progression from $G_0/_1$ to S phase. These results suggest that, in this hepatic system, PCDH9 serves its growth-inhibition role through the upregulation of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and cyclin E proteins.

In summary, the present study demonstrated, to the best of our knowledge for the first time, that PCDH9 acts as a potential tumor suppressor gene, and that the loss of PCDH9 expression appears be a driver in a significant fraction of hepatocarcinogenesis cases.

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