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Original Article

Down-regulation of PPM1H in papillary thyroid cancer inhibits cancer cell proliferation and invasion

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Abstract: Recent studies have confirmed that aberrant expression of protein phosphatase 1H (PPM1H) plays multiple roles in various types of cancers, but a potential role in papillary thyroid cancer (PTC) has not been studied. This study aimed to explore the clinical characteristics of PPM1H in PTC and its roles in the proliferation and invasion of PTC cells. Down-regulation of PPM1H expression was frequently detected in primary PTC tumor tissues compared with those in non-tumor tissues, and it was significantly associated with tumor size (P = 0.048), lymph node metastasis (P = 0.016), and clinical stage (P = 0.033). Knockdown of PPM1H in SW579 cells, a human PTC cell line, decreased E-cadherin level, while increasing expression of Vimentin. After PPM1H inhibition, SW579 cells exhibited morphological change from an epithelial cobblestone phenotype to an elongated fibroblastic phenotype. Wound healing and transwell assays were used to analyze the migratory and invasive ability of cells, and it was found that SW579 cells had increased migratory and invasive ability after PPM1H knockdown. Furthermore, MTT and colony formation assays showed that down-regulation of PPM1H promoted cell proliferation (P<0.05). These results suggest that low expression of PPM1H is associated with increased cancer cell proliferation and invasion and that PPM1H may act as a tumor suppressor in PTC.

Keywords: PPM1H, papillary thyroid cancer, proliferation, metastasis, tumor suppressor

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence is increasing globally [1]. Papillary thyroid cancer (PTC) is the most frequently observed thyroid carcinoma, accounting for over 80% of all thyroid carcinoma cases, results in sustained growth, and lymph node metastasis [2, 3]. The development of metastasis continues to be the most significant cause of thyroid cancer mortality [4, 5]. Growing evidence shows that PTC metastasis is a complex and multi-step process, which is associated with various genetic and epigenetic changes [6]. Therefore, it is of great significance to uncover the genes governing growth and metastasis for improving the prognosis of PTC patients.

Protein serine/threonine phosphatase 2C (PP-2C) is a defining member of the PPM ($Mg2^{+}$- or $Mn2^{+}$-dependent protein phosphatase) family, characterized by a monomeric form, dependence on divalent cations, and insensitivity to okadaic acid [7, 8]. In recent years, the PP2C family members grown steadily, with at least 18 different PP2C genes having been identified in the human genome [9] since their discovery. PP2Cs have been predominantly linked to cell growth and cellular stress signaling and they are implicated in various intracellular processes including apoptosis and cell cycle regulation that are relevant to cancer [10]. For instance, ILKAP (PP2Cδ) negatively affects proliferation and malignant transformation [11, 12] and PHLPP promotes apoptosis and inhibits tumor growth and motility [13-15]. PPM1H (NERPP-2C) is a novel member of the PP2C family, originally identified as a negative regulator of neurite outgrowth [16]. Previously, PPM1H was found to be up-regulated in colon adenocarcinomas compared with normal colon tissues. PPM1H controls cell cycle and proliferation of colon cancer cells potentially through dephos-

phorylation of CSE1L [17]. More recently, Lee-Hoeflich and colleagues have found PPM1H as a potential suppressor of trastuzumab resistance in breast cancer cells, and loss of PPM1H triggers increased phosphorylation and degradation of p27, which leads to increased cell proliferation [18]. Therefore, a different role for PPM1H in cancer cell proliferation may be due to tissue specificity, but expression of PPM1H in PTC proliferation and metastasis has never been studied.

In the present report, we demonstrate a significant correlation between down-regulation of PPM1H expression and larger tumor size, presence of lymph node and clinical stage metastasis of PTC patients, we further examined its role on proliferation, migration, and invasion in PPM1H knockdown SW579 cells. With a focus on its role in tumor progress, we demonstrate that PPM1H acts as a potential tumor suppressor gene.

Materials and methods

Patients and tissue specimens

Surgical specimens (105 tumor tissues and 60 their adjacent normal tissues) were obtained from patients in Jingzhou central hospital (Jingzhou, China). Histological diagnosis was verified by an experienced pathologist. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until later used. No patient received chemotherapy or radiation therapy treatment before surgery. The Ethics Committee approved this procedure. Informed consent for the experimental use of surgical specimens was obtained from all patients in written form according to the hospital's ethical guidelines.

Cell culture and siRNA transfection

Human PTC cell line SW579 was obtained from Nanjing KeyGen Biotech. Co. Ltd (Nanjing, China). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. PPM1H siRNAs and negative control (NC) were chemically synthesized by RiboBio Biotechnology (Guangzhou, China), PPM1H siRNA1 (5'-CACGCUUCUUUACCGAGA-3' and 5'-UCUCGGUAAAGAAGCGUG-3', PPM1H siRNA2 (5'-AAUCUUCUUCUCGGUAAAGAA-3' and 5'-CUUUACCGAGAAGAAGAUUCC-3'). SW579 cells were plated in 6-well plates at 40% confluence the day before transfection. The next day, 50 nM of PPM1H siRNAs or NC siRNAs were transfected into SW579 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Total RNA or proteins were extracted at 48 or 72 hours post-transfection and used for quantitative reverse transcription (qRT-PCR) or Western blot analysis respectively.

RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen, USA), and reverse-transcribed to cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. Relative gene expression was detected by SYBR green qPCR assay (Fermentas, USA) on ABI 7500 system (Applied Biosystems). β-actin was used as control to normalize the starting quantity of RNA. Primers used for PPM1H and β-actin are as follows: PPM1H, 5'-CTGGCTACGCAGAGGTTATCA-3' (forward), 5'-GGAGGACCGTCTCTTGGATGA-3' (reverse); β-actin, 5'-AGTGTGACGTGGACATCCGCAAAG-3' (forward), 5'-ATCCACATCTGCTGGAAGGTGGAC-3' (reverse). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Scratch wound healing study

SW579 cells transfected with PPM1H siRNAs or control siRNA were seeded into 6-well plates (1×10^6 cells per well) and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours. Cells were incubated with serum-free medium for 6 hours before the scrape line was made. Wounds were generated on the surface of confluent monolayer using a sterile pipette tip. After washing away suspended cells, the cells were incubated with serum free medium. Wound closure was photographed immediately and 24 hours after wounding at the same spot. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area (n = 3).

Cell migration and invasion assays

Cell migration was studied using 6.5 mm Costar transwell chambers with 8 μm pores (Corning). At 48 hours post-transfection, cells were tryp-

sinized and resuspended in 0.1% serum containing DMEM media with 0.5% bovine serum albumin. A total of 10^5 cells in 0.3 mL media were plated in the upper chambers in duplicate filters. DMEM medium with 15% serum was used as a chemoattractant in the lower chamber. Cells were allowed to migrate in the 5% CO_2 incubator at 37°C. After 24 hours, the non-migrating cells were removed from the upper surface of each transwell by a cotton swab. Transwell membranes were then stained with crystal violet. Cells that migrated through the membrane to the lower surface were counted by light microscopy. To study cell invasion, the upper chambers were coated with ECM gel (Sigma, USA). FBS was added to the lower chamber as described for the cell migration experiments.

Western blot

Total protein was extracted from SW579 cells using RIPA Buffer (Sigma, USA). The protein concentration was determined with BCA Protein Assay Kit (Beyotime Biotechnology, China). For performing polyvinylidene fluoride membrane blotting, 50 μg cell lysates were separated on a 12% SDS-PAGE. The blotted membranes were blocked with 5% BSA in TBST for 1 hour at room temperature, and incubated with each primary antibody overnight at 4°C. Anti-PPM1H antibody was obtained from GenTex Biotechnology (GenTex, USA), Anti-E-cadherin (sc-710-09), Vimentin (sc-66001) and β-actin (sc-811-78) were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence using ECL detection reagent

Immunohistochemistry

Sample sections were deparaffinated in xylene and rehydrated in graded ethanol, followed by incubated in 3% hydrogen peroxide for 10 minutes to quench endogenous peroxides. The samples were heated in 0.01 mol/L citrate buffer for 10 minutes at 100°C and then put at room temperature for 60 minutes. After cooling, the samples were blocked with 5% normal goat serum in phosphate-buffered saline (PBS) for 30 minutes to block anti-genic epitopes then incubated with PPM1H antibody (1:100 dilution) at 4°C overnight. After washing with PBS for three times, the samples were incubated with system-labeled HRP anti-mouse secondary antibody at room temperature for 30 minutes. The sections were then incubated in DAB and dehydrated in alcohol and xylene. The substitution of primary antibody with PBS was used as negative control. The staining of the samples were observed and scored under microscope.

Evaluation of the staining reaction was performed in accordance with the immunoreactive score (IRS) proposed by Remmele and Stegner (1987) [19]: IRS = SI (staining intensity) × PP (percentage of positive cells). SI was determined as 0 is negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0 is negative; 1, 10% positive cells; 2, 11-50% positive cells; 3, 51-80% positive cells; and 4, more than 80% positive cells. IRS results in a 13 point scale ranging from 0 to 12, tumor slices scoring >4 points in our study were classified immunoreactive, indicating PPM1H high expression, tumor slices scoring ≤4 points were classified as low expression.

Colony formation assay

Cells were trypsinized and replated into 6-well plates at a density of 5×10^2 cells per well. After two weeks, the cells were washed twice with PBS and fixed with methanol for 15 minutes. The fixing solution was removed and the cells were stained with 0.3% crystal violet for 10 minutes. The number of colonies was counted.

Cell proliferation assay

Thiazolyl blue tetrazolium bromide (MTT) assay was performed to detect the proliferation capacity change after PPM1H knockdown in SW579 cells according to the manufacturer's protocol (Sigma-Aldrich, USA). A total of 8×10^3 trypsin-dispersed cells in 0.1 mL culture medium was seeded into each well of a 96-well plate, and cultured for 24 h. Next, 10 μL of MTT was added to each well and incubated for an additional 4 hours at 37°C. Culture medium was then replaced with 100 μL of dimethyl sulfoxide (DMSO) and the absorbance rate was determined using an ELISA reader at 570 nm. Cell proliferation inhibition rate was calculated as (the value of experimental group OD/the value of control group OD) × 100%.

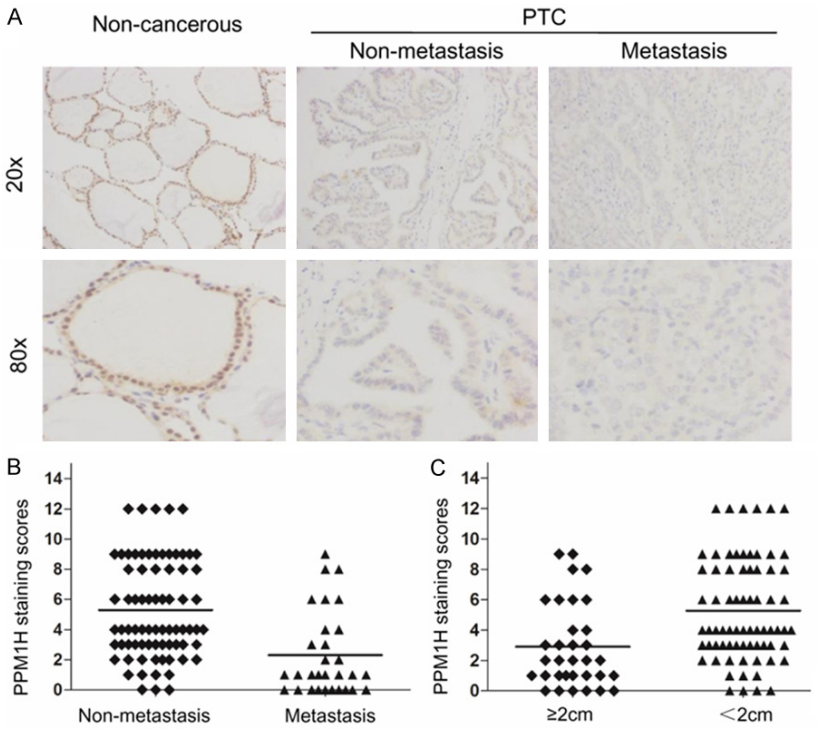

Figure 1. PPM1H expression in PTC tissues and matched non-cancerous adjacent tissues (NCAT). A. Immunohistochemistry determined the presence and expression of PPM1H in NCAT and PTC specimens with or without Metastasis. B. Immunoreaction scores of PPM1H expression in PTC specimens with or without Metastasis. C. PPM1H expression scores in PTC specimens with tumor size lager or less than 2 cm.

Table 1. Immunohistochemical analysis of PPM1H expression in tissue specimens

Group	No. of cases	PPM1H expression		χ^2	*P*
		Low	High		
PTC	105	65 (61.9%)	40 (38.1%)	6.307	0.012
Non-cancerous adjacent tissues	60	25 (41.7%)	35 (58.3%)		

Statistical analysis

Results are expressed as mean ± SD and representative of at least three independently performed experiments. For statistical analysis, the Chi square test (χ^2 test) for non-parametric variables and Student t test for parametric variables were used to compare the means of two groups. The Pearson χ^2 test was used to analyze the association of PPM1H expression with clinicopathologic parameters. All tests were two-sided and $P<0.05$ was considered statistically significant. Analysis was performed using SPSS software (version 18) or GraphPad Prism 5.0.

Results

PPM1H expression and its correlation with clinicopathologic variables in PTC

To explore the effect of PPM1H on PTC, immunohistochemistry was performed to determine the presence and expression of PPM1H in 105 PTC specimens and 60 matched non-cancerous adjacent tissues (NCAT). The result showed that PPM1H was mainly present in the cytoplasm and nucleus (**Figure 1A**). Compared to NCAT, PPM1H was significantly down-regulated in PTC tissues ($P<0.05$; **Table 1**). Further analysis of the clinicopathological characteristics in the 105 tissues specimens showed that PPM1H expression significantly correlated with tumor size ($P = 0.048$), lymph node metastasis ($P = 0.016$), and clinical stage ($P = 0.033$), but not related to other clinical characteristics, including age, sex, pathological grading and clinical stage (**Table 2**). PTC patients who developed metastasis or lager tumor size (≥2 cm) showed significantly lower immunoreaction scores for PPM1H than those without metastasis or small tumor size (<2 cm) (**Figure 1B**, **1C**).

Knockdown PPM1H induces EMT-like changes in SW579 cells

Cancer cell EMT is indispensable for cancer metastasis. To confirm whether or not PPM1H knockdown induces EMT-like changes in SW579 cells, we employed the siRNA approach. SW579 cells were transfected with PPM1H siRNA-1, siRNA-2 or control siRNA. qRT-PCR and Western blot was used to detect the inter-

Table 2. Association of PPM1H expression with the clinicopathological characteristics of PTC

Feature	No. of cases	PPM1H expression		χ^2	*P*
		Low	High		
Sex					
Male	30	19 (63.3)	11 (36.7)	0.036	0.849
Femal	75	46 (61.3)	29 (38.7)		
Age					
<45	53	29 (54.7)	24 (45.3)	2.345	0.126
≥45	52	36 (69.2)	16 (30.8)		
Tumor size (cm)					
<2	72	40 (55.6)	32 (44.4)	3.916	0.048
≥2	33	25 (75.8)	8 (24.2)		
Number of lesions					
Single	49	30 (61.2)	19 (38.8)	0.018	0.893
Multiple	56	35 (62.5)	21 (37.5)		
Lymph node metastasis					
Absent	75	41 (54.7)	34 (45.3)	5.832	0.016
Present	30	24 (80.0)	6 (20.0)		
Clinical stage					
Stage I-II	71	39 (54.9)	32 (45.1)	4.524	0.033
Stage III-IV	34	26 (76.5)	8 (23.5)		

ference efficiency, and both two interference fragments significantly reduced the PPM1H expression (**Figure 2A**, **2B**). We next evaluated the alterations of morphology after PPM1H knockdown. As shown in **Figure 2C**, SW579 cells transfected with PPM1H siRNA-1 and siRNA-2 acquired a more spindle-shaped fibroblast-like appearance and generally lost cell contact, which always appeared in mesenchymal-like cell lines. Interestingly, comprehensive analysis western blot data showed reduced expression of E-cadherin, but elevated expression of Vimentin in SW579 cells after PPM1H knockdown (**Figure 2D**). These results were in accordance with the phenotypic EMT-like changes of SW579 cells. Taken together, these data suggest that PPM1H knockdown promotes EMT changes in SW579 cells.

PPM1H decreases SW579 cells migration and invasion

Increased invasion and migration ability are important steps for metastasis. To assess whether PPM1H induced EMT involved in cancer cells metastasis, migration and invasion assays were performed. As shown in **Figure 3A**, migration was increased in SW579 cells transfected with PPM1H siRNA-1 and siRNA-2, as the number was 60 ± 5 versus 130 ± 8 and 155 ± 6 (n = 5, *P*<0.01). Similar results were also obtained in the monolayer wound healing assay, SW579 cells transfected with PPM1H siRNA-1 and siRNA-2 showed a 66.3 ± 3.0% and 75 ± 4.2% healing rate, significantly higher than that in the control group, 36.1 ± 2.5% (n = 3, *P*<0.01) (**Figure 3B**). To analyze invasiveness, another important feature of malignant cells, we performed transwell invasion assays using cell culture inserts covered by extracellular matrix components. SW579 cells transfected with PPM1H siRNA-1 and siRNA-2 had strong invasive ability resulted in a significant increase in invasion cell (**Figure 3A**). These results indicate that down-regulation of PPM1H increase the migration and invasion of SW579 cells.

PPM1H inhibits SW579 cells proliferation and colony formation

As mentioned above, PPM1H may be correlated with tumor size. Thus, we next asked whether down-regulation of PPM1H induced cells proliferation. As shown in **Figure 4A**, MTT proliferation assay indicated that the cell proliferation was promoted in both of the SW579 cells transfected with PPM1H siRNA-1 and siRNA-2 compared with control cells. Colony formation assay also suggested an increased colony-forming activity of SW579 cells with down-regulated PPM1H expression (**Figure 4B**). These results indicate that PPM1H inhibits cell proliferation and colony formation in PTC cells.

Discussion

Thyroid cancer is the most common primary endocrine neoplasm in adults. The latest data show that thyroid cancer is the most common cancer in Chinese women younger than 30 years old (6.1/1000) [20]. Papillary thyroid cancer (PTC) is the most common type of thyroid cancer making up to 80% of all thyroid cancer cases [21]. Thyroid cancer is generally very treatable, however, papillary thyroid tumor often spreads to the cervical lymph nodes, and

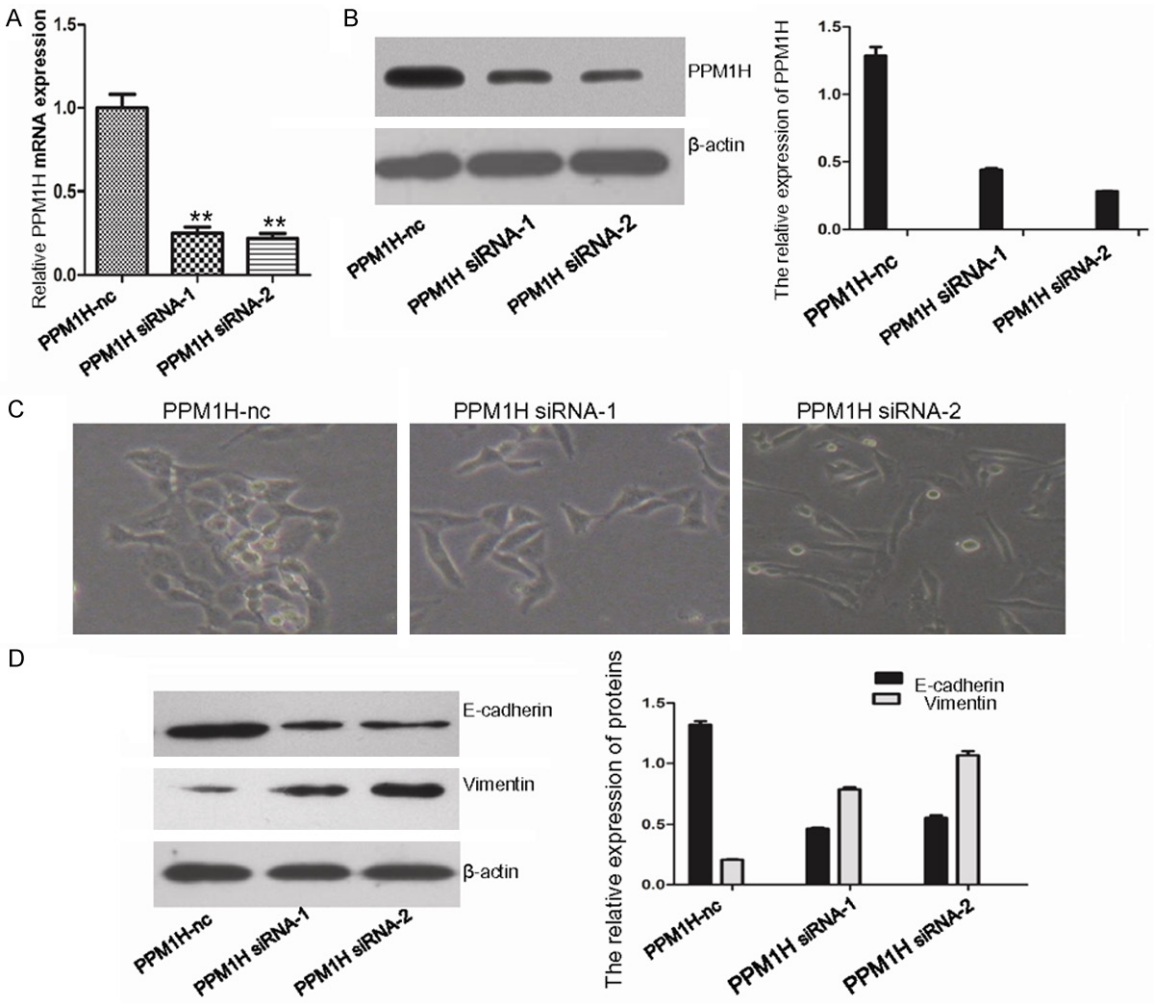

Figure 2. PPM1H inhibition induces EMT-like changes in SW579 cells. Interference efficiency of PPM1H was examined by qRT-PCR (A) and Western blot (B). (C) The alterations of morphology in SW579 cells transfected with PPM1H siRNA-1 and siRNA-2, SW579 cells transfected with PPM1H-nc were as control. (D)Western blot detected the EMT markers E-cadherin and Vimentin.

this metastasis is the major cause of fatal outcome. Therefore, efforts are urgently needed to identify metastasis associated molecules and to better understand the mechanisms behind the metastasis of PTC.

Metastasis is the movement or spreading of cancer cells from one organ or tissue to another. However, it is a complex and multistep process including proliferation, detachment, migration, angiogenesis, immuneescape, and homing to target organs [22]. During this process, increased proliferation and invasion abilities are essential features [23]. Identifying the molecules and pathways that control cell proliferation and invasion is critical to understanding cancer metastasis.

The type 2C family of protein phosphatases (PP2C) is one of the eight major protein serine/threonine phosphatase families, it is a structurally and functionally distinct family of phosphatase enzymes that currently contains at least 18 different family members in the human genome encoding for at least 22 different isozymes. The family is conserved throughout evolution with 7 PP2C family members existing in yeast (PTC1-7) [24]. The better characterized family members in humans include PPM1A and PPM1B, which are negative regulators of growth, have been shown to dephosphorylate CDK2 and inhibit signaling through cellular stress pathways [9]. PPM1H is a novel member of the PP2C family, it has been previously suggested to regulate neuronal signaling pathways,

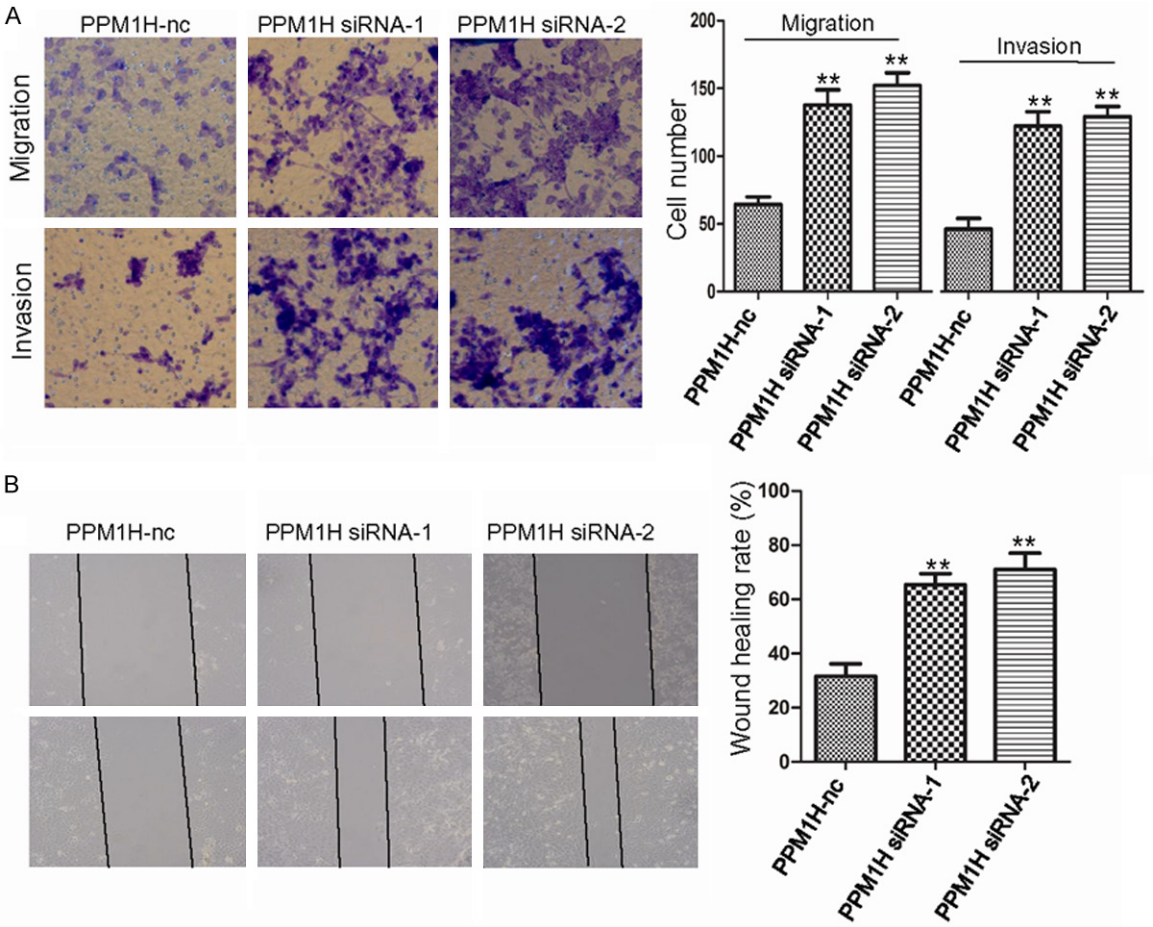

Figure 3. A. Transwell assays were used to analyze migration and invasion in SW579 cells transfected with PPM1H siRNA-1, siRNA-2 and control, $P<0.01$. B. Monolayer wound healing assay detected the migration ability in different cells, $P<0.01$.

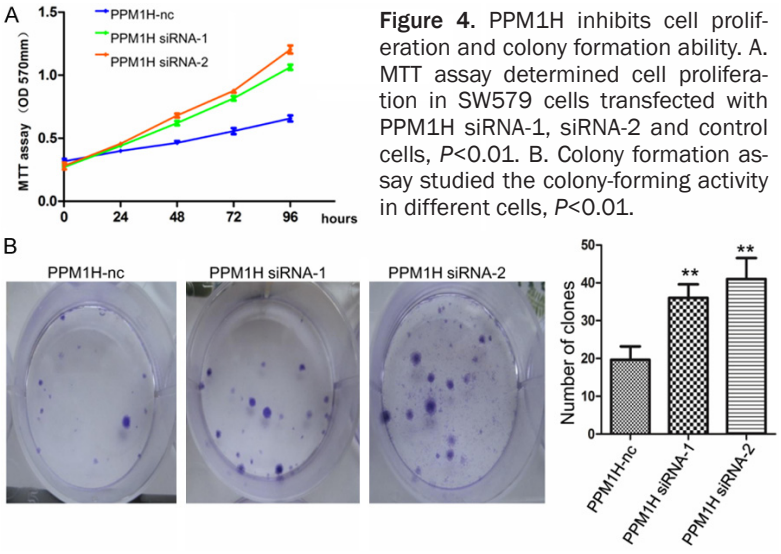

Figure 4. PPM1H inhibits cell proliferation and colony formation ability. A. MTT assay determined cell proliferation in SW579 cells transfected with PPM1H siRNA-1, siRNA-2 and control cells, $P<0.01$. B. Colony formation assay studied the colony-forming activity in different cells, $P<0.01$.

dephosphorylate CSE1L in colon adenocarcinoma and dephosphorylate p27 in breast cancer [17, 18]. However, to date, the subcellular localization and precise physiological functions of PPM1H are still unclear, especially in PTC.

In this study, we examined PPM1H expression in human PTC samples using immunohistochemistry assay, and evaluated association of PPM1H expression with several clinicopathological indicators of PTC. We also tested the role of PPM1H in the proliferation and metastasis of PTC cells by siRNAs in SW579 cells. The results demonstrate that PPM1H is located both in the cytoplasm and nucleus, it was down-regulated in

PTC tissues, and there is a statistically significant correlation between PPM1H expression and tumor size, lymph node metastasis, and clinical stage. Moreover, knockdown PPM1H expression in SW579 cells induced EMT-like changes, including the alterations of morphology and cellular architecture, increased invasion, and migration ability. PPM1H knockdown also promoted SW579 cell proliferation and colony-forming activity. These results are in accordance with the observation in PTC tissues, suggesting that PPM1H might be a tumor suppressor in PTC.

Although an increasing number of studies have indicated that PPM1H may be involved in some cancers [25, 26], its role and mechanism in metastasis are rarely known. Using mass spectrometry analysis of co-immunoprecipitated proteins, Sagiura et al. have identified CSE1L as a potential substrate of PPM1H, CSE1L is a growth and apoptosis-related molecule that has recently been shown to associate with a subset of p53 target promoters; down-regulating CSE1L reduced p53-mediated transcription and lowered apoptosis [25]. In addition, Shen et al. discovered that PPM1H directly interacted with Smad1/5/8 by its Smad-binding domain, then dephosphorylated phospho-Smad1/5/8 in the cytoplasm. Overexpression of PPM1H attenuated BMP signaling, whereas loss of PPM1H activity or expression obviously enhanced BMP-dependent gene regulation and mesenchymal differentiation [27, 28]. As mesenchymal differentiation is very important in cancer cell metastasis as known as epithelial-mesenchymal transition (EMT) [29], PPM1H may decrease metastasis by inhibiting cancer cell proliferation and EMT.

In conclusion, our results reveal that PPM1H could participate in the proliferation and invasion of PTC by inhibiting proliferation and EMT change. These findings support the notion that inhibiting PPM1H expression and function may be a potential therapy strategy for PTC.

Disclosure of conflict of interest

None.

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