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Original Article

Clinical and biologic roles of PDGFRA in papillary thyroid cancer: a study based on immunohistochemical and in vitro analyses

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Abstract: Background: Platelet-derived growth factor receptor alpha (PDGFRA) plays essential roles in several malignant tumors. Nevertheless, its clinical function in papillary thyroid cancer (PTC) is still unclear. This study aimed to examine the clinicopathologic implication and potential molecular underpinning of PDGFRA in PTC. Material and methods: Relative PDGFRA expression levels in eight cases of normal thyroid tissue, 15 cases of benign thyroid disease, and 90 cases of PTC were examined by immunohistochemistry (IHC). The prognostic value of PDGFRA was assessed by data mining of The Cancer Genome Atlas dataset. LV-PDGFRA overexpression and negative control CON220 lentivirus vectors were constructed and transfected into a PTC cell line. The capacity for cell proliferation, status of the cell cycle, efficiency of colony-forming, and migration ability of the PTC cells after PDGFRA were detected by multiple assays including methyl thiazolyl tetrazolium, flow cytometry, colony formation, transwell assay, and wound healing. Furthermore, bioinformatics analyses were conducted to determine the potential biologic mechanisms of PDGFRA. Results: Results of IHC showed that PDGFRA expression was significantly upregulated in PTC samples and was associated with an advanced pathologic stage. Furthermore, patients with PDGFRA overexpression showed poor survival. Ectopically overexpressed PDGFRA accelerated the migration and invasion of PTC cells. Results of the bioinformatics analyses suggested that PDGFRA was involved in several cell proliferation-related pathways. Conclusion: Collectively, our results indicate that PDGFRA overexpression is associated with the poor survival of patients with PTC and that PDGFRA is a potent oncogene in PTC because it significantly increases PTC cell migration and invasion. Thus, PDGFRA may be a promising novel biomarker and therapeutic target for treating PTC.

Keywords: Platelet-derived growth factor receptor alpha, papillary thyroid cancer, migration

Introduction

Thyroid cancer remains the most frequent cancer that originates from the endocrine system. The prevalence has increased quickly with the regular usage of imaging diagnosis such as ultrasound [1, 2]. Thyroid cancer is predicted to be the cause of 53,990 new cases and 2060 deaths in 2018 in the United States [3]. The occurrence of thyroid cancer has even increased in children and teenagers, and has markedly grown recently in many districts [4, 5]. Several tumors with adverse clinical characteristics, including distant metastasis, progress to functional diseases [6]. Notably, thyroid cancer shows various biologic and clinical behaviors. Differentiated papillary thyroid cancer (PTC), the most common subtype of thyroid cancer, accounts for nearly 70% of the whole thyroid cancer population. PTC is usually indolent and distinct compared to other fatal undifferentiated subtypes [7, 8]. Molecular factors frequently participate in tumor cell growth, proliferation, and angiogenesis and may provide novel insight for developing precision medicine [9, 10]. Therefore, a comprehensive awareness of the molecular indicators contributing to the PTC growth and development remains urgent.

Platelet-derived growth factor receptor alpha (PDGFRA), an indispensable protein contributing to cell growth, binds to PDGF family members [11]. PDGFRA plays an essential role in regulating various biologic processes, including embryonic development, cell proliferation, and tumor progression [11, 12]. Remarkably, PDG-

FRA mutation status is used clinically for diagnosing and selecting treatment options for gastrointestinal stromal tumors (GISTs) [13, 14]. Furthermore, clinical application of PDGFRA-targeting tyrosine kinase inhibitors such as imatinib and sunitinib has provided promising results [15]. Because of the indispensable role of PDGFRA in many tumors, a detailed analysis of its role in PTC may provide novel insight. Chen et al. reported that PDGFRA mRNA and protein expression levels dramatically increased in thyroid cancer cells [16]. Additionally, single nucleotide polymorphisms in the PDGFRA promoter are jointly related to the high-risk of PTC [17], suggesting that PDGFRA may play a vital part in the growth and progression of PTC.

Herein, we observed the expression levels and its clinicopathologic implications of PDGFRA in PTC by conducting immunohistochemistry (IHC) and high throughput data mining. Furthermore, we performed functional assays to determine the relationship between PDGFRA overexpression and several malignant phenotypes of PTC cells. Finally, we performed bioinformatics analyses to determine pathways affected by PDGFRA at the whole-genome level.

Material and methods

Immunohistochemistry

Two tissue microarrays with eight cases of normal thyroid tissues, 15 cases of benign thyroid disease, and 90 cases of PTC were used for the IHC detection. IHC was performed using mouse polyclonal primary antibodies against PDGFRA and COL5A1, one of the co-expressed genes of PDGFRA (Sigma, CA, USA). Slides treated with the antibodies were reviewed and scored by two pathologists (GC and KLW) who were blinded to each other. Staining intensity was determined using an immunoreactivity scoring system (IRS) [18, 19], with 0 indicating no PDGFRA or COL5A1 expression, 1 indicating weak expression, 2 indicating medium expression, and 3 indicating strong expression. Staining rate was scored as follows: 0, no protein expression in tumor cells; 1, expression in < 10% tumor cells; 2, expression in 11%-50% tumor cells; 3, expression in 51%-80% tumor cells; and 4, expression in > 80% tumor cells. Finally, an IRS score was achieved by multiplying the staining intensity with positive staining rates. Based on the IRS score, patients with PTC were divided into -positive (IRS > 6) and -negative (IRS ≤ 6) groups for performing further analysis. The difference of protein expression levels between two groups were analyzed by student T-test and relationship between PDGFRA and COL5A1 expression levels were assessed by Spearman analysis. *P* value less than 0.05 was considered significant.

Cell culture

PTC cell line B-CPAP was cultured with Dulbecco's modified Eagle's medium (DMEM, 10-013-CVR; Corning) supplemented with 5% fetal bovine serum (FBS, VS500T; Ausbian) in a stable humidified atmosphere of 5% CO_2 at 37°C.

Packaging of lentiviruses

The PTC cell line B-CPAP was transfected with LV-PDGFRA (24987-J3) overexpression (OE) or negative control CON220 (NC) lentivirus vectors. The lentivirus titer was 2E+8 transducing units (TU)/mL for the OE group and 1E+9 TU/mL for the NC group. Next, the prepared lentiviruses were added to the B-CPAP cells. The B-CPAP cells were cultivated at a density of 4×10^5 cells/well in six-well culture plates for 12 h. After 72 h, the cells were harvested and the number of green fluorescent protein-positive cells was determined under a fluorescence microscope. Fluorescence rate was used as a positive infection rate.

Quantitative RT-PCR and western blotting analysis

Primers were designed based on PDGFRA sequence, and GAPDH was used as a control. The sequences of the designed primers were as follows: PDGFRA forward: 5'-GAACATTGTAAACTTGCTGG-3', PDGFRA reverse: 5'-GACCAAATCTCCATAGAAGC-3', GAPDH forward: 5'-TGACTTCAACAGCGACACCCA-3', and GAPDH reverse: 5'-CACCCTGTTGCTGTAGCCAAA-3'. Total RNA was extracted with Trizol reagent (Pufei Biotechnology, Shanghai, China), as manufacturer's instruction described. Complementary DNA was synthesized using M-MLV reverse transcriptase (Promega) following the manufacturer's instruction. Next, quantitative PCR was performed using SYBR master mixture (TAKARA, Beijing, China) and LightCycler 480 Real-Time PCR System (Roche Molecular Systems, Inc. Basel, Switzerland). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

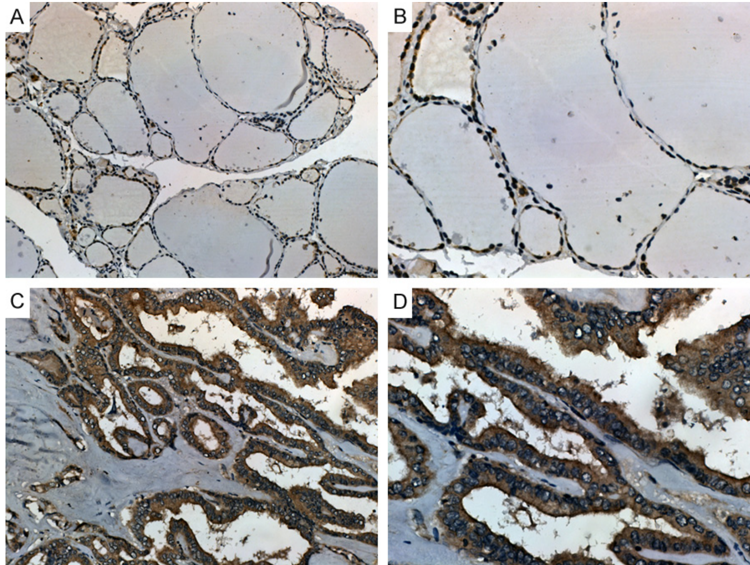

Figure 1. PDGFRA protein expression levels in non-cancerous thyroid tissues (A, B) and PTC (C, D). PDGFRA protein expression levels were detected by immunohistochemistry. Magnification: × 200 (A and C), and × 400 (B and D).

Next, PTC B-CPAP cells were lysed using RIPA lysis buffer. Total proteins were extracted, and the concentration was measured by enhanced BCA protein assay kit (Beyotime, Shanghai, China). Proteins were resolved by performing SDS-PAGE on a 10% gel and were transferred to a PVDF membrane. Next, the membrane was immunoblotted using the primary polyclonal mouse anti-PDGFRA antibody and anti-GAPDH antibody (Santa Cruz, MA, USA). Density of protein bands was determined by performing enhanced chemiluminescence with a Pierce western blotting substrate kit (Millipore, USA).

Functional assays

Cell proliferation was determined by methyl thiazolyl tetrazolium (MTT) assay. After the transfection, B-CPAP cells were seeded into 96-well plates at a density of 2000 cells/well. Next, the cells were stained with 20 μL MTT (5 mg/mL) for 4 h to produce formazan in living cells. Subsequently, the supernatants were discarded, and the cells were incubated with 150 μL DMSO. Absorbance was measured at 490 nm by using infinite M2009PR microplate reader (Tecan, Männedorf, Switzerland). Each experiment was repeated three times.

Cell cycle was detected by propidium iodide (Sigma) staining assay. Samples were analyzed using a flow cytometry analyzer (Guava easyCyte HT system; Millipore, Darmstadt, Germany).

B-CPAP cell scratch test was performed at 24 h after the transfection once the cells reached 90% confluency in a 96-well plate. A scratch was created using a sterile 200 mL micropipette tip, and the cells were incubated further in an incubator at room temperature. The wound healing area was observed in different groups and at different time points. We used an inverted microscope to photograph three randomly selected views (BD Biosciences, CA, USA).

PTC B-CPAP cells in the exponential growth phase were grown in six-well plates at a density of 500 cells/well. Subsequently, the cells were transfected with the lentivirus vectors and were cultured until they formed ideal amount of clones (50 cells/clone). Afterwards, the cells were fixed with methanol and stained with Giemsa. Finally, the clone-containing cells were directly visualized under a microscope and were counted.

Cell migration assay was performed using a 24-pore transwell chamber (3422; Corning, NY, USA). The cells were cultured for another hours at 37°C in a CO_2 incubator. The cells with serum-free DMEM were transferred to the upper chamber with Matrigel, and 600 μL 30% FBS was added to the lower chamber. Non-migrated cells were removed. Migrated cells were stained with Giemsa for 4 min and were visually counted under a microscope.

Bioinformatics analysis

Pathways associated with PDGFRA were determined by performing a series of bioinformatics analyses. Genes showing an expression pattern similar to PDGFRA in thyroid cancer tissues were obtained from an online database GEPIA (http://gepia.cancer-pku.cn/index.html) [20]. Next, gene functional enrichment analysis was performed using clusterProfiler package of R software [21]. The protein-protein interaction (PPI) was performed with those genes enriched in a selected pathway. The mRNA expression

Table 1. Relationships between PDGFRA expression and clinicopathologic features in PTC

Clinicopathological feature	*n*	PDGFRA expression		Chi square value	*P*-value
		negative	positive		
Tissues					
Non-cancerous tissue	23	20	3	13.294	< 0.001
PTC tissue	90	40	50		
Age					
< 45	50	31	19	14.042	< 0.001
≥ 45	40	9	31		
Gender					
Male	29	9	20	3.116	0.078
Female	61	31	30		
T					
T1-T2	61	24	27	5.485	0.019
T3-T4	29	6	23		
N					
N0	68	31	37	0.147	0.701
N1	22	9	13		

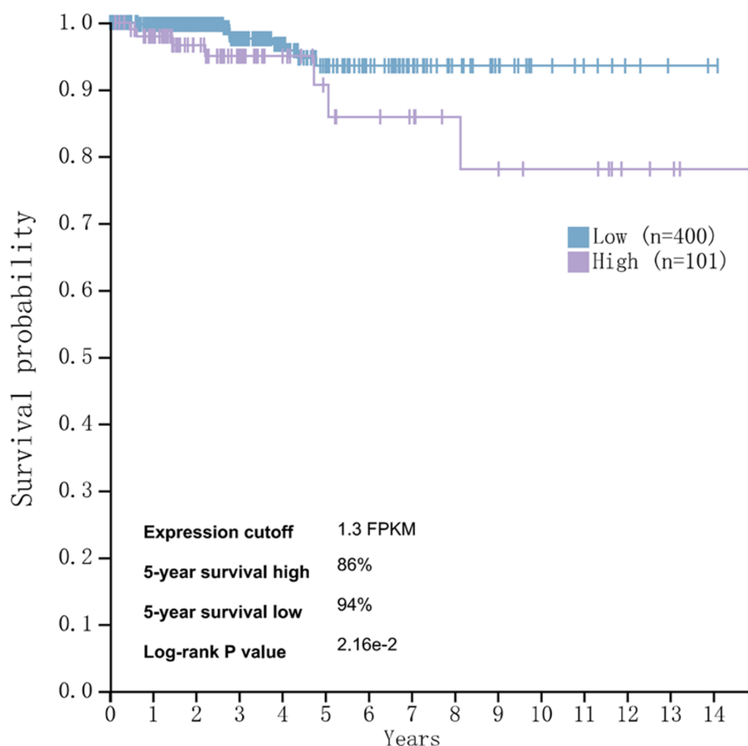

Figure 2. Survival analysis of patients with PTC according to PDGFRA expression based on RNA-sequencing data. Patients with high *PDGFRA* mRNA expression showed poor overall survival compared with patients with low *PDGFRA* mRNA expression (P = 0.0216).

levels of the PDGFRA co-expressed genes, and the relations with PDGFRA were analyzed with TCGA RNA-sequencing data. Biologically significant categories and pathways were identified when *P*-value and FDR were < 0.5.

Results

PDGFRA upregulation is related to clinical stage and survival status

This study included 29 cases of males and 61 cases of females, whose mean age was 42.6 years (range, 11-80 years). In all, 61 patients had T1 or T2 stage disease and 29 patients had T3 or T4 stage disease. Lymph node metastases were observed in 22 patients. IHC detected high PDGFRA expression in 50 out of 90 patients, and positive samples were significantly more than non-cancerous tissues (3/23, P < 0.001; **Figure 1**). Statistical analysis of the correlation between PDGFRA expression and clinicopathologic variables indicated that high PDGFRA expression was closely correlated with old age (P < 0.001) and advanced tumor stage (P = 0.019), but was not related to sex and lymph node metastasis (**Table 1**). Kaplan-Meier analysis with TCGA data revealed that the cases with higher PDGFRA expression showed a more unfavorable survival (P = 0.0216; **Figure 2**).

PDGFRA expression is associated with epigenetic changes and patient survival

By using the CBioPortal database, altered PDGFRA expression was observed in 30 (6%) out of 508 patients with thyroid cancers (**Figure 3A**), with PDGFRA mRNA upregulation being the only type of alteration. Evaluation of the association between altered PDGFRA expression and patient survival showed that patients with

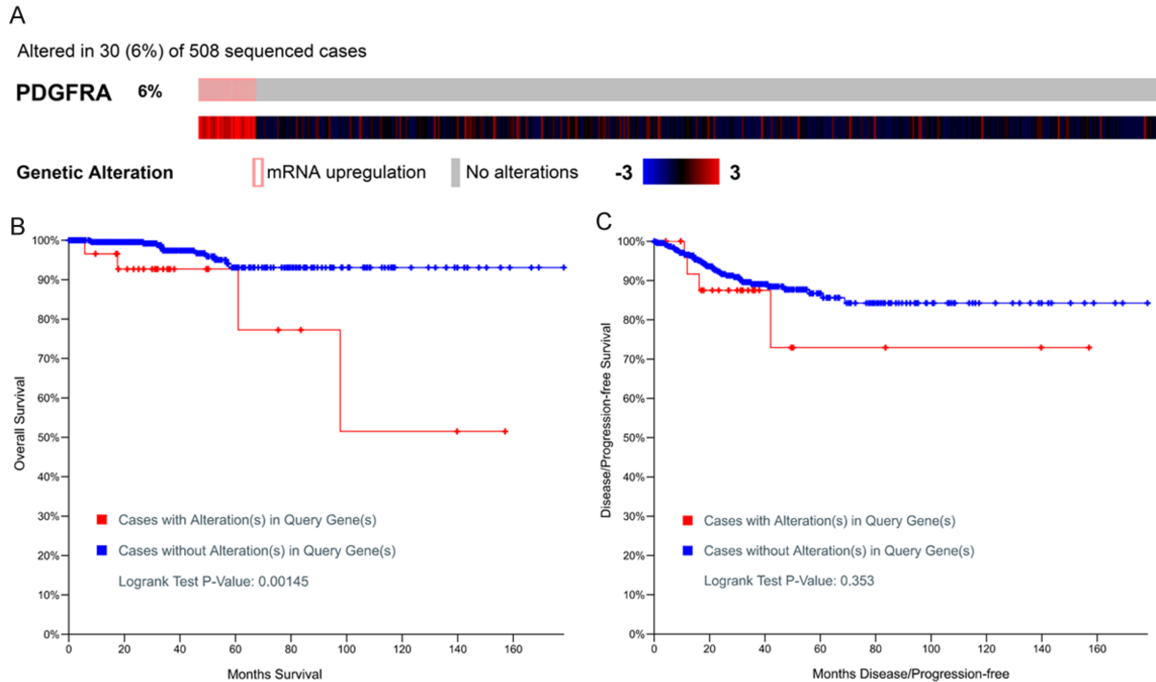

Figure 3. Relationship between altered PDGFRA expression status and patient survival based on the data from cBioPortal. A. Determination of altered PDGFRA status in 508 sequenced patients. In all, 30 out of 508 patients showed *PDGFRA* mRNA upregulation. B. Patients with *PDGFRA* mRNA upregulation showed poor overall survival. C. No difference was observed between patients with and without altered PDGFRA expression.

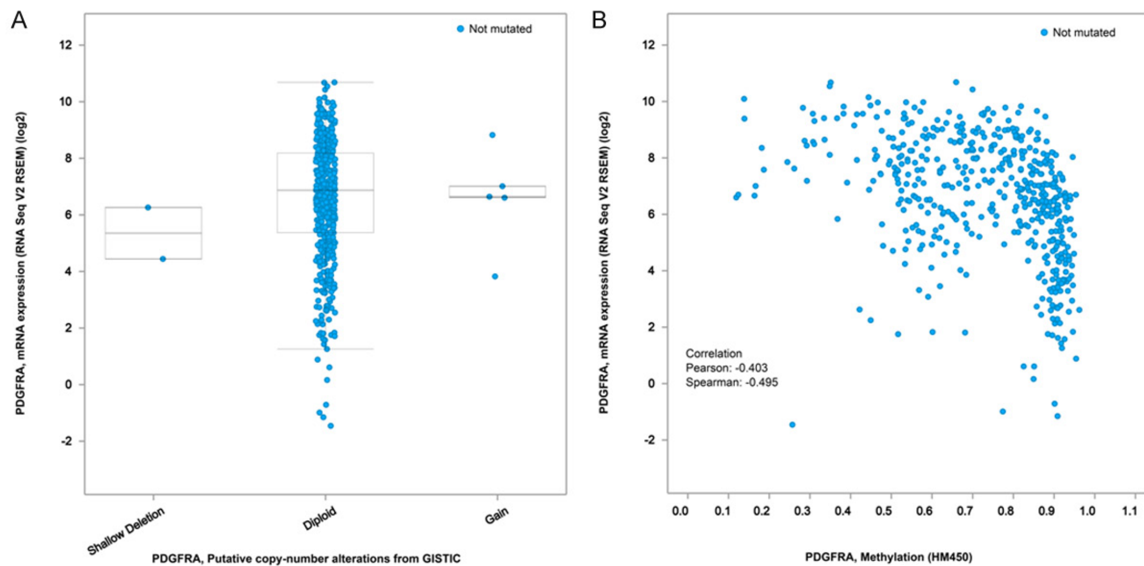

Figure 4. Relationship between PDGFRA expression levels and DNA methylation and copy-number variations based on RNA-sequencing data. A. Diploidy was the main type of copy number alteration. B. *PDGFRA* mRNA expression level was significantly inversely correlated with *PDGFRA* methylation level.

altered PDGFRA expression showed poor overall survival (**Figure 3B**). However, no difference was observed in disease-free survival between patients with and without altered PDGFRA expression (**Figure 3C**). Notably, DNA methylation level changes and copy number variations were markedly associated with altered PDGFRA mRNA expression (**Figure 4**).

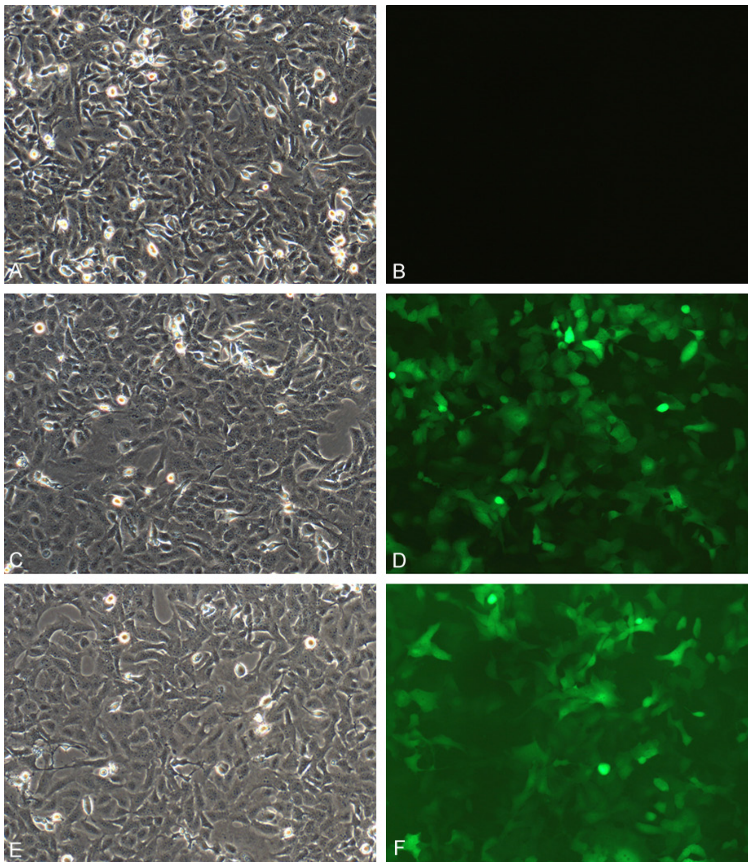

Figure 5. PDGFRA overexpression in PTC cells using the LV-PDGFRA-expressing lentivirus vector. Control group (A, B); negative group (C, D); knockdown group (E, F). Light microscope (A, C, E); Fluorescent microscope (B, D, F), Magnification: × 400.

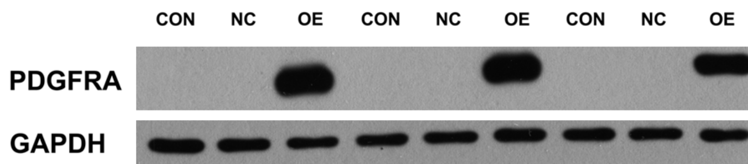

Figure 6. PDGFRA protein expression level in control, negative control, and overexpression groups. Western blot analysis demonstrating the overexpression of PDGFRA protein in transfected PTC cells.

PDGFRA upregulation accelerates PTC cell migration

The siRNA targeting PDGFRA was transfected into B-CPAP cells. At 24 h after the transfection of the lentivirus vectors, green fluorescence was detected using an inverted fluorescence microscope, which indicated successful transfection (**Figure 5**). Quantitative RT-PCR disclosed that PDGFRA mRNA expression levels were markedly higher in B-CPAP cells transfected with the OE lentivirus vector than in those transfected with the CON and NC lentivirus vectors. Furthermore, western blot displayed consistently that PDGFRA protein level rose significantly in the B-CPAP cells transfected with PDGFRA compared to controls (**Figure 6**). These data indicated that PDGFRA transfection efficiency was high. PDGFRA overexpression significantly promoted the migration of both B-CPAP cells in the transwell chamber (**Figure 7**). These results suggest that PDGFRA upregulation accelerates the migration and invasion of PTC cells. However, results of the MTT, wound healing, cell cycle, and colony-forming assays showed no difference between B-CPAP cells in the NC or control and OE groups.

Potential molecular functions of PDGFRA

To study the biologic functions of PDGFRA in PTC, PDGFRA-related genes were sent to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. Results of these analyses showed that “extracellular structure organization”, “extracellular matrix organization”, and “skeletal system development” were the most significantly enriched biologic processes (**Figure 8A**); “proteinaceous extracellular matrix”, “extracellular matrix component”, and “endoplasmic reticulum lumen” were the most significantly enriched cellular components (**Figure 8B**); and “glycosaminoglycan binding”, “extracellular matrix structural constituent”, and “sulfur compound binding” were the most significantly enriched molecular functions of the PDGFRA-related genes (**Figure 8C**). Moreover, “phagosomes” was the most significantly enriched KEGG pathway of the PDGFRA-related genes. Interestingly, several essential pathways involved in PTC, such as ECM-receptor interaction, were also

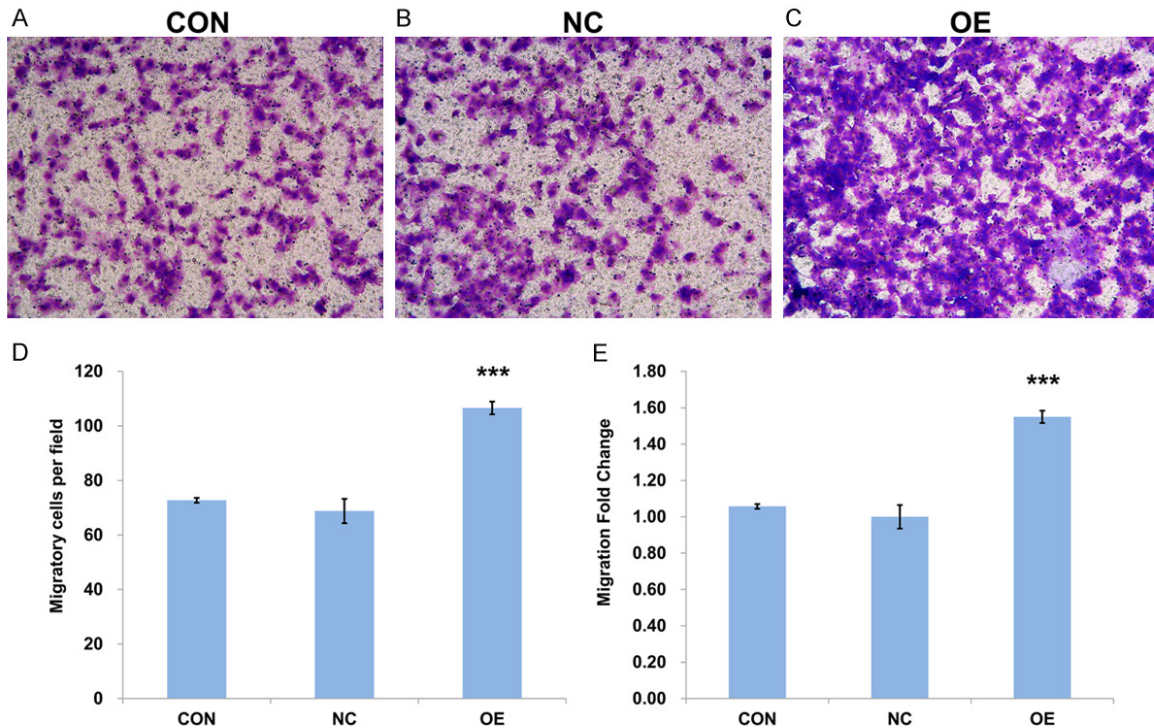

Figure 7. PDGFRA overexpression elevated the migration capacity of PTC cells. PTC cells in control (A), negative control (B), and overexpression groups (C) were treated with melatonin for 24 h and were analyzed by performing the transwell matrix penetration assay. Quantification of migratory cells per field (D) and migration fold change (E) in the three groups. Magnification: × 400.

enriched significantly (**Figure 8D**). We selected the pathway of extracellular matrix components to show the PPI (**Figure 9**), including 14 genes: DCN, SERPINF1, LUM, THBS2, LAMA2, FBLN1, COL6A3, ELN, FBLN5, MFAP5, MFAP4, COL3A1, COL5A2, and COL5A1 (**Figures 10-12**). The up-regulated protein level of one candidate, COL5A1, was also confirmed by in-house immunohistochemistry (**Figure 13**). There is also a positive correlation between the expression levels of PDGFRA and COL5A1, supporting that COL5A1 was indeed co-expressed with PDGFRA in THCA tissues. These results indicate that PDGFRA is potentially involved in cell proliferation, binding, and migration.

Discussion

PDGFRA performs various important clinical roles; however, its role in PTC oncogenesis and progression is poorly understood. PDGFRA mutation status can be used as a marker for diagnosing and predicting the survival of patients with GISTs [22, 23]. In this study, we carried out IHC to examine high PDGFRA expression in PTC tissues compared to non-cancerous thyroid, and found that PDGFRA upregulation was related to advanced tumor TNM clinical stage. Furthermore, analysis of the TCGA dataset showed that patients with high PDGFRA expression showed poor overall survival. Moreover, we found that PDGFRA overexpression at the transcriptional level enhanced PTC cell migration. Interestingly, subsequent bioinformatics analyses based on big data showed that PDGFRA significantly affected several cell proliferation and migration pathways. These findings indicate the potential diagnostic and prognostic value of PDGFRA and highlight possible molecular mechanisms, thus providing novel insights into the clinical management and molecular mechanisms of PTC.

The mechanism of action and clinical relevance of PDGFRA in PTC clinical samples remain unknown. Prominently, studies have revealed that PDGFRA is activated in and can act as a latent therapeutic target in several malignancies [24, 25]. Determination of the role of PDGFRA in PTC may provide new horizons for developing treatment strategies. Clinical specimens obtained from patients with PTC showed

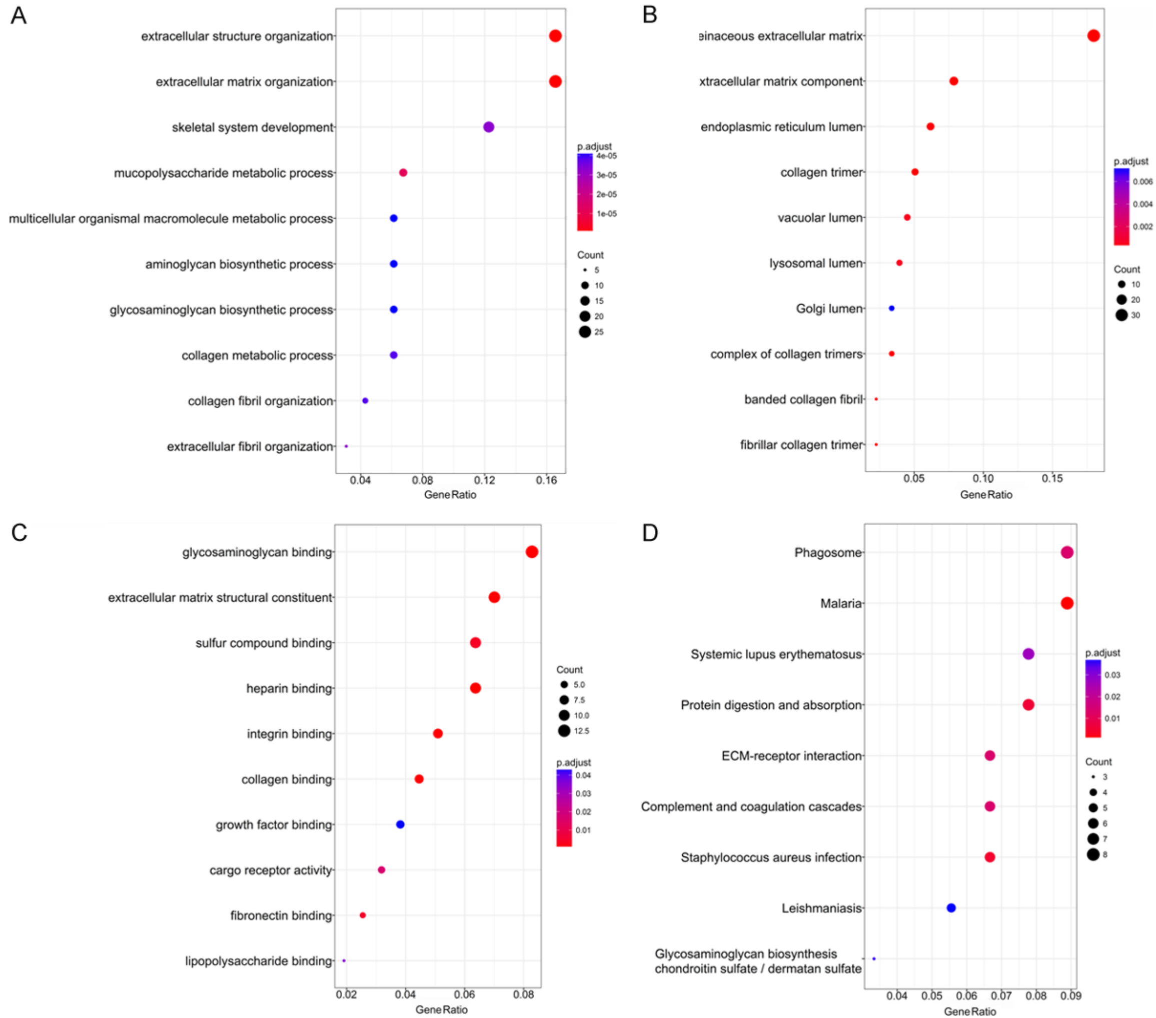

Figure 8. Functional enrichment GO and KEGG pathways of PDGFRA-related genes generated from RNA-sequencing data. (A) Biologic processes, (B) cellular components, (C) molecular functions, and (D) KEGG pathways.

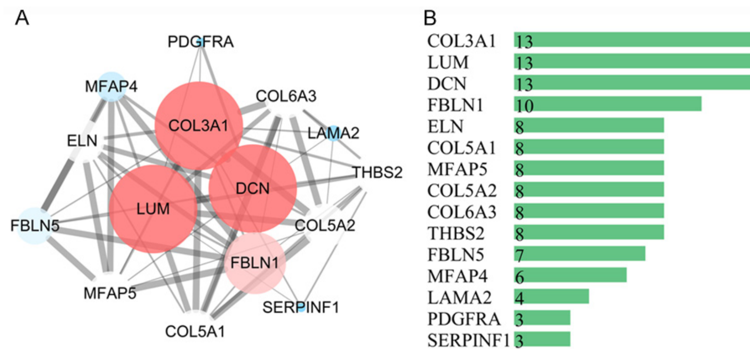

Figure 9. Protein-protein interaction (PPI) network of PDGFRA related genes enriched in extracellular matrix component pathways. A. PPI network was generated using STRING online tool and Cytoscape software. B. Plots of genes included in the PPI network. The number in each block represents the frequency of gene connection with other nodes.

high PDGFRA expression, suggesting the crucial role of PDGFRA in PTC onset. Previous studies have shown the same expression pattern of PDGFRA in several other cancers, including liver [26], prostate [27], oral [28], and colorectal cancers [29]. Interestingly, high PDGFRA expression is correlated with advanced tumor stage, suggesting its involvement in PTC progression and its importance for designing novel inhibitors for treating advanced PTC. PDGFRA expression is elevated in PTC showing lymph node metastasis compared to that in PTC not showing lymph node metastasis [30]. However, we did not observe this in the present study probably because of the

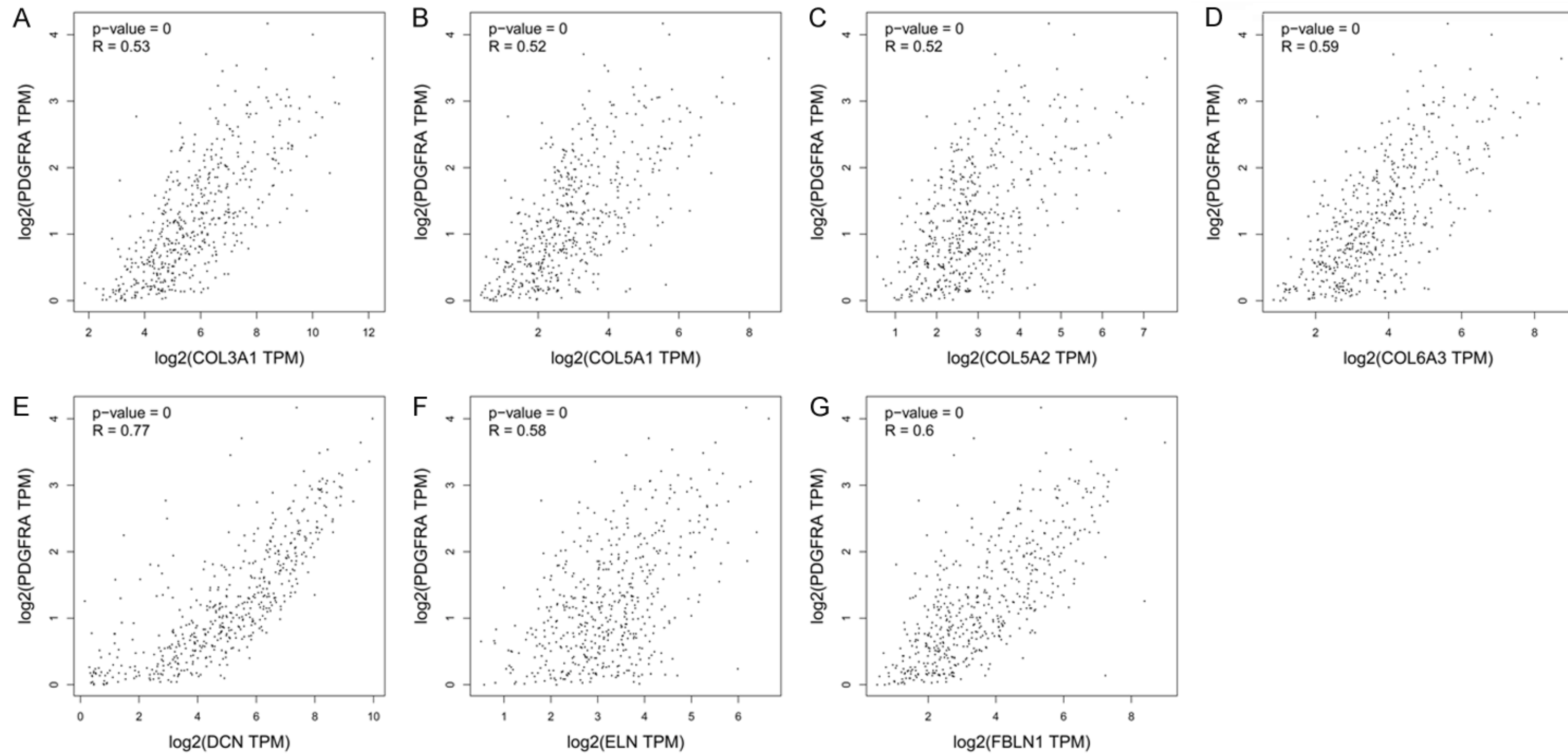

Figure 10. Scatter plots show the correlations between PDGFRA expression and the genes involved in the extracellular matrix component pathways. A. COL3A1; B. COL5A1; C. COL5A2; D. COL6A3; E. DCN; F. ELN; G. FBLN1.

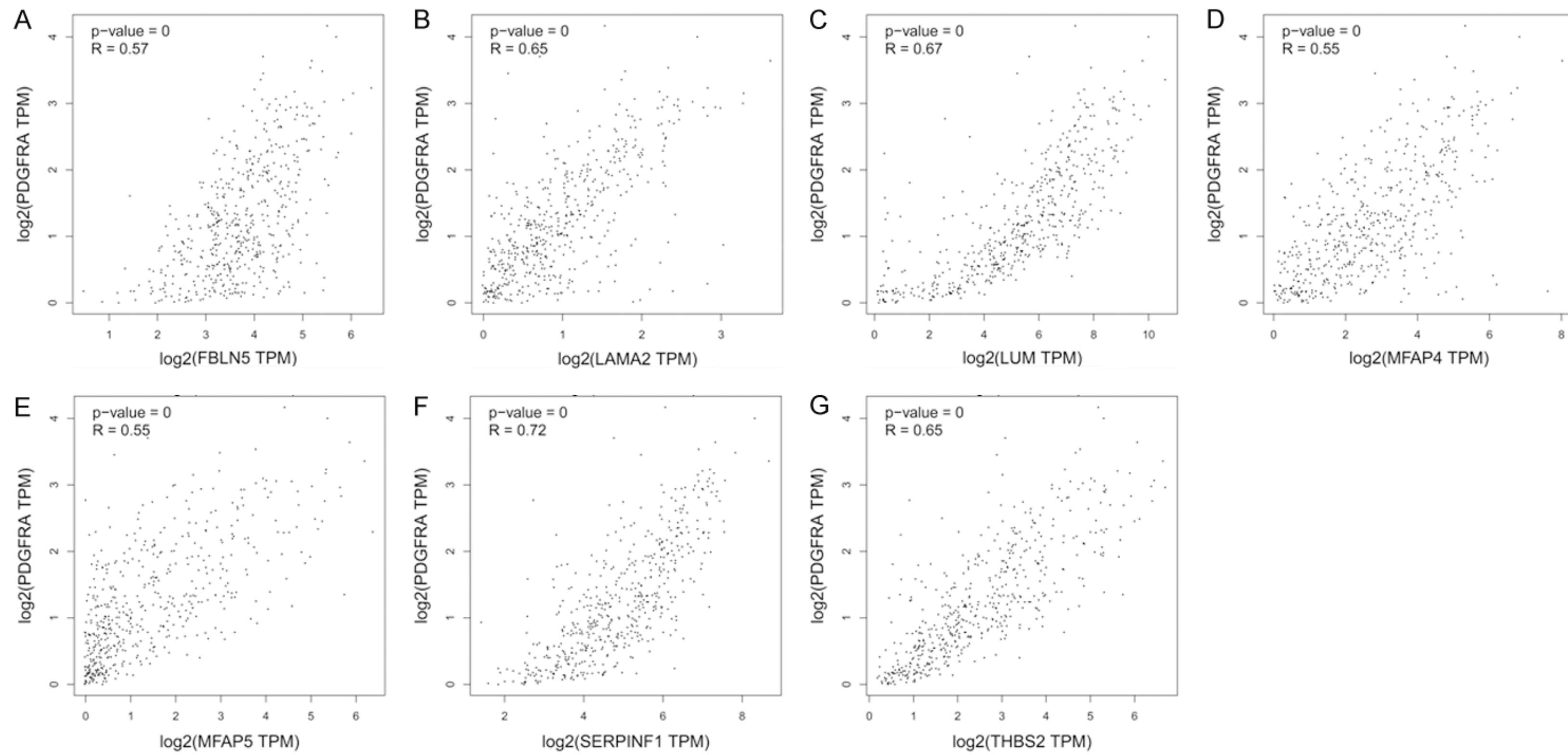

Figure 11. Scatter plots show the correlations between PDGFRA expression and another seven genes involved in the extracellular matrix component pathways. A. FBLN5; B. LAMA2; C. LUM; D. MFAP4; E. MFAP5; F. SERPINF1; G. THBS2.

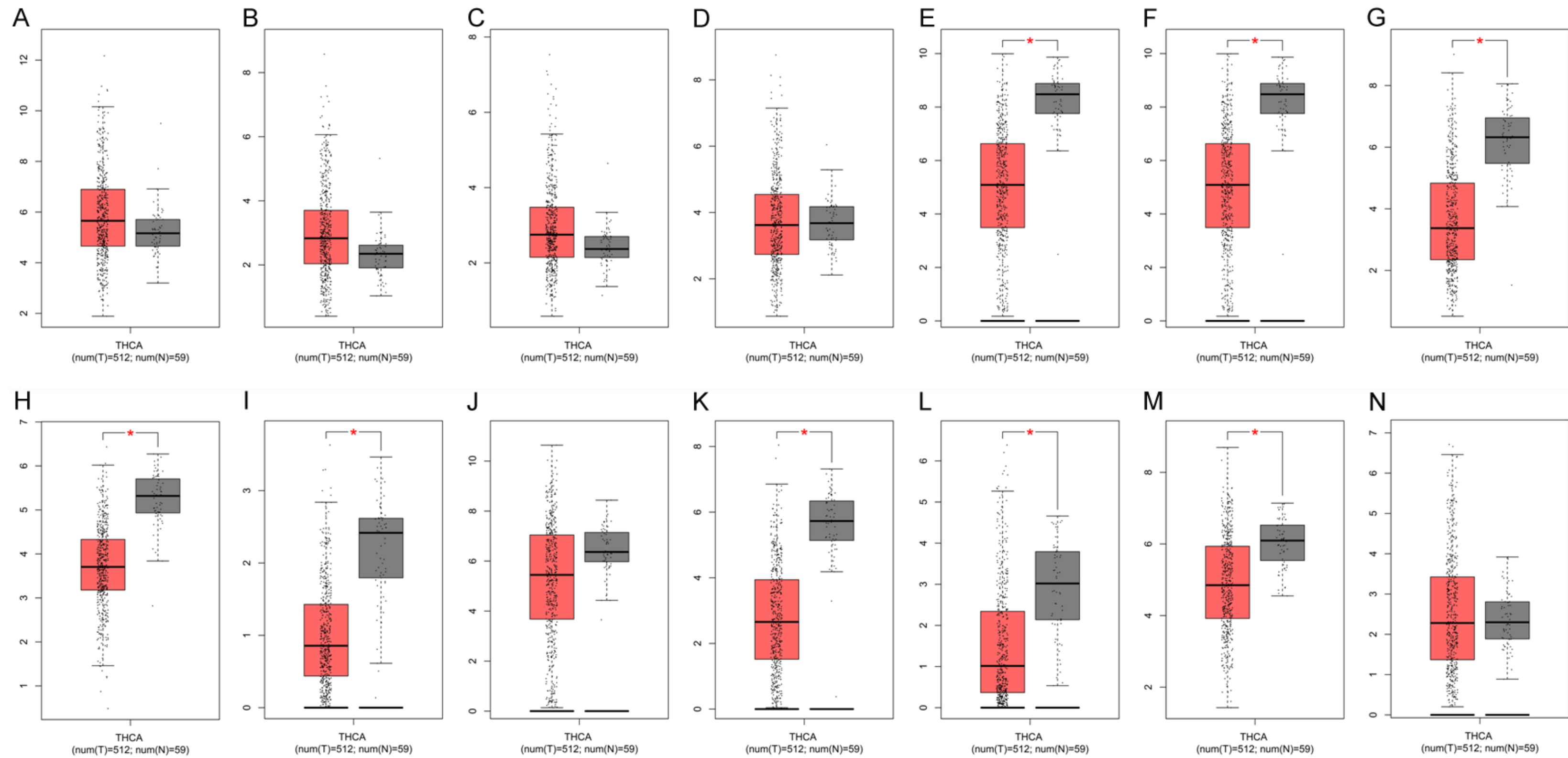

Figure 12. Different expression levels of genes involved in the extracellular matrix component pathways based on RNA-sequencing data. A. COL3A1; B. COL5A1; C. COL5A2; D. COL6A3; E. DCN; F. ELN; G. FBLN1; H. FBLN5; I. LAMA2; J. LUM; K. MFAP4; L. MFAP5; M. SERPINF1; N. THBS2.

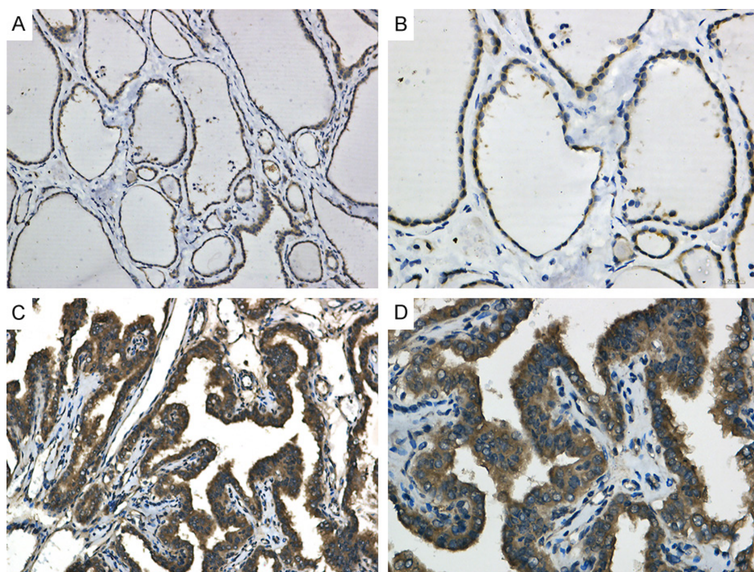

Figure 13. COL5A1 protein expression levels in non-cancerous thyroid tissues (A, B) and PTC (C, D). The COL5A1 protein expression levels were detected by immunohistochemistry. Magnification: × 200 (A and C), and × 400 (B and D).

use of a limited number of samples. Therefore, future studies involving a large sample size will yield more accurate results. As expected, PDGFRA overexpression showed a moderate ability to predict the poor survival of patients with PTC, as determined by analyzing the TCGA dataset. Because PDGFRA mutation status is commonly used clinically for diagnosing and predicting the survival of cancer patients, especially patients with GISTs [31, 32], we determined the relationship between altered PDGFRA expression and patient survival. As expected, patients with altered PDGFRA expression showed poor survival. Moreover, PDGFRA mRNA expression level can be modulated through DNA methylation. Although a few studies have assessed the molecular mechanism of PDGFRA in PTC development at multiple levels, no study has evaluated the role of epigenetic modulation. These observations suggest that PDGFRA contributes to the maintenance of a tumor-invasive phenotype and promotes cancer development by maintaining the migration capacity of tumor cells.

In the present study, we verified the functional phenotype of PDGFRA in PTC cells by performing a series of functional assays. Results of *in vitro* experiments showed that elevated PDGFRA expression promoted PTC cell migration, suggesting that PDGFRA enhanced the invasive potential of PTC cells. These discoveries are consistent with the clinical implication of PDGFRA. However, the biologic functions of PDGFRA underlying the elevated invasive capacity of PTC cells are largely unknown. Clinical data of PTC, including tumor infiltration, reflect the invasive potential of PTC. We analyzed the functional phenotypes of PDGFRA in PTC to obtain novel insights into its oncogenic role *in vitro*.

Our findings obtained using clinical samples and by performing *in vitro* experiments suggest that PDGFRA acts as a facilitator in PTC. PDGFRA is suggested to exert its oncogenic effect through various mechanisms. Genes mainly exert their biologic effect by affecting or interacting with other molecules. Therefore, we used PDGFRA-related genes to perform functional enrichment analysis. Our results suggest that PDGFRA exerts its effect through several pathways such as the ECM-receptor interaction pathway, which involves SV2, collagen, and HLA. Moreover, all these genes participate in tumor invasion and migration [33-35]. Moreover, we found that PDGFRA may participate in the formation of cellular components. These findings suggest that PDGFRA is mainly involved in PTC cell proliferation and migration.

Conclusion

Collectively, our findings suggest that elevated PDGFRA expression promotes the proliferation and metastasis of and increases the invasive potential of PTC cells. Moreover, our findings suggest that PDGFRA enhances PTC cell migration through several cancer-related pathways and that PDGFRA expression can be modulated through epigenetic regulation.

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Disclosure of conflict of interest

None.

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