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PCDH10 inhibits invasion of lymphoma cells by regulating β-catenin

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of protocadherin 10 (PCDH10) on the invasive potential of lymphoma cells by regulating matrix metalloproteinase-7 (MMP7) and MMP9 via targeting β-catenin.

MATERIALS AND METHODS: The mRNA and protein expressions of PCDH10, β-catenin, MMP7 and MMP9 in lymphoma cell lines were examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. Raji cells with low expression of PCDH10 were transiently transfected with pCMV5-HA-PCDH10 or pcDNA5-His-β-catenin. Meanwhile, HUT-78 cells with high expression of PCDH10 were transfected with PCDH10-shRNA or β-catenin-shRNA. Subsequently, the expression levels of β-catenin, MMP7 and MMP9 in transfected lymphoma cells were determined as well. In addition, the regulatory effects of PCDH10 on the invasive potential of lymphoma cells were explored by transwell assay.

RESULTS: PCDH10 expression was negatively correlated with the expressions of β-catenin, MMP7 and MMP9 in several lymphoma cell lines. Transfection of HA-PCDH10 in human-derived malignant B lymphoma cell line Raji markedly down-regulated the protein levels of β-catenin, MMP7 and MMP9. However, the mRNA level of β-catenin was not influenced by PCDH10. Interference with PCDH10 in HUT-78 cells with high expression of PCDH10 significantly increased the protein expressions of β-catenin, MMP7, and MMP9. However, no significant changes were observed in the mRNA expression of β-catenin. In addition, knockdown of β-catenin in cells with high expression of PCDH10 remarkably down-regulated the expression levels of MMP7 and MMP9.

CONCLUSIONS: PCDH10 overexpression in lymphoma cells downregulates β-catenin expression, as well as inhibits the expressions of MMP7 and MMP9, eventually inhibiting the invasive potential of lymphoma cells.

Key Words:
PCDH10, MMP7, MMP9, Lymphoma, β-catenin

Introduction

Lymphoma is a malignant tumor originating from lymph nodes and lymphoid tissues. Statistical analysis has shown that lymphoma incidence ranks ninth and tenth in male and female malignancies, respectively[1]. As a kind of malignant tumor with the fastest growth rate, lymphoma seriously threatens human health. Therefore, explorations on the metastasis of lymphoma are of great significance. Protocadherin-10 (PCDH10) is a newly discovered tumor-suppressor gene in recent years. PCDH10 belongs to the δ2 protocadherin family and is mainly involved in calcium-dependent, cell-specific adhesion and signaling pathways[2]. Due to hypermethylation in PCDH10 promoter, epigenetic silence of PCDH10 has been identified in gastric cancer, breast cancer, bladder cancer, lymphoma and endometrial cancer[3-5]. Previous studies[6-8] have found that PCDH10 can be used as a biochemical marker to evaluate the recurrence and prognosis of diseases. It has been reported that PCDH10 overexpression markedly inhibits the invasion and metastasis of tumor cells. However, its specific mechanism remains unclear currently.

Matrix metalloproteinases (MMPs) are closely related to tumor invasion and metastasis. It has been demonstrated that overexpression of MMPs accelerates the invasion and metastasis of tumor cells[9]. MMP7 and MMP9 are important members of the MMP family, which can degrade extracellular matrix including basement membrane. Eventually, this may destroy tumor cell abilities to invade and metastasize[10]. It has been reported[11] that PCDH10-induced β-catenin downregulation inhibits the invasion of multiple myeloma cells. Therefore, the aim of this study was to investigate the regulatory effects of PCDH10 on the expressions of MMP7 and MMP9, as well as the invasi-

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ve potential of lymphoma cells, and to explore its underlying mechanism.

Materials and Methods

Cell Culture and Transfection

Lymphoma cell lines (Ramos, U397, Raji, Daudi, HUT-78 and SUK-6) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in α-Modified Eagle's Medium (α-MEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained in a 5% CO_2 incubator at 37°C.

Cell transfection was performed according to the instructions of Lipofectamine^{TM} LTX (Invitrogen, Carlsbad, CA, USA) and Plus Reagent in serum-free α-MEM. 4-6 h after transfection, the complete medium was replaced. Total RNA and protein were extracted 48 h after transfection for subsequent experiments.

Construction of pCMV5-HA-PCDH10 and PcDNA5-His-β-Catenin

Primers were designed based on gene sequences downloaded from Genbank (Table I). Total RNA was extracted from Raji and Daudi cells, and was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). Polymerase Chain Reaction (PCR) amplification was carried out, and the full-length β-catenin fragment and pcDNA5-His empty vector were ligated with T4 DNA ligase. Meanwhile, full-length PCDH10 fragment and pCMV5-HA vector were ligated. Subsequently, ligation products were transformed into DH5α E-coli and amplified to extract plasmid DNAs. Finally, enzyme digestion and sequencing were conducted.

Construction of pCMV5-HA-PCDH10-Raji Cells

Raji cells in the logarithmic growth phase were first inoculated into 6-well plates (2×10^5/well). Subsequently, the cells were transfected with pCMV5-HA-PCDH10 plasmid or negative control until 70%-80% of confluence. After transfection for 48 h, 1000 μg/mL G418 was applied for screening positive clones. Labeled monoclonal cells were picked up under a fluorescence microscope, screened and purified by limiting dilution method in 96-well plates. During the process of monoclonal cell culture, G418 was applied for screening. The expressions of green fluorescent proteins were observed under a fluorescence microscope, indicating cells overexpressing PCDH10. Raji cells stably overexpressing PCDH10 were named as pCMV5-HA-PCDH10-Raji.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from lymphoma cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA was reverse transcribed into cDNA using Primescript^{RT} kit (TaKaRa, Otsu, Shiga, Japan). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed in strict accordance with SYBR Premix Ex TaqII kit (TaKaRa, Otsu, Shiga, Japan) on an ABI Prism 7500 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Specific qRT-PCR conditions were as follows: denaturation at 95°C for 30 seconds, annealing at 95°C for 5 seconds, and extension at 60°C for 30 s, for a total of 40 cycles. The relative expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: PCDH10, F: 5'-GCATGGAACG-GCTCCTTACTC-3', R: 5'-GCCTAGGGATGT-GTCTTAGGA-3'; β-catenin, F: 5'-GACTTGTAAC-

Table I. PCR primer sequences, production size and annealing temperature.

Gene	Primer sequence	Product length (bp)	Annealing temperature
PCDH10	Sense: 5'-CTGGTGTCCTTGCTGATAC-3' Antisense: 5'-TGTCTTGGTCGTTGTCTTC-3'	184	55.4
β-catenin	Sense: 5'-ACCTGAGACTGGATGTAaGAA-3' Antisense: 5'-GCTGGAATGACAACTGGAT-3'	158	53.2
MMP7	Sense: 5'-AGGCTCAGGACTATCTCAA-3' Antisense: 5'-CCACTGTAATATGCGGTAAG-3'	276	51.9
MMP9	Sense: 5'-GGAAGATGCTGCTGTTCA-3' Antisense: 5'-CCACCTGGTTCAACTCAC-3'	210	57.8
GAPDH	Sense: 5'-CATCACCATCTTCCAGGAG-3' Antisense: 5'-AGGCTGTTGTCATACTTCTC-3'	213	56.1

GAATCCTACTG-3', R: 5'-CCATTGCGTGTCGA-GAGTCG-3'; MMP7, F: 5'-GCAACAGCGCATTT-GAGCGGA-3', R: 5'-AATCAGCGTGCGACAGT-TGCT-3'; MMP9, F: 5'-GAGGAGCGCAGCTGA-CA-3', R: 5'-CGCGTGCGGCGACAGTTTACT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Total protein in cells was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). After determination of protein concentration, extracted protein samples were separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After washing with Tris-Buffered Saline with Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with the corresponding secondary antibody at room temperature for 2 h. Immunoreactive bands were exposed by the enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) method and analyzed by Image Software. Tubulin was utilized as an internal control.

Transwell Invasion Assay

Transwell chamber pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was first inserted in 24-well plates. 2×10^5 cells were seeded into the upper chamber. Meanwhile, 500 μL of medium with 10% FBS was added to the lower chamber. At 24 h, the chambers were taken out. Penetrating cells were fixed with 5% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 20 min. 4 fields were randomly selected for each sample. Finally, the number of penetrating cells was counted under a microscope (magnification 200×).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were represented as mean ± Standard Deviation (SD). *t*-test was used to compare the differences between the two groups. Differences among multiple groups were analyzed by Tukey or Dunnett's T3. $p<0.05$ was considered statistically significant.

Results

Expression Levels of PCDH10, β-Catenin, MMP7 and MMP9 in Lymphoma Cell Lines

The protein and mRNA expression levels of PCDH10, β-catenin, MMP7 and MMP9 in Ramos, U397, Raji, Daudi, HUT-78 and SUK-6 cells were determined by Western blot and qRT-PCR, respectively. It was found that lymphoma cells with relatively high expression of PCDH10 showed significantly lower protein levels of β-catenin, MMP7 and MMP9. Conversely, lymphoma cells with low expression of PCDH10 showed markedly higher protein levels of β-catenin, MMP7 and MMP9 (Figure 1A). However, no significant expression patterns were observed at the mRNA level (Figure 1B). Therefore, it was speculated

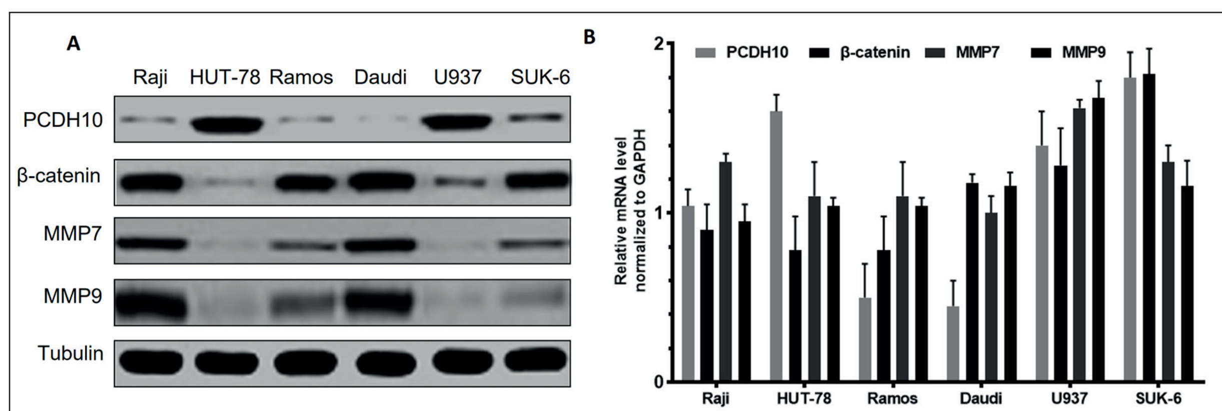

Figure 1. Expression levels of PCDH10, **β**-catenin, MMP7 and MMP9 in lymphoma cell lines. ***A,*** Western blot analyses of PCDH10, β-catenin, MMP7 and MMP9 expressions in Raji, HUT-78, Ramos, Daudi, U937, and SUK-6 cells. ***B,*** The mRNA levels of PCDH10, β-catenin, MMP7 and MMP9 in Raji, HUT-78, Ramos, Daudi, U937, and SUK-6 cells.

that the protein level of PCDH10 was negatively correlated with the expressions of β-catenin, MMP7 and MMP9 in lymphoma cells.

PCDH10 Downregulated β-Catenin, MMP7 and MMP9 in Lymphoma Cells

Raji cells were transfected with negative control, pCMV5-HA-PCDH10, pcDNA5-His-β-catenin or pcDNA5-His-β-catenin+pCMV5-HA-PCDH10, respectively. Western blot indicated that the protein levels of β-catenin, MMP7 and MMP9 significantly decreased in cells overexpressing PCDH10 (Figure 2A). Identically, the mRNA levels of MMP7 and MMP9 were markedly down-regulated in cells transfected with HA-PCDH10 (Figure 2B). However, no significant change was observed in the mRNA level of β-catenin. Meanwhile, the protein and mRNA levels of MMP7 and MMP9 in cells overexpressing β-catenin were remarkably higher than those in the blank control group. However, no significant changes in PCDH10 expression were observed. Moreover, the protein and mRNA levels of MMP7 and MMP9 markedly decreased in cells co-overexpressing PCDH-10 and β-catenin compared with those overexpressing β-catenin. The above results indicated that overexpression of PCDH10 significantly down-regulated the protein levels of β-catenin, MMP7 and MMP9. More importantly, overexpression of PCDH10 could restore the effect of β-catenin overexpression on the expression patterns of MMP7 and MMP9.

Interference with PCDH10 or β-Catenin Regulated Expressions of MMP7 and MMP9 in Lymphoma Cells

HUT-79 cells with high expression of PCDH10 were transfected with negative control, PCDH10-shRNA, β-catenin-shRNA or PCDH10-shRNA+β-catenin-shRNA, respectively. Subsequent Western blot results showed that the protein levels of β-catenin, MMP7 and MMP9 were remarkably up-regulated in HUT-79 cells transfected with PCDH10-shRNA. Conversely, transfection of β-catenin-shRNA in HUT-79 cells significantly downregulated the protein levels of β-catenin, MMP7 and MMP9. Meanwhile, co-transfection of PCDH10-shRNA and β-catenin-shRNA remarkably increased the protein levels of β-catenin, MMP7 and MMP9 compared with those transfected with β-catenin-shRNA (Figure 3A). However, no significant change was found in the mRNA level of β-catenin (Figure 3B). Hence, it was suggested that PCDH10 knockdown upregulated the protein levels of β-catenin, MMP7 and MMP9. Furthermore, PCDH10 could restore the effect of β-catenin interference on the expression levels of MMP7 and MMP9.

PCDH10 Regulated Invasive Potential of Lymphoma Cells through β-Catenin

Transwell assay revealed fewer penetrated pCMV5-HA-PCDH10-Raji cells (48±2.74) than controls (90±6.63, $p<0.05$), indicating significantly inhibited invasive ability. However, in the PC-

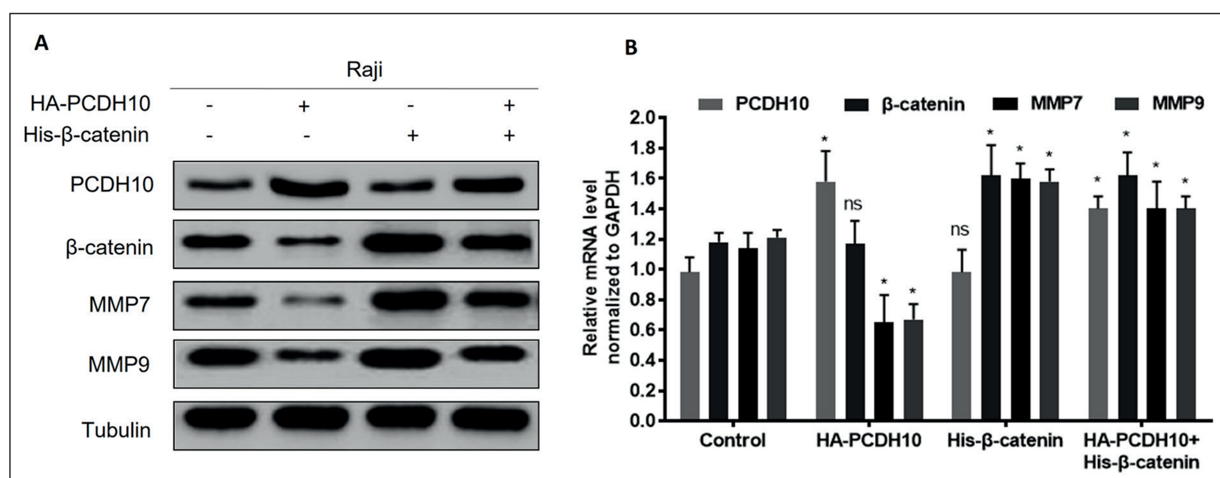

Figure 2. PCDH10 overexpression downregulated **β**-catenin, MMP7 and MMP9 in lymphoma cells. Raji cells were transfected with negative control, pCMV5-HA-PCDH10, pcDNA5-His-β-catenin or pcDNA5-His-β-catenin+pCMV5-HA-PCDH10, respectively. ***A,*** Western blot analyses of PCDH10, β-catenin, MMP7 and MMP9 expressions in Raji cells. ***B,*** The mRNA levels of PCDH10, β-catenin, MMP7 and MMP9 in Raji cells. *$p<0.05$; NS, no significance.

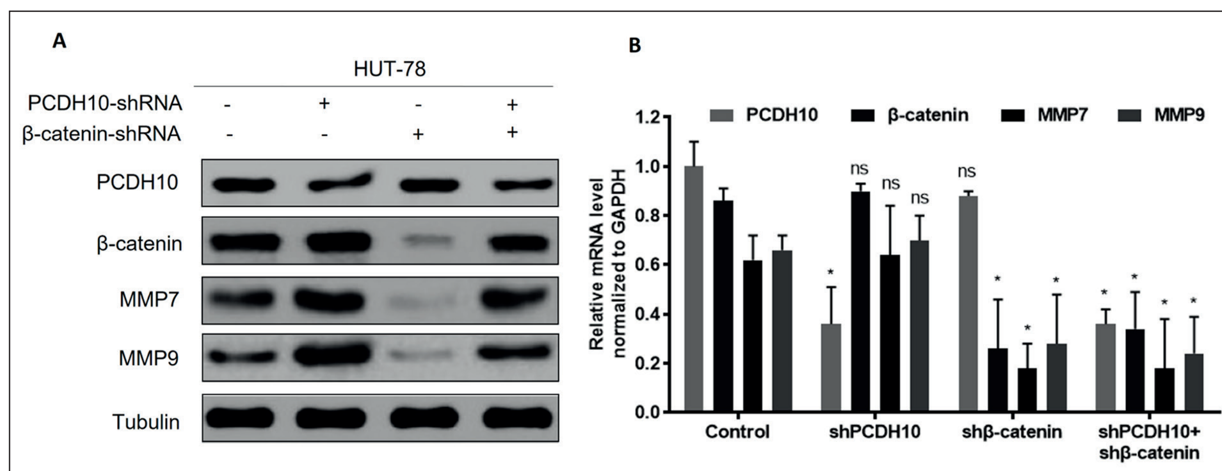

Figure 3. PCDH10 knockdown upregulated the expressions of **β**-catenin, MMP7 and MMP9 in lymphoma cells. HUT-78 cells were transfected with negative control, PCDH10-shRNA, β-catenin-shRNA, or PCDH10-shRNA+β-catenin-shRNA, respectively. ***A,*** Western blot analyses of PCDH10, β-catenin, MMP7 and MMP9 expressions in HUT-78 cells. ***B,*** The mRNA levels of PCDH10, β-catenin, MMP7 and MMP9 in HUT-78 cells. *$p<0.05$; NS, no significance.

DH10 interference group (PCDH10-shRNA-Raji), the number of penetrating cells (128±2.74) was markedly higher than that of the control group ($p<0.05$, Figure 4).

Discussion

Globally, the incidence of malignant lymphoma has reached 20/100,000 in the past 30 years. Lymphocytes are distributed throughout the body, leading to difficulties in the surgical treatment of lymphoma. Currently, radiotherapy and chemotherapy are the major treatments for lymphoma[12]. Meanwhile, biotherapy has been widely applied for lymphoma in recent years. However, high infiltrated and metastatic rates are the leading causes of poor prognosis and death of lymphoma patients. In this study, we mainly elucidated the functional role of PCDH10-β-catenin-MMP7/MMP9 axis in the pathogenesis of lymphoma. The results found that PCDH10 markedly downregulated the protein level of β-catenin, while the mRNA level of β-catenin was not significantly altered. The results showed that PCDH10 did not affect the transcription of β-catenin, but markedly degraded its cellular expression.

Previous studies have reported that invasive tumor cells gradually infiltrate and invade into the extracellular matrix (ECM). Tumor cells and stromal cells degrade ECM by secreting proteases, thereby impairing basement membrane. Tumor cells are metastasized to blood vessels and distant organs in such way[13]. ECM

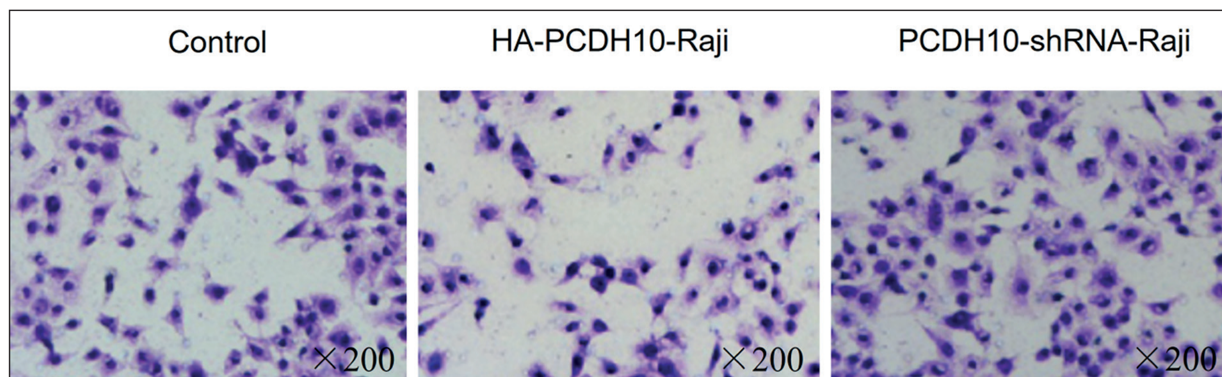

Figure 4. PCDH10 regulated invasive potential of lymphoma cells through **β**-catenin. Transwell assay showed invasive ability of control cells, pCMV5-HA-PCDH10-Raji cells and PCDH10-shRNA-Raji cells (magnification 200×).

degradation in tumor cells or tumor stroma tissues mainly depends on MMPs. It is known to all that MMP7 and MMP9 are important members of the MMPs family[14]. PCDH10 is silenced in multiple myeloma due to promoter methylation, thus accelerating proliferation, migration, and angiogenesis[15]. Demethylation of PCDH10 can reverse its cellular behaviors. PCDHGC3, PCDHs and PCDH-P are members of the protocadherin family, whose biological mechanisms have been observed to be related to the Wnt/β-catenin pathway[16-18]. Recent studies[19] have found that abnormally activated Wnt/β-catenin pathway is widely involved in malignant performances and drug resistance of tumor cells. In addition, the Wnt/β-catenin pathway exerts a vital role in the occurrence and development of lymphoma[19].

Numerous studies have demonstrated that PCDH10 binds to important genes related to the Wnt/β-catenin pathway, thus affecting cellular functions. β-catenin is a classical cadherin-catenin complex in the Wnt pathway. Its abnormal accumulation accelerates the malignant progression of tumors[20,21]. As a key molecule in regulating β-catenin degradation, PCDH10 maintains the low activity of the Wnt pathway, and regulates cell proliferation and apoptosis[22,23]. These findings are consistent with our results. In this work, we found that PCDH10 was involved in tumor inhibition by degrading β-catenin. The correlation between PCDH10 with MMP7 and MMP9 have rarely been reported. In the present study, we found for the first time that invasion-related genes, MMP7 and MMP9 were significantly downregulated after PCDH10 overexpression in Raji cells with low expression of PCDH10. Besides, the invasive potential of cells was markedly inhibited, but could be further reversed by β-catenin overexpression. Transfection of PCDH10-shRNA in Raji cells stably overexpressing PCDH10 upregulated the protein levels of MMP7 and MMP9, leading to elevated invasive ability. However, enhanced invasion of lymphoma cells was inhibited by β-catenin knockdown. Therefore, we speculated that β-catenin served as a link between PCDH10 with MMP7 and MMP9 in lymphoma.

ECM degradation is a vital process for tumor cell invasion and metastasis. MMPs are important proteolytic enzymes that can degrade ECM, resulting in tumor cell invasion and metastasis[24-26]. This study demonstrated for the first time that PCDH10 regulated lymphoma cell invasion by downregulating the protein levels of MMP7 and MMP9 *via* targeting β-catenin. Our results might provide a novel theoretical and experimental basis for targeted therapy of lymphoma.

Conclusions

We showed that the PCDH10 overexpression downregulated the expression of β-catenin in lymphoma cells, and inhibited the expressions of MMP7 and MMP9, eventually inhibiting the invasive potential of lymphoma cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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