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Journal of Cellular Biochemistry 116:1766–1775 (2015)

Journal of Cellular Biochemistry

Protocadherin20 Acts as a Tumor Suppressor Gene: Epigenetic Inactivation in Nasopharyngeal Carcinoma

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ABSTRACT

Genetic alterations of 13q21 (*PCDH 8,9,17*, and *20*) are frequently observed in multiple tumors, suggesting the presence of critical tumor suppressor genes (TSGs). P*rotocadherin20* (*PCDH20*), located at 13q21.2, belongs to the δ1-protocadherins, which constitutes one of the largest subgroup within the adherin superfamily. Frequent downregulation/silencing of *PCDH20* was found in NPC cell lines using semiquantitative PCR. *PCDH20* mRNA was broadly expressed in normal nasopharyngeal tissues and cell lines. Promoter methylation of *PCDH20* was observed in 80% (4/5) of NPC cell lines and 78.4% (40 of 51) of primary tumors by methylation-specific PCR, but rarely in normal nasopharygeal tissues and nasopharyngeal epithelial cell line (NP69). The silencing of *PCDH20* can be reversed by pharmacological demethylation, indicating an epigenetic mechanism. Restoration of PCDH20 expression in NPC cells strongly suppressed cell numbers and colony formation. Overexpression of PCDH20 antagonized Wnt/β-catenin signaling pathway and promoted β-catenin to translocate from nucleus to cytoplasm and membrane. PCDH20 significantly inhibited the migration and invasion ability of NPC cells. PCDH20 also inhibited epithelial–mesenchymal transition (EMT) through enhanced expression of E-cadherin. Our study identified PCDH20 as a functional tumor suppressor and an important antagonist of Wnt/β-catenin signaling and EMT, with frequent epigenetic inactivation in NPC. J. Cell. Biochem. 116: 1766–1775, 2015.

KEY WORDS: EPIGENETIC INACTIVATION; EPITHELIAL–MESENCHYMAL TRANSITION; METHYLATION; NASOPHARYNGEAL CARCINOMA; PROTOCADHERIN 20; TUMOR SUPPRESSOR GENE; Wnt/β-CATENIN

Nasopharyngeal carcinoma (NPC) is the most prevalent head and neck cancer in southern China. It is a highly malignant disease with a 5-year overall survival rate of approximately 70%. Epidemiologic data suggest that NPC formation is a multistep process involving dietary and environment factors, Epstein-Barr virus (EBV) infection, and genetic susceptibility [Chou et al., 2008]. However, the molecular mechanism underlying NPC tumorigenesis is still unclear [Tao and Chan, 2007]. Although NPC is extremely sensitive to radiation and chemotherapy, almost 30–40% of patients develop distant metastasis within 4 years [Le et al., 2003]. Effective therapeutic strategies are urgently needed.

Cadherin is a calcium-dependent adherin that constitutes a large family of cellular adhesion molecules. Cadherins are characterized by the presence of extracellular cadherin repeats of about 110 amino acid residues, and classified into three subfamilies: the classical cadherins, desmosomal cadherins, and protocadherins (*PCDHs*) [Nollet et al., 2000; Halbleib and Nelson, 2006]. Recently, different *PCDHs* (*PCDH8, PCDH9, PCDH10, PCDH17*, and *PCDH20*, on

Tao Chen contributed equally to this work.
Conflict of interest: none.

Grant sponsor: National Key Clinical Specialties Construction Program of China, [2012]649; Grant sponsor: National Natural Science Foundation; Grant numbers: 81372898, 81101492, 813732238.

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Manuscript Received: 11 February 2014; Manuscript Accepted: 6 February 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 March 2015
DOI 10.1002/jcb.25135 • © 2015 Wiley Periodicals, Inc.

chromosome 13q21) have been reported as candidate tumor suppressor genes (TSGs) [Kim et al., 2011]. Promoter CpG island (CGI) methylation is frequently involved in epigenetic alteration of TSGs. Extensive study of promoter CGI methylation and discovery of novel TSGs reveals the epigenetic mechanism of tumorogenesis. It also enables the discovery of epigenetic biomarkers for early detection of NPC [Tao and Chan, 2007]. Gene silencing has been observed to reduce the expression the following *PCDHs*: *PCDH8* in hematologic and breast cancer [Yu et al., 2008; Leshchenko et al., 2010]; *PCDH9* in glioblastoma [de Tayrac et al., 2009]; *PCDH10* in lung, hepatocellular, nasopharyngeal, esophageal, breast, colorectal, cervical, testicular, gastric, and hematologic cancers [Miyamoto et al., 2005; Ying et al., 2006, 2007; Yu et al., 2009; Narayan et al., 2009; Wang et al., 2009; Cheung et al., 2010; Yu et al., 2010]; *PCDH17* in esophageal squamous cell carcinoma and gastric and colorectal cancers [Haruki et al., 2010; Hu et al., 2013]; And *PCDH20* in non-small-cell lung cancers [Imoto et al., 2006]. Mechanisms of promoter hypermethylation, and re-expression of *PCDH8*, *PCDH10, PCDH17* suppresses tumor cell proliferation, inhibits cell migration and induces cell apoptosis, and autophagy [Yu et al., 2008, 2009; Haruki et al., 2010; Hu et al., 2013]. It suggests that promoter CpG methylation-mediated silencing is an important regulatory mechanism disrupting protocadherin members in tumorigenesis. P*rotocadherin20* (*PCDH20*) belongs to the δ1 subgroup of the protocadherin subfamily. The human *PCDH20* gene, also known as *PCDH13*, is located at 13q21.2. Current literature shows that aberrant DNA methylation and reduced expression of the *PCDH20* gene have been found in non-small cell lung cancer (NSCLC). Restoration of *PCDH20* expression reduces cell numbers in colony formation and anchorage-independent assay [Imoto et al., 2006]. However, whether this gene is subject to epigenetic silencing in NPC remains unclear. In the present study, we examined the expression and promoter methylation of *PCDH20* in NPC cell lines and primary NPC tumors. On the basis of the research, we found that promoter methylation was the key mechanism to inactivate *PCDH20* expression in NPC. Furthermore, over-expression of PCDH20 in NPC cells resulted in inhibition of cell clonogenicity via Wnt/β-catenin signaling pathway, and down-regulation of cell motility and invasion. However, the detailed mechanism remained undiscovered. In conclusion, our data here demonstrated that PCDH20 may act as a candidate TSG in NPC.

MATERIALS AND METHODS

PATIENTS, TUMOR TISSUES, AND CELL LINES

A total of 51 patients (39 males, 12 females) were used in this study, with a median age of 51.5 years (range 28–75 years) admitted to our Institution between 2011 and 2013. All specimens were obtained from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and after approval of the ethics committee of Chongqing Medical University. The diagnosis was also confirmed by a biopsy and established according to standard procedures. The five NPC cell lines used were C666-1, CNE-1, HK-1, HNE-1, and HONE-1. One immortalized but nontransformed nasopharyngeal epithelial cell line (NP69) was used as controls. These cell lines were maintained in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum. NP69 was cultured as described [Tsao et al., 2002].

BISULFITE TREATMENT OF DNA AND METHYLATION ANALYSIS

DNA was extracted from cell pellets and tissues by TriReagent or the QIAamp DNA Mini kit (Qiagen, Dusseldorf, Nordrhein-Westfalen, Germany). Following one set of primers for amplification of methylated (M) DNA and one set of primers for unmethylated (U) DNA spanning the CpG Islands of *PCDH20*, cDNA clones were designed:

PCDH20-MF: 5′-TTCGGCGATTTGGTATTCGC-3′
PCDH20-MR: 5′-CTACAACTTATCGAAATCGCG-3′
PCDH20-UF: 5′-GTGTTTGGTGATTTGGTATTTGT-3′
PCDH20-UR: 5′-AATACTACAACTTATCAAAATCACA-3′.

The bisulfite-modified DNA was then amplified using primers that specifically amply either methylated or unmethylated sequences of the PCDH20 gene. Methylation-specific PCR (MSP) was performed using AmpliTaq Gold (methylation-specific primer: annealing temperature 60°C, 40 cycles, and unmethylated-specific primer: annealing temperature 58°C, 40 cycles).

5-AZA-2′-DEOXYCYTIDINE AND TRICHOSTATIN A TREATMENT

Cells were treated with 5-aza-2′-deoxycytidine (Aza; Sigma-Aldrich, St Louis, MO) as described earlier [Qiu et al., 2004]. For the treatment combining Aza and trichostatin A (TSA; Cayman Chemical Co, Ann Arbor, MI), cells were treated with Aza (10 μM) for 3 days and subsequently with TSA (100 ng/ml) for an additional 24 h [Ying et al., 2005]. Cells were then harvested for DNA and RNA extraction.

IMMUNOHISTOCHEMISTRY

A ChemMate EnVision Detection Kit (Dako, Carpinteria, CA) was used. The sections were incubated with PCDH20 antibody (1:50 dilution; sc-84558, Santa Cruz, USA) overnight at 4°C. The ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent was used to treat the sections, followed by application of ChemMate DAB + chromogen included in the kit. The slides were lightly counterstained with hematoxylin.

PCDH20 protein expression was scored, based on the intensity of membrane and cytoplasmic staining, using a four-point system: 0, negative; 1, weak; 2, moderate; and 3, strong. To examine the association of PCDH20 expression with clinicopathological features, patients were divided into two groups: low expression (0 and 1, −) or high expression (2 and 3, +). Immunostaining was scored independently on separate occasions by two investigators who were blinded to the clinical information of the patients.

TOTAL-RNA EXTRACTION AND SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). The quantities and qualities of the RNA were measured by electrophoresis using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit. The cDNA was then synthesized using Go-Tap (Promega, Madison, WI) and random hexamer primers. *GAPDH* served as a control for RNA integrity. *PCDH20* expression was analyzed by reverse transcriptase-PCR (RT-PCR). The primers used were:

PCDH20-F: 5′-ACCAGCTACAGGAACCTGC-3′
PCDH20-R: 5′-GTCTAGGGTCACGTACTGG-3′

GAPDH-F: 5′- GGATGACCTTGCCCACAGGGT-3′

GAPDH-R: 5′-GTTGGGGGTTCTGGGGACTGGC-3′.

RT-PCR was performed for 32 cycles with an annealing temperature of 55°C.

CONSTRUCTION OF *PCDH20*-EXPRESSING VECTOR AND GENERATION OF STABLE CELL LINES

The *PCDH20*-expressing plasmid was gifted of professor Q Tao. CNE-1 cells were seeded into 6-well plates and this expression plasmid transfected using Lipofectamine 2000 (Invitrogen). Forty-8 h later, we changed the cell culture in RPMI-1640 media supplemented with 10% fetal bovine serum and containing G418 (300 μg/ml) for 21–28 days to establish stable *PCDH20*-expressing CNE-1 cells.

COLONY FORMATION ASSAY

Briefly, cells (2 × 105/well) were cultured overnight in RPMI-1640 medium and 10% FBS on a 12-well plate and transfected with *PCDH20*-expressing plasmid and empty vector plasmid (2 μg each) using Lipofectamine 2000 (Invitrogen). Cells were re-plated in 6-well Plate 48 h post-transfection, and selected after 2–3 weeks with G418 (300 μg/ml). Surviving colonies were stained with Gentian Violet after methanol fixation and visible colonies (>50 cells/colony) were counted. Total RNA and protein from the transfected cells was extracted to confirm the ectopic expression of *PCDH20*. All the experiments were performed in triplicate.

CELL CYCLE ANALYSIS

PCDH20-CNE-1, Vector-CNE-1 or CNE-1 cells were cultured in RPMI-1640 medium and 10% FBS with G418 (300 μg/ml). These cells were harvested and fixed in ice-cold 75% ethanol overnight. The fixed cells were then treated with 50 μg/ml RNase and stained with 50 μg/ml propidium iodide at room temperature for 20 min. The cell cycle profiles were assayed by the Elitle ESP flow cytometer and data were analyzed with the CellQuest software (BD Biosciences, Ann Arbor, MI).

PROTEIN EXTRACTION AND WESTERN BLOTTING ANALYSIS

The total cellular extracts were obtained by lysis of cells in a lysis buffer and a protease inhibitor cocktail, followed by centrifugation at 13,000*g* for 15 min at 4°C. We added 1/4 volume of 5× sodium dodecyl sulfate (SDS) loading buffer, and the samples were boiled for 5 min. The filters were blocked with Tris–HCl buffer saline containing Tween-20 buffer (pH 8.0, 10 mM Tris–HCl buffer, 0.15 M NaCl and 0.05% Tween-20). Briefly, membranes were blocked with 5% skimmed milk for 1 h and subsequently incubated with primary antibodies (dilution 1:1,000) for 1.5 h at room temperature or overnight at 4°C followed by incubation with a secondary antibody. The antibodies used were CCND1, c-myc, CD44, p21, monoclonal PCDH20, E-cadherin, total β-catenin, Vimentin, Snail, phosphor-GSK3β, phosphor-β-catenin, active-β-catenin, anti-mouse IgG. Anti-rabbit IgG, and β-actin, The signals were detected with an ECL Kit (Themor Chemical) and the blots were developed using a imaging system.

Immunofluorescence cytochemistry *PCDH20*-expressing-CNE-1 cells were plated onto sterile cover slips in a 12-well plate. Sixteen hours after plating, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 30 min at room temperature. Cells were washed for 4 min× 5 min in PBS, permeabilized for 30 min in buffer A 3% bovine serum albumin (BSA), and incubated with primary antibodies against PCDH20, β-catenin, E-cadherin, or Vimentin in buffer A. Cells were washed in PBS, and incubated with Alexa Fluor 5 94- (Invitrogen Molecular Probes) or FITC-conjugated (Dako) secondary antibody against mouse or rabbit IgG and counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.15 μg/ml in water) and imaged with an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) and Leica TCS SP5 confocal microscope (Leica Micro systems CMS GmbH, Mannheim, Germany). Confocal images of 2048 × 2048 pixels were acquired and assembled.

SCRATCH WOUND ASSAY AND MATRIGEL INVASION ASSAYS

We cultured stably transfected CNE-1 cells in 6-well plates until confluent. The cell layers were carefully wounded using a sterile 20 μl tip, washed twice with fresh medium and cultured for 24 h (Fig. 7B). Images of the wound monolayers were acquired on a phase contrast microscope linked to a charge-coupled device camera, and the wound area was measured using CT-AS software (Nikon, Tokyo, Japan) counting the number of pixels, after the photographs were converted to TIFF images. Transwell matrigel invasion assays were carried out in 24-well modified Boyden chambers (BD Transduction, Franklin Lakes, NJ) as described previously (22). The experiments were performed in triplicate.

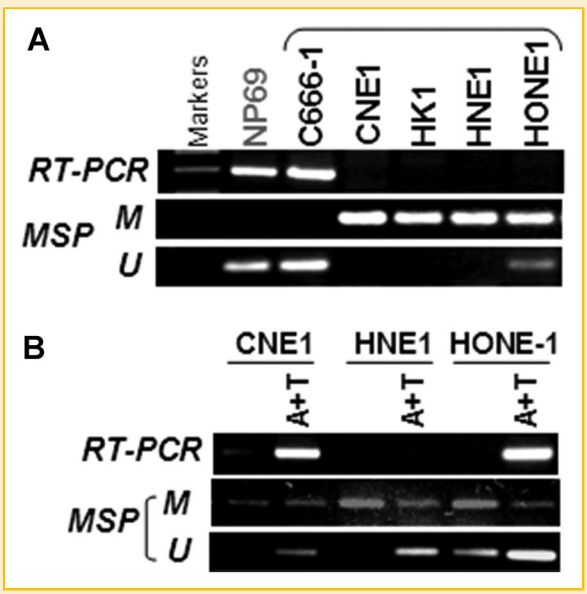

Fig. 1. (A) *PCDH20* expression and methylation status in NP69, and NPC cell lines were determined by semiquantitative reverse transcriptase-PCR and MSP. M, methylated; U, unmethylated. (B) Pharmacological demethylation with 5-aza-2′-deoxycytidine (Aza) alone or combined with trichostatin A (TSA)-induced *PCDH20* expression in NPC cell lines. *PCDH20* expression before and after drug treatment was determined by RT-PCR (upper panel), and demethylation was confirmed by MSP (lower two panels).

PCDH20 REGULATED EPITHELIAL–MESENCHYMAL TRANSITION

Epithelial–mesenchymal transition (EMT) plays a critical role in tumor cell metastasis by reducing cell–cell contact and increasing motility. The effect of PCDH20 expression on EMT regulation in NPC cells was further assessed. Epithelial and mesenchymal markers were examined to determine whether PCDH20 negatively regulated EMT. *PCDH20*-expressing cells exhibited a reversed EMT phenotype, including upregulated epithelial marker E-cadherin and down-regulated mesenchymal marker Vimentin, which was further confirmed by immunofluorescence.

RESULTS

PROMOTER METHYLATION OF *PCDH20* IN NPC

The expression of *PCDH20* was evaluated among the five NPC cell lines, 4/5 of the NPC cell lines (CNE-1, HK-1, HNE-1, and HONE-1) showed complete silencing of *PCDH20* expression, and expression in C666-1. In contrast, *PCDH20* was readily detected in the immortalized nasopharyngeal epithelial cell line (NP69) (Fig. 1A). To further assess whether CGI methylation directly mediated *PCDH20* downregulation, the three NPC cell lines (CNE-1, HNE-1, and HONE-1) were treated with the DNA methyltransferase inhibitor Aza, together with or without the histone deacetylase inhibitor TSA. After drug treatment, *PCDH20* expression was significantly reactivated in CNE-1 and HONE-1 cell lines (Fig. 1B). These results indicated that PCDH20 promoter methylation directly mediated its transcriptional silencing in NPC.

PCDH20 WAS HIGHLY EXPRESSED IN NORMAL NASOPHARYNGEAL TISSUES AND FREQUENTLY DOWN-REGULATED AND METHYLATED IN NASOPHARYNGEAL CARCINOMA TISSUES

The expression of *PCDH20* was evaluated in normal nasopharyngeal tissues and NPC tissues by semiquantitative RT-PCR. PCDH20 was strongly expressed in normal nasopharyngeal tissues, and was moderately or weakly expressed in NPC tissues, representative data were showed in Figure 2A. To investigate whether the promoter methylation of the *PCDH20* gene could be detected in primary NPC tumors, we further used MSP to examine *PCDH20* promoter

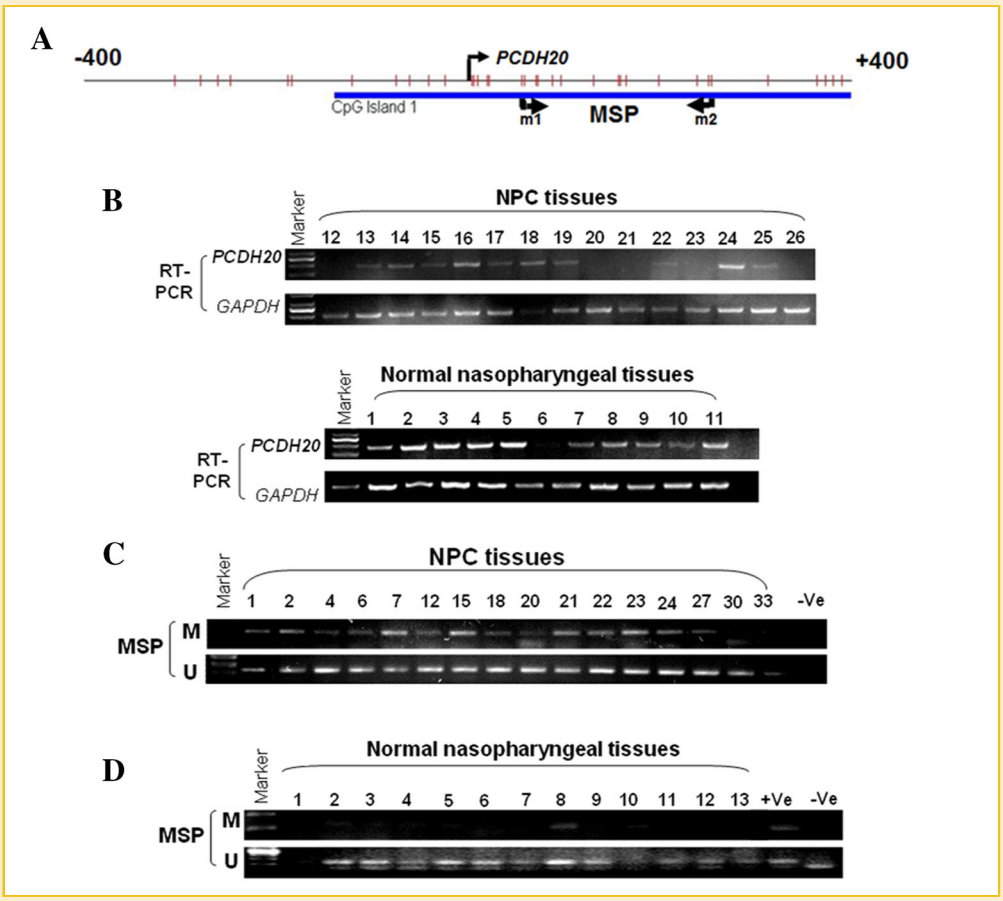

Fig. 2. (A) Schematic structure of the PCDH20 promoter CpG island (CGI), methylation-specific polymerase chain reaction (MSP) regions indicated. The transcription start site is indicated by a curved arrow. (B) *PCDH20* expression in NPC tissues and normal nasopharyngeal tissues was determined by semiquantitative reverse transcriptase-PCR. (C and D) Methylation-specific PCR analysis of *PCDH20* CpG in primary nasopharyngeal carcinoma (NPC) tumors and normal nasopharyngeal tissues. Methylation-specific polymerase chain reaction (MSP) results of 16 primary tumor tissues and 13 normal nasopharyngeal tissues are shown. M, methylated; U, unmethylated. +Ve, Positve control; −Ve, Negative control.

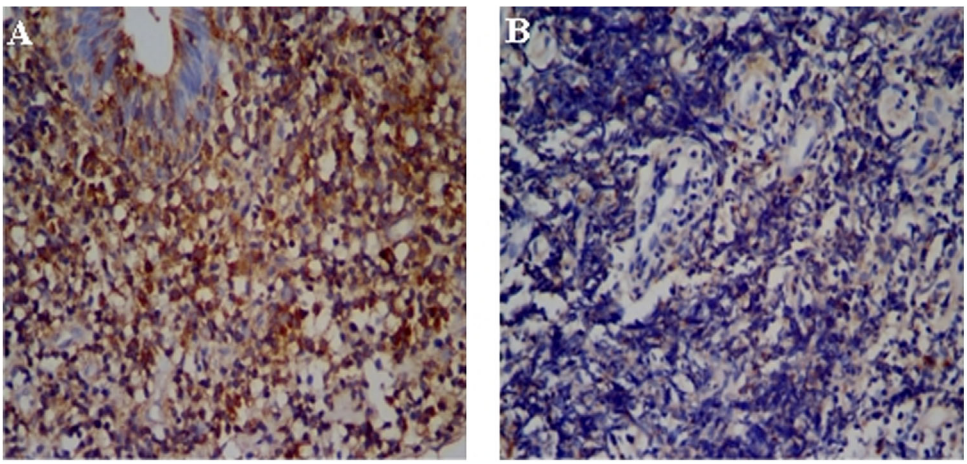

Fig. 3. PCDH20 expression in human NPC and normal nasopharyngeal tissues. NPC tissues show low immunohistochemical staining of PCDH20. Clinical specimens were collected and examined by immunohistochemical staining with PCDH20 antibody. Examples of PCDH20 staining were presented. normal nasopharyngeal tissue displayed high immunohistochemical staining (A), whereas NPC showed low staining (B) (original magnification, 400×).

methylation in additional primary NPC tumors and normal nasopharyngeal tissues. Aberrant methylation was detected in 40/51(78.4%) NPC tissues, but barely in normal nasopharyngeal tissues, representative data were showed in Figure 2B.

PCDH20 protein expression was further investigated in NPC by immunohistochemistry. PCDH20 was highly expressed in the membrane and cytoplasm of normal nasopharyngeal tissues, but was low to undetectable in NPC tissues (Fig. 3, Table I).

CLINICOPATHOLOGICAL SIGNIFICANCE OF *PCDH20* PROMOTER METHYLATION

Statistical analyses of the correlation between *PCDH20* promoter methylation and clincopathological parameters were summarized in Table II. No significant differences were observed between patients with methylated and unmethylated *PCDH20* promoters with regard to age, sex, lymph node metastasis, or the Tumor Node Metastasis stage (Table II).

OVEREXPRESSION OF *PCDH20* INHIBITED CLONOGENICITY OF CNE-1 CELLS AND INDUCES G1 CELL CYCLE ARREST

To assess the role of *PCDH20* as a TSG in NPC, we sought to establish whether overexpression of *PCDH20* inhibited tumor cell clonogenicity. We successfully obtained a stable overexpression of PCDH20 as shown by Western blotting. Results of colony formation assays showed that ectopic expression of *PCDH20* significantly suppressed the number of CNE-1 cell colonies to <50% of the control cells,

TABLE I. Summary of PCDH20 Immunohistochemistry Results of Normal Nasopharyngeal Tissues and NPC Tissues

Samples	Number (n)	High (%)	Low (%)	*P*-value
PCHD20 immunostaining				
Normal nasophyngeal tissues	13	13 (100)	0 (0)	<0.01
Nasopharyngeal carcinoma	51	9 (17.6)	42 (82.4)	<0.01

TABLE II. Correlations Between *PCDH20* Promoter Methylation and Clinicopathological Indices of Nasopharyngeal Carcinoma Patients

	Number of patients (n = 51)	Methylated (%)	Unmethylated (%)	*P*-value
Age (years)				
<60	37	31 (83.8)	6 (16.2)	0.131
≥60	14	9 (64.3)	5 (34.7)	
Sex				
Male	39	31 (79.5)	8 (20.5)	0.741
Female	12	9 (75.0)	3 (25.0)	
Lymph node metastasis				
Negative	22	15 (68.2)	7 (31.8)	0.388
Positive	29	24 (82.8)	5 (17.2)	
Tumor node metastasis				
I–III	44	34 (77.3)	10 (22.7)	0.614
IV	7	6 (85.7)	1(14.3)	

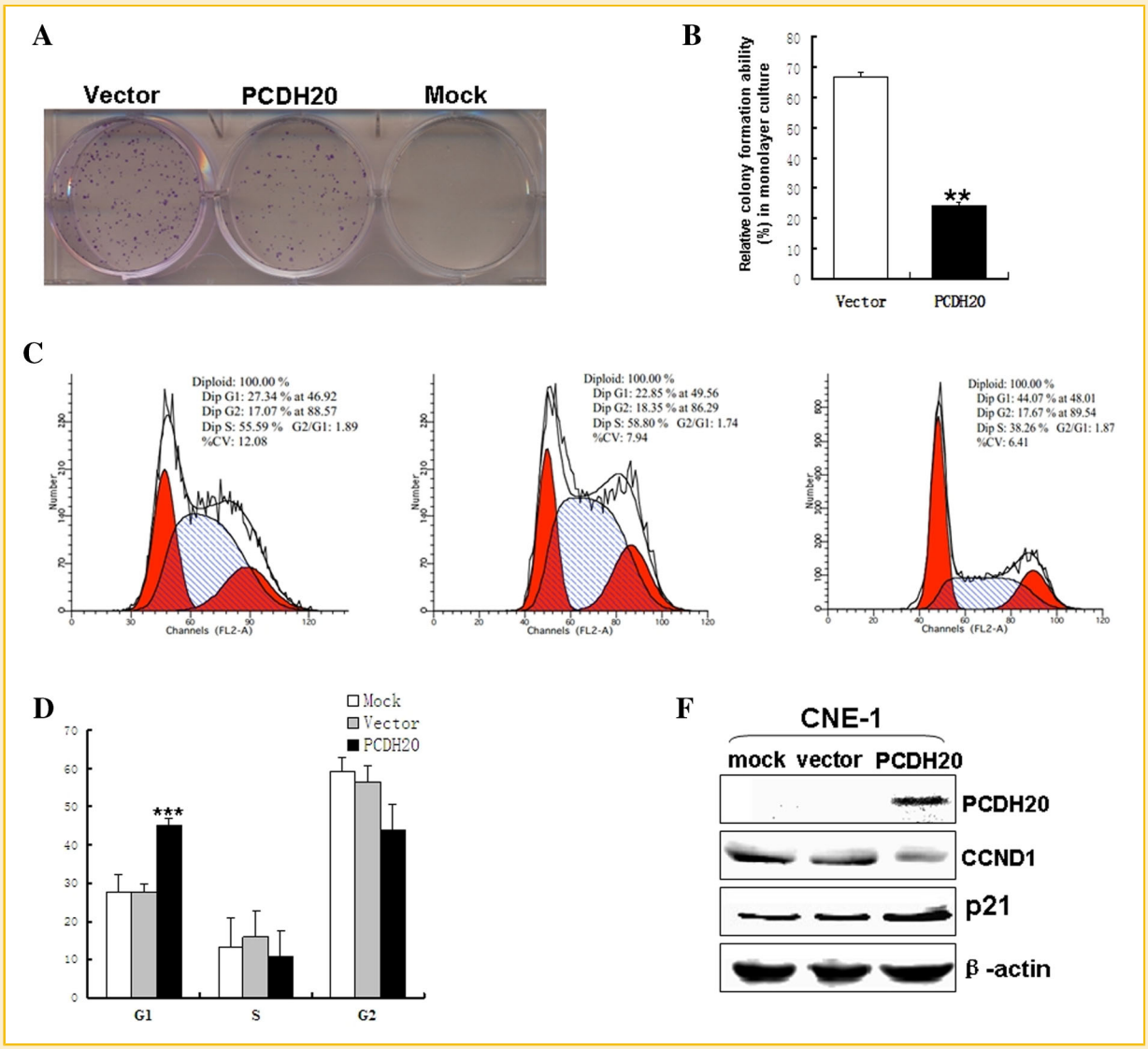

Fig. 4. (A and B) PCDH20 inhibits tumor cell growth. Representative colony formation assays. Quantitative analyses of colony numbers are shown in the right as values of mean ± s.d. $^{*}P < 0.05$ (C and D) Overexpression of PCDH20 inhibit CNE-1 cells clonogenicity and induces G1 cell cycle arrest. Quantitative analyses of cell cycle are shown in the below as values of mean ± s.d. $^{*}P < 0.05$. And (F) decreases the expression of cyclin D1, and up-regulate p21 protein.

indicating that *PCDH20* functions as a TSG in CNE-1 cells (Fig. 4A). We further explored the mechanism by which *PCDH20* suppresses colony formation by examining the effect of *PCDH20* on cell cycle distribution using flow cytometry. The percentage of cells in the G1 phase was increased in *PCDH20*-overexpressing CNE-1 cells compared with control vector cells and CNE-1 cells ($P < 0.05$; Fig. 4C). Western blotting showed that overexpression of PCDH20 decreased the expression of CCND1 (cyclin D1), and upregulated p21 protein (Fig. 4F).

PCDH20 ANTAGONIZED WNT/β-CATENIN SIGNALING PATHWAY

We explored the molecular mechanisms underlying silencing of PCDH20 in NPC, using a Western blotting to determine if PCDH20 neutralized Wnt/β-catenin signaling. We found that in *PCDH20*-overexpressing CNE-1 cells, downstream target genes of Wnt/β-catenin signaling pathway, c-Myc, CCND1 (cyclin D1) and CD44 were significantly decreased compared with control vector cells and CNE-1 cells (Fig. 5).

Nuclear β-catenin staining was also detected in tumor cells of NPC xenografts and primary NPC specimens [Haruki et al., 2010]. β-catenin plays a significant role as an activator of the transcription factor TCF/LEF. We investigated the expression and localization of the β-catenin by Western blotting and immunofluorescence cytochemistry. Western blotting analysis showed no significant change in totalβ-catenin protein levels. However, levels of active and phosphorylated β-catenin (Ser552) dramatically decreased in *PCDH20*-expressing CNE-1 cells compared with control cells, accompanied by downregulated GSK-3β phosphorylation. However,

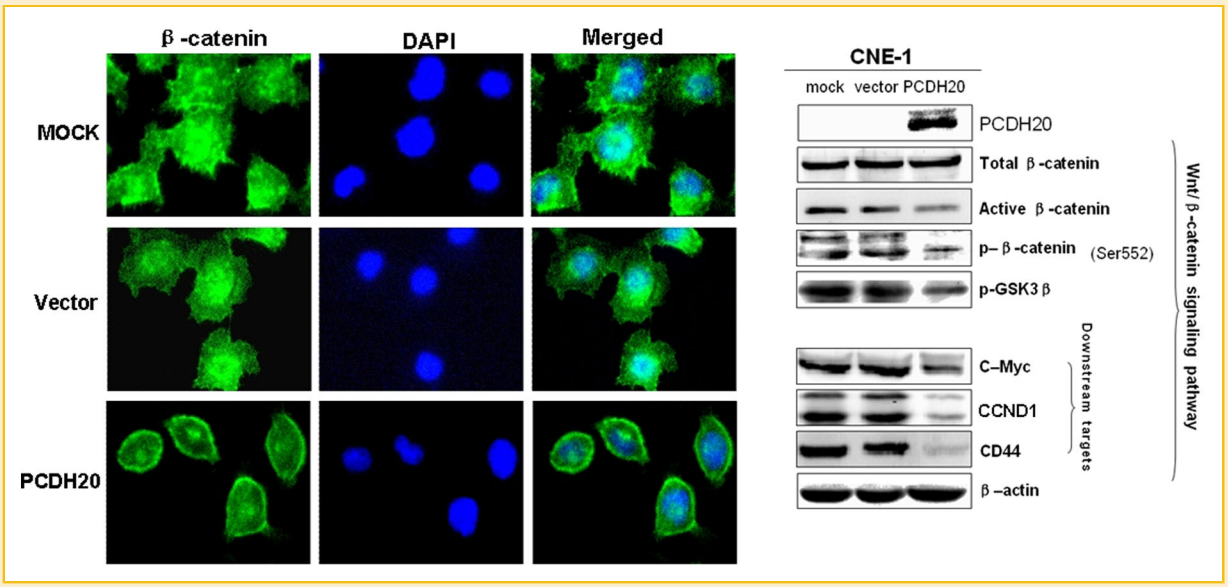

Fig. 5. Overexpression of PCDH20 disrupted Wnt/β-catenin signaling pathway. Western blotting was performed using antibodies against total β-catenin, active β-catenin, phosphor-β-catenin(Ser552),phospho-GSK3 β, CCND1, c-Myc and CD44. Beta-actin was used as a control. PCDH20 translocated nuclear β-catenin to membrane. Endogenous PCDH20 was visualized in *PCDH20*- and PCMV-transfected CNE-1 cells by indirect immunofluorescence. Original magnification, 400×.

immunofluorescence cytochemistry revealed that in *PCDH20*-transfected CNE-1 cells, β-catenin translocated from nucleus to cytoplasm and membrane (Fig. 5). The results indicated that the extranuclear sequestration and inactivation of β-catenin regulated by PCDH20 contributed to reduce β-catenin/TCF-mediated transcriptional activity.

EVALUATION THE EFFECT OF PCDH20 ON CELL MIGRATION AND INVASION

We wondered whether overexpression of PCDH20 altered cell migration and invasion. First, we carried out a wound-induced migration assay to assess the contribution of *PCDH20* to cellular motility. *PCDH20*-Overexpressing cells exhibited reduced ability to migrate away from the monolayer relative to empty vector control cells and CNE-1 cells (Fig. 6B). Further, we studied cell invasiveness with the use of a Matrigel invasion chamber system. As shown in Figure 6A, overexpressing *PCDH20* cells exhibited significantly suppressed invasiveness in this assay than empty vector control cells, suggesting that overexpression of *PCDH20* downregulated invasiveness of CNE-1 cells. These results indicate that *PCDH20* overexpression inhibited CNE-1 cell motility and invasiveness.

PCDH20 RESTORES EXPRESSION OF EPITHELIAL MARKERS

Epithelial–mesenchymal transition (EMT) is a key initial step during tumor invasion, and plays an important role in tumor cell metastasis by reducing cell–cell contact and increased motility. Western blotting analysis and immunofluorescence staining showed an altered expression of epithelial and mesenchymal markers: E-cadherin was increased markedly and Vimentin was decreased in *PCDH20*-overexpressing CNE-1 cells. Further, Western blotting analysis showed that Snail, an E-cadherin transcription suppressor, was reduced in *PCDH20*-overexpression CNE-1 cells (Fig. 7).

DISCUSSION

PCDH20 was first identified as a candidate tumor suppressor in NSCLC. However, the exact role in human tumorigenesis is not well defined. Protocadherins are not simple cell adhesion proteins involved in homophilic interactions. Their heterophilic interaction with other molecules may be more important physiologically [Weiner and Jontes, 2013].

Increased DNA methylation in the promoter region of genes is well established as one of epigenetic mechanisms for the silencing of tumor-suppressor genes in human carcinomas [Herman et al., 1994]. In this study, we found that *PCDH20*, located at an important tumor suppressor locus 13q21, was widely expressed in normal nasopharyngeal tissues. However, it was frequently silenced or down-regulated in our panel of NPC cell lines and tissues, accompanied with hypermethylation of CpG sites, indicating that promoter methylation is one of the mechanisms associated with *PCDH20* inactivation in NPC. However, the frequency of *PCDH20* methylation in primary tumors was not as high as that in cell lines, indicating that other mechanisms such as lack of transcriptional activators, histone modification, or the presence of transcriptional repressors, could further silence gene expression, especially in primary tumors. Pharmacologic demethylation resulted in the demethylation and restoration of *PCDH20* expression. In the 51 primary NPC tumors, the methylation status of the *PCDH20* promoter examined by MSP demonstrated no association with any clinicopathological variables. No significant differences were

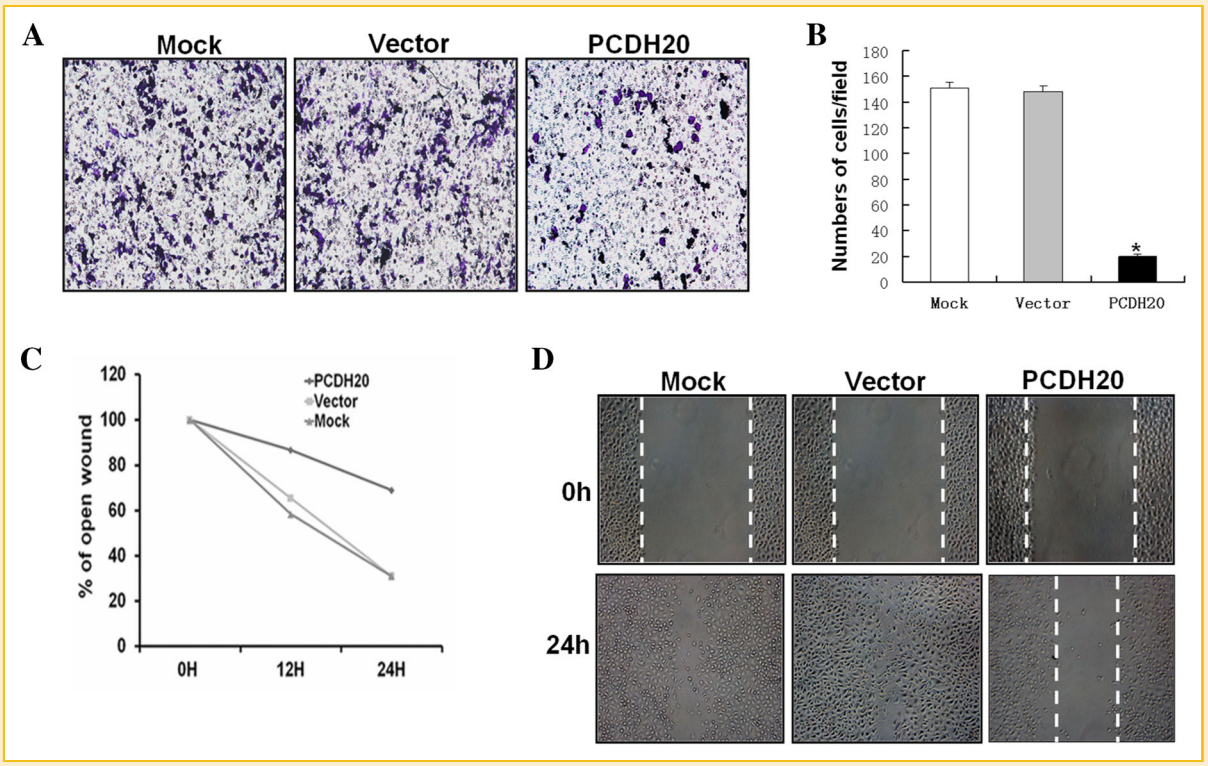

Fig. 6. (A) PCDH20 inhibited the invasive activity of tumor cells. Original magnification, 400×. (B) Quantitative analyses of cell numbers are shown in the right as values of mean ± s.d. $^{*}P < 0.05$. (C and D) Wound-healing assay of tumor cells transfected with either vector, *PCDH20* or CNE-1 cells. Pictures were taken at 0, 24 h after scratching the cell surface. All experiments were performed in triplicate and a representative image is shown. Quantitative analyses are shown in the left as values of mean ± s.d. $^{*}P < 0.05$.

observed between the methylation of *PCDH20* and clinicopathological indices. Further, we found that *PCDH20* functions as an anti-proliferative tumor suppressor. Stable *PCDH20*-transfected cells demonstrated cell cycle arrest at the G1-S checkpoint, without inducing apoptosis of CNE-1 cells.

Adhesion properties and cytoplasmic partners of non-clustered PCDHs are still poorly understood. Most of the cadherin superfamily proteins show calcium-dependent homophilic adhesion activities [Takeichi, 1995; Shapiro and Colman, 1999]. Nevertheless, in other protocadherins, various tumor suppression mechanisms have been

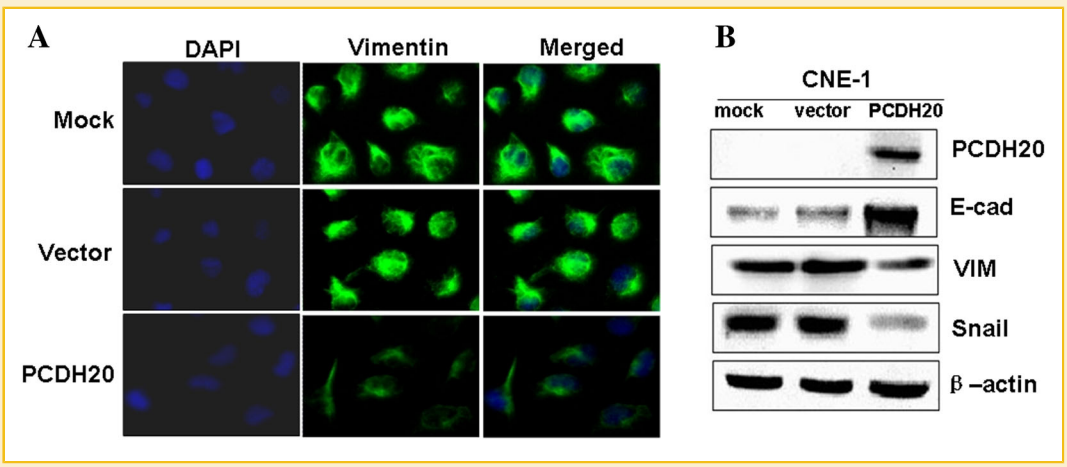

Fig. 7. PCDH20 can restore the expression of epithelial markers. (A) Indirect immunofluorescence detecting the expression of Vimentin in *PCDH20*-overexpression, control vector cells and CNE-1 cells. (B) Western blotting showing the expression of E-cadherin. Vimentin and the transcription suppressor Snail in *PCDH20*-overexpression, control vector cells and CNE-1 cells. Beta-actin was used as a loading control.

reported: PCDH10 inhibits cell proliferation and induces apoptosis via multiple genes including p21 (13). PCDH24 inhibits the expression of downstream targets of β-catenin including cyclin D1 [Ose et al., 2009]. PCDH17 induces apoptosis and autophagic proteins Atg5–Atg12 (19). In addition, PCDH8 and PCDH10, members of the heterogeneous subgroup of protocadherins, are also identified as TSGs for NPC, mediating the inhibition of cell growth, colony formation, migration and invasion, indicating the crucial role of PCDH family members in NPC [Ying et al., 2006; He et al., 2012]. Carcinogenesis is a multi-step process involving the accumulation of multiple genetic and epigenetic alterations mediated by oncogenes and TSGs.

Increasing evidences suggest that aberrant activation of the WNT/β-catenin signaling pathway is frequently involved in a broad spectrum of human malignancies [Suzuki et al., 2004; Baylin and Ohm, 2006]. It has been reported that some protocadherins such as PCDHGA, PCDHGC3, PCDH20, and PCDH24 could affect the expression of some downstream targets of Wnt signaling pathway and growth properties [Junghans et al., 2005; Ose et al., 2009; Dallosso et al., 2012; Lv et al., 2014]. In our study, PCDH20 was found to antagonize Wnt/β-catenin signaling in CNE-1 cell line via suppressing their abilities to inactive GSK-3β. As expected, we found that nuclear β-catenin translocated to cell membrane, and several markers of Wnt/β-catenin targeted genes like c-Myc, CCND1, and CD44 were also downregulated in *PCDH20*-overexpressed cells. Further studies should be conducted to investigate which intracellular moleculer mechanisms were induced by PCDH20.

EMT is a process in which epithelial cells lose cell-to-cell adhesion characterized by repression of membrane proteins such as β-catenin and E-cadherin, and undergo remarkable cytoskeletal remodeling [Grunert et al., 2003]. EMT disrupts intercellular contacts and enhances cell motility. Detachment of cells from the parental epithelial tissue leads to tumor invasion. Although the molecular bases of EMT have not been completely illuminated, several interconnected transduction pathways and a number of signaling molecules potentially involved have been identified. These include Ras, growth factors, beta-catenin. Most of these pathways assemble on the down-regulation of the epithelial molecule E-cadherin, an critical event in tumor invasion and in EMT [Guarino et al., 2007]. The E-cadherin/β-catenin signaling axis have also demonstrated a key role for EMT involving epithelial cells. Cytoplasmic β-catenin has a role in the normal cell by binding to the intracellular domain of E-cadherin to maintain cellular adhesion. Loss of membrane E-cadherin/β-catenin and gain of vimentin and N-cadherin have been considered as key events in EMT of squamous carcinoma cells and also associated with high levels of Snail in the nucleus [Kalluri and Weinberg, 2009]. Interestingly, we clearly demonstrated that overexpression of PCDH20 translocated nuclear β-catenin to membrane, and increased membrane expression of epithelial marker E-cadherin in NPC cells. Overexpression of membrane E-cadherin/β-catenin decreased the expression levels of Snail. The up-regulation of E-cadherin and down-regulation of Snail induced by PCDH20 provide a possible role of PCDH20 to suppress CNE-1 cell migration and invasion (Fig. 7). Therefore, PCDH20 may have several tumor-suppressive functions potentially contributing to growth control, signal transduction and cell–cell adhesion. Further study of the underlying mechanisms of PCDH20 and other EMT events are needed.

Taken together, our data identifies that PCDH20 can inhibit cell proliferation, migration and invasion, through antagonizing Wnt/β-catenin signaling pathway and EMT in NPC. Our results provide an opportunity for future target-guided therapy. Additional studies are required to unravel the consequences of PCDH20 loss of function in human nasopharyngeal carcinogenesis.

ACKNOWLEDGMENTS

The authors thank Prof. Qian Tao (the Chinese University of Hong Kong, Hong Kong, China) for generously providing NPC cell lines, plasmid and primers, technical assistance, and help with the whole design of the experiment. We also thank the Molecular Oncology and Epigenetics Laboratory, the First Affiliated Hospital of Chongqing Medical University for technical assistance and encouragement. Our paper is supported by National Key Clinical Specialties Construction Program of china, No. [2012]649, and grants from National Natural Science Foundation (#81372898, #81101492, #813732238).

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