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LncRNA PWRN2 stimulates the proliferation and migration in papillary thyroid carcinoma through the miR-325/DDX5 axis

C.-H. XIN[1], Z. LI[2]

[1]Department of Endocrinology and Metabolism, The Fourth People's Hospital of Shenyang, Shenyang, China
[2]Department of Internal Medicine-Cardiovascular, The First Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, Shenyang, China

Abstract. – OBJECTIVE: The objective of this study was to illustrate the role of long non-coding RNA (lncRNA) PWRN2 in the development of papillary thyroid carcinoma (PTC) and the potential mechanism.

PATIENTS AND METHODS: Expression levels of PWRN2, miR-325 and DDX5 in 32 PTC tissues and paired normal ones were detected. The interaction in the PWRN2/miR-325/DDX5 axis was assessed by Luciferase assay. At last, the roles of the PWRN2/miR-325/DDX5 axis in regulating proliferative and migratory potentials in PTC were examined.

RESULTS: It was found that PWRN2 was upregulated and miR-325 was downregulated in PTC tissues and cell lines. MiR-325 level was negatively correlated with PWRN2 level in PTC samples, and the overexpression of PWRN2 stimulated proliferative and migratory potentials in PTC cells, which were partially abolished by overexpression of miR-325. In addition, DDX5 was the target gene binding to miR-325, and its level was negatively regulated by miR-325. Moreover, Luciferase assay and rescue experiments confirmed that the PWRN2/miR-325/DDX5 axis aggravated the development of PTC.

CONCLUSIONS: LncRNA PWRN2 stimulates proliferative and migratory potentials in PTC through sponging miR-325 to upregulate DDX5.

Key Words:
Papillary thyroid carcinoma, PWRN2, MiR-325, DDX5, Proliferation, Migration.

Introduction

Thyroid cancer is a common malignancy in endocrine organs, and its morbidity and mortality have persistently increased for many years. Based on pathological features, thyroid cancer derived from follicular cells is divided into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and anaplastic thyroid carcinoma (ATC). PTC is the major subtype, accounting for 80% of all thyroid cancer cases[1]. The adverse prognostic factors of PTC mainly include advanced age at diagnosis, gender, large tumor size, and extrathyroid growth[2]. Most PTC patients have a good prognosis after active treatment, and their 5-year survival is up to 90%[3]. However, the pathogenic cause of PTC is still unclear, and exploring the underlying mechanisms contributes to improving early detective rate and therapeutic efficacy of PTC[4]. Molecular markers are highly specific for identifying malignant tumors. Therefore, it is of significance to seek biomarkers of PTC[5].

Long non-coding RNAs (LncRNAs) are evolutionarily conserved non-coding RNAs with over 200 nt long, and they are extensively involved in chromatin dynamics, embryonic development, differentiation and carcinogenesis[6,7]. Accumulating evidence has shown the participation of lncRNAs in tumor cell behaviors[8,9]. Abnormally expressed lncRNAs are discovered in PTC, which directly or indirectly influence tumor cell growth. For example, lncRNA HOTTIP and lnc00152 are involved in the development of PTC[10,11]. Notably, differentially expressed lncRNAs may serve as tumor suppressors in PTC, which can be utilized as therapeutic targets[12].

LncRNA-Prader-Willi region nonprotein coding RNA 2 (PWRN2) was initially discovered to be upregulated after meiosis during spermatogenesis[13]. PWRN2 sponges miR-92b-3p to upregulate TREM120B and thus influences oocyte maturation in polycystic ovary syndrome[14].

Corresponding Author: Zheng Li, MD; e-mail: lnzylizheng@cumt.edu.cn

In this paper, it was found that PWRN2 was upregulated in PTC samples and miR-325 was the target gene binding PWRN2. Our findings reveal the role of PWRN2 in the development of PTC.

Patients and Methods

Sample Collection

A total of 24 paired PTC samples and adjacent normal ones were surgically resected from PTC patients treated in The Fourth People's Hospital of Shenyang from June 2016 to December 2018, and then they were preserved at -80°C. Patients were selected according to the NCCN guidelines (2016). This investigation was approved by the Ethics Committee of The Fourth People's Hospital of Shenyang and conducted after informed consent was obtained from each subject.

Cell Culture and Transfection

PTC cell lines (BHP5-16, TPC1, K1 and BHP2-7) and normal thyroid cell line (Nthyori3-1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Cells were inoculated in a 6-well plate one day prior to transfection and transfected with 50-100 nM plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Original medium was replaced with a fresh one at 6 h.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 3×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

200 μL of suspension (5×10^5 cells/ml) were inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Finally, migratory cells were counted in 10 random fields per sample.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). PCR system (20 μL) was prepared, including 10 μL of SYBR GreenMaster Mix, 1 μL of forward plasmid, 1 μL of reverse plasmid, 0.4 μL of Rox Dye, 2 μL of cDNA and 5.6 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 as internal controls, the relative level of the target was calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed as follows: PWRN2: forward: 5'-TAATAGCCAGCCACAACT-3' and reverse: 5'-AAGCCAAGACAGGTTACT-3', miR-325: forward: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACACUUAC-3' and reverse: 5'-ACACTCCAGCTGGGCCUAGUAGGUGUCCAGU-3', DDX5: forward: 5'-ATGTCGGGTTATTCGAGTGACC-3' and reverse: 5'-TGTGCGCCTAGCCAAATCAG-3', GAPDH: forward: 5'-TCAAGATCATCAGCAATGCC-3' and reverse: 5'-CGATACCAAAGTTGTCATGGA-3', and U6: forward: 5'-ATACAGAGAAAGTTAGCACGG-3' and reverse: 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Luciferase Assay

Wild-type and mutant Luciferase vectors were constructed based on the predicted binding sequences. Cells were co-transfected with wild-type/mutant vectors and miR-325 mimics/NC, respectively using Lipofectamine 2000. 24 hours later, cells were lysed and subjected to luciferase activity measurement (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for data analysis. Data were expressed as mean ± SD (standard deviation). Differences between groups were compared using the *t*-test. Pearson correlation test was conducted to assess the relationship between two gene expressions. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression Levels of PWRN2 and MiR-325 In PTC

Upregulated PWRN2 (Figure 1A) was found in PTC samples than normal ones, and PWRN2 was

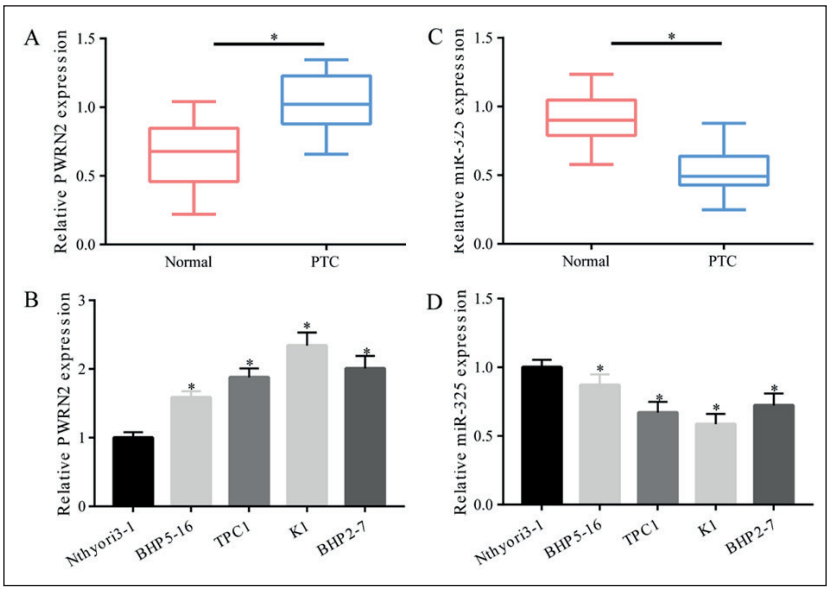

Figure 1. Expression levels of PWRN2 and miR-325 in PTC. **A,** PWRN2 levels in normal tissues and PTC tissues. **B,** PWRN2 levels in human normal thyroid cell line and thyroid carcinoma cell lines. **C,** MiR-325 levels in normal tissues and PTC tissues. **D,** MiR-325 levels in human normal thyroid cell line and thyroid carcinoma cell lines. **p*<0.05, ***p*<0.01, ****p*<0.001.

highly expressed in PTC cell lines (Figure 1B). Downregulated miR-325 (Figure 1C) was found in PTC samples than normal ones, and miR-325 was lowly expressed in PTC cell lines (Figure 1D).

PWRN2 Stimulated Proliferative and Migratory Potentials in PTC

pcDNA-PWRN2 was constructed, and its transfection efficacy was tested in K1 and TPC1 cells (Figure 2A, 2B). Overexpression of PWRN2 markedly increased viability in K1 and TPC1 cells (Figure 2C, 2D). Moreover, migratory cell number was remarkably elevated in PTC cells overexpressing PWRN2 (Figure 2E, 2F). It is suggested that PWRN2 promotes proliferative and migratory potentials in PTC.

MiR-325 Was the Target Gene of PWRN2

Binding sequences in the 3'UTR of miR-325 and PWRN2 were predicted using bioinformatics method (Figure 3A). Subsequently, Lucifer-

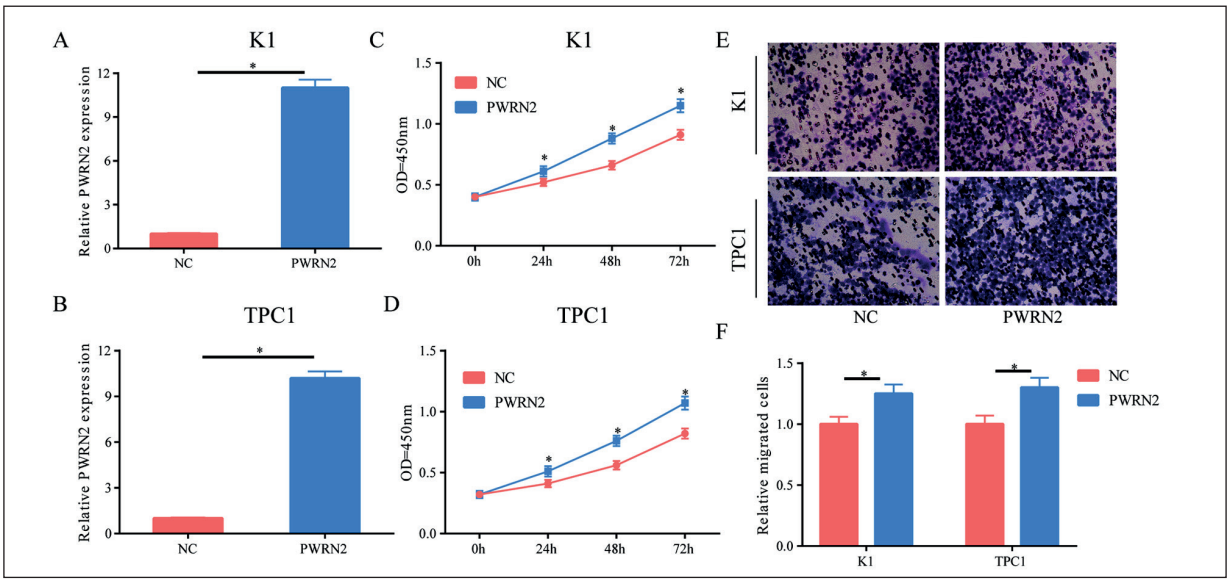

Figure 2. PWRN2 stimulates proliferative and migratory potentials in PTC. **A, B,** Transfection efficacy of pcDNA-PWRN2 in K1 (**A**) and TPC1 cells (**B**). **C, D,** Viability in K1 (**C**) and TPC1 cells (**D**) transfected with NC or pcDNA-PWRN2, respectively. **E, F,** Migration in K1 (**E**) and TPC1 cells (**F**) transfected with NC or pcDNA-PWRN2, respectively. (magnification: 40×) **p*<0.05, ***p*<0.01, ****p*<0.001.

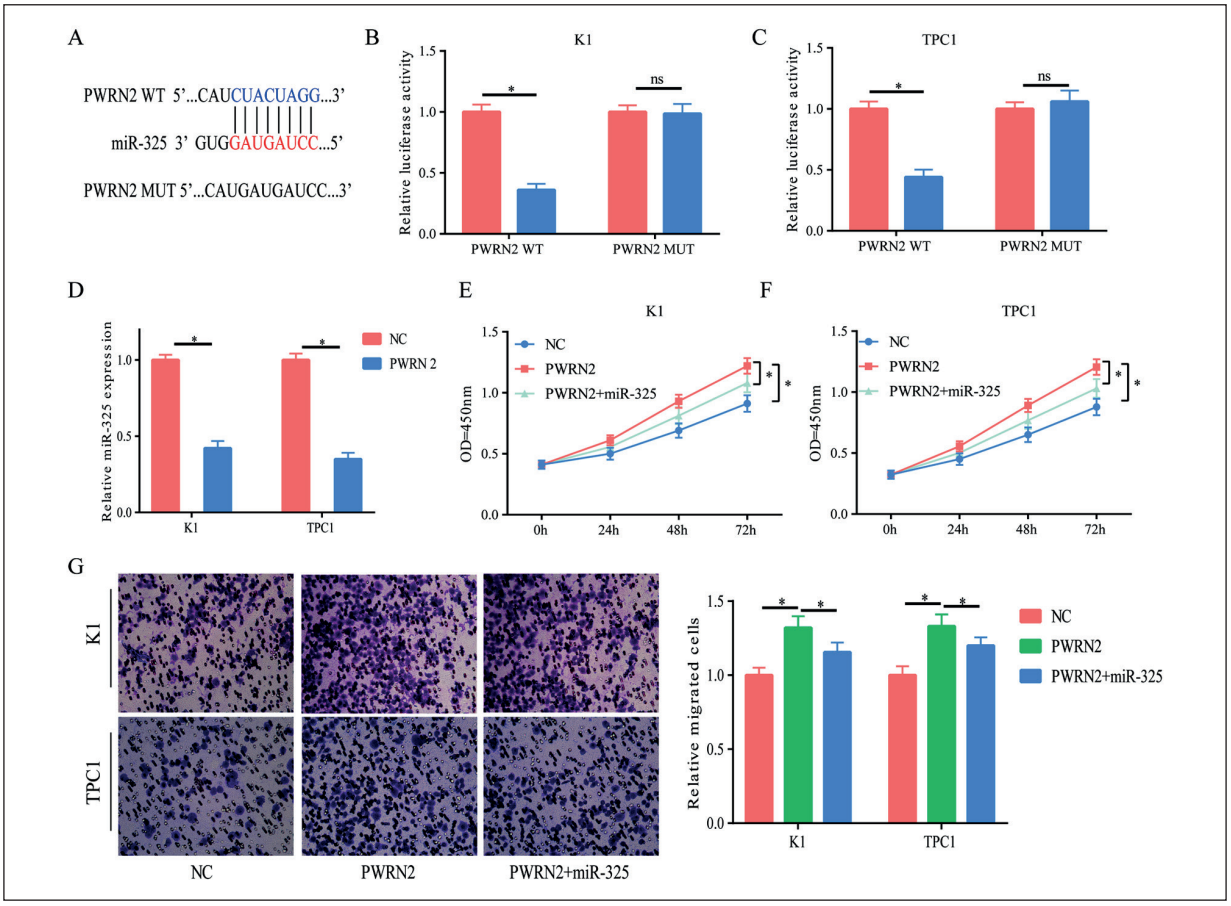

Figure 3. MiR-325 is the target gene of PWRN2. **A,** Binding sequences in the 3'UTR of PWRN2 and miR-325. **B, C,** Luciferase activity in K1 (**B**) and TPC1 cells (**C**) co-transfected with PWRN2-WT/PWRN2-MUT and miR-325 mimics/NC, respectively. **D,** MiR-325 level in K1 and TPC1 cells transfected with NC or pcDNA-PWRN2, respectively. **E, F,** Viability in K1 (**E**) and TPC1 cells (**F**) transfected with NC, pcDNA-PWRN2 or pcDNA-PWRN2+miR-325 mimics, respectively. **G,** Migration in K1 and TPC1 cells transfected with NC, pcDNA-PWRN2 or pcDNA-PWRN2+miR-325 mimics, respectively. (magnification: 40×) *p<0.05, **p<0.01, ***p<0.001.

ase assay showed that overexpression of miR-325 remarkably quenched Luciferase activity in wild-type PWRN2 vector, further confirming the binding between miR-325 and PWRN2 (Figure 3B, 3C). In K1 and TPC1 cells, overexpression of PWRN2 downregulated miR-325 level (Figure 3D). Notably, increased viability in PTC cells overexpressing PWRN2 was partially reversed by co-overexpression of miR-325 (Figure 3E, 3F). Moreover, improved migratory potential in PTC cells transfected with pcDNA-PWRN2 was also abolished by co-overexpression of miR-325 (Figure 3G).

DDX5 Was the Target Gene of MiR-325

In a similar way, DDX5 was predicted to be the potential target gene of miR-325 (Figure 4A). Luciferase assay further verified the binding relationship between DDX5 and miR-325 (Figure 4B, 4C). Subsequently, miR-325 mimics were constructed and its transfection efficacy in PTC cells was tested (Figure 4D, 4E). Besides, overexpression of miR-325 was markedly downregulated DDX5 in both K1 and TPC1 cells (Figure 4F, 4G). Compared with normal tissues, DDX5 was identified to be highly expressed in PTC tissues (Figure 4H). Moreover, DDX5 level was negatively correlated with miR-325 level in PTC samples (Figure 4I). As a result, these findings demonstrate that the PWRN2/miR-325/DDX5 axis is involved in the development of PTC.

Discussion

Atypical symptoms and signs of PTC result in difficulties in the detection of early stage PTC. In addition, fine-needle aspiration for thyroid mass-

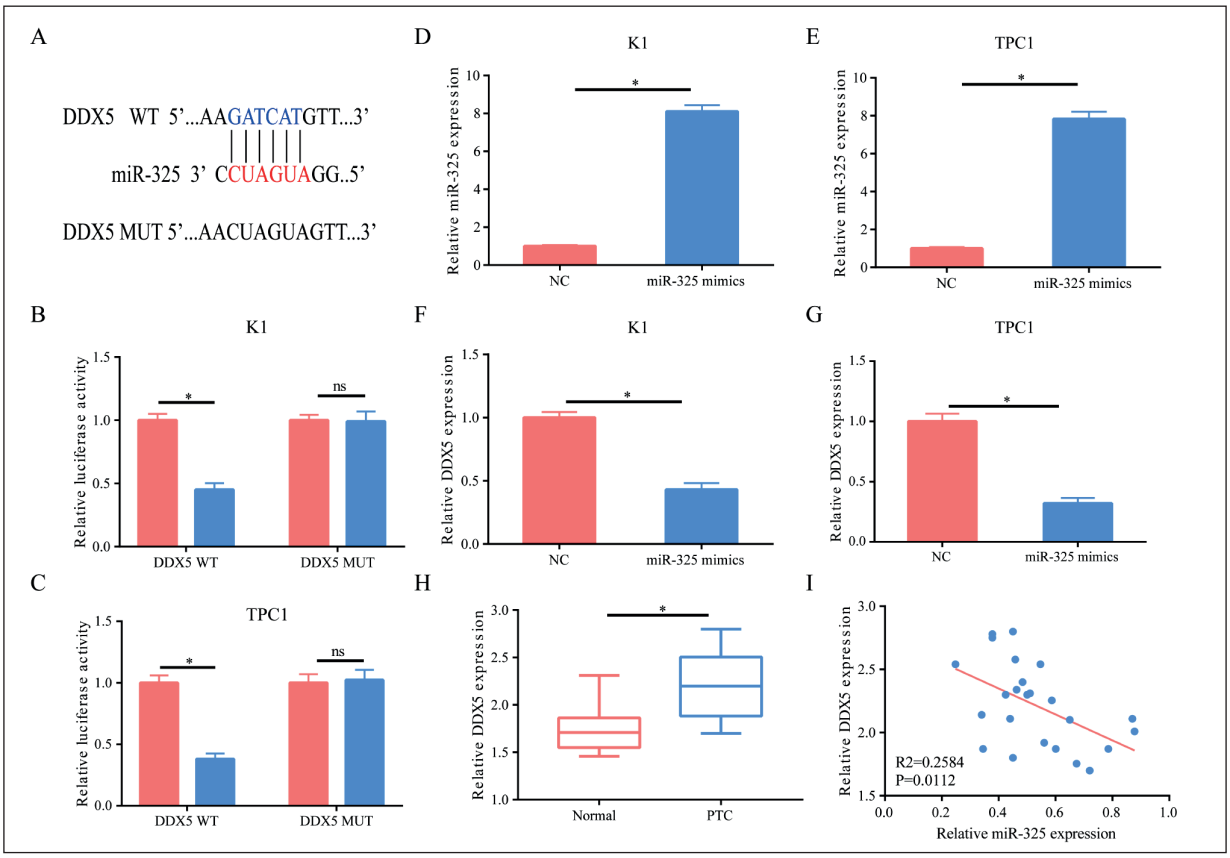

Figure 4. DDX5 is the target gene of miR-325. **A,** Binding sequences in the 3'UTR of miR-325 and DDX5. **B, C,** Luciferase activity in K1 (**B**) and TPC1 cells (**C**) co-transfected with DDX5-WT/DDX5-MUT and miR-325 mimics/NC, respectively. **D, E,** Transfection efficacy of miR-325 mimics in K1 (**D**) and TPC1 cells (**E**). **F, G,** DDX5 level in K1 (**F**) and TPC1 cells (**G**) transfected with miR-325 mimics or NC, respectively. **H,** DDX5 levels in normal tissues and PTC tissues. **I,** A negative correlation between expression levels of DDX5 and miR-325 in PTC tissues. **p*<0.05, ***p*<0.01, ****p*<0.001.

es is an invasive procedure that is not extensively acceptable. Effective biomarkers for determining malignant level and invasive degree of PTC are lacked. Although PTC is a well prognosed disease, tumor metastasis will markedly decrease survival rate[15].

So far, several lncRNAs have been identified to participate in the development of PTC[16]. LncRNA FOXD2-AS1 is involved in the development of thyroid cancer by regulating TERT level[17]. LncRNA LUCAT1 is considered as a tumor biomarker of PTC[18]. In this paper, PWRN2 was highly expressed in PTC samples and cell lines, and its overexpression greatly stimulated proliferative and migratory potentials in PTC cells. It is suggested that PWRN2 may be an oncogene involved in the development of PTC.

A novel theory proposed in recent years suggested that lncRNAs can serve as ceRNAs to sponge target miRNAs, thus regulating downstream gene expressions and functions[19,20]. Bioinformatics analysis verified that miR-325 was the target gene binding to PWRN2. In addition, miR-325 level was negatively regulated by PWRN2. The ceRNA theory indicated that lncRNAs are important regulators in tumor development through forming an interaction in lncRNA-miRNA-mRNA network[21].

DDX5 (DEAD-Box Helicase 5), also known as RNA Helicase P68, is widely expressed in human cells and involved in cell phenotypes, embryogenesis, adipogenesis, etc[22,23]. DDX5 has been identified to be abnormally expressed in many types of solid tumors, serving as a biomarker in diagnosing and predicting the prognosis of tumor diseases[24]. In a similar way, DDX5 was proved to be the target gene binding to miR-325. DDX5 was highly expressed in PTC samples, and its level was negatively regulated by miR-325. To sum up, PWRN2 sponged miR-325 to upregulate DDX5, thus aggravating the development of PTC.

Conclusions

Taken together these results showed that lncRNA PWRN2 stimulates proliferative and migratory potentials in PTC through sponging miR-325 to upregulate DDX5, and it may be used as a promising biomarker in screening and treatment of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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