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Overexpression of G Protein-Coupled Receptor Kinase 6 (GRK6) Is Associated with Progression and Poor Prognosis of Papillary Thyroid Carcinoma

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Background: Approximately 20% of patients with papillary thyroid carcinoma (PTC) will develop cancer recurrence, but no clinically available biomarker has been identified. Our study aimed to evaluate the prognostic value of G protein-coupled receptor kinase 6 (GRK6) in PTCs.

Material/Methods: We retrospectively enrolled 108 PTC patients in this study, and explored the expression of GRK6 in resected tumor samples by RT-qPCR and immunohistochemistry (IHC). The clinical data were interpreted by chi-square test, univariate analysis, and multivariate analysis. To investigate the functional mechanisms of GRK6 in regulating PTC progression, we also performed overexpression and silencing experiments in TPC-1 cells, a cell line generated from PTC tissues.

Results: RT-qPCR results showed a higher level of GRK6-mRNA in PTCs than in adjacent thyroid tissues. IHC revealed a distinct protein expression pattern of GRK6 among PTCs. Accordingly, we classified patients into low-GRK6 and high-GRK6 groups. The chi-square test showed that a higher GRK6 was associated with larger tumor size ($P=0.045$) and advanced TNM stage ($P=0.001$). Kaplan-Meier survival curve and log rank test demonstrated that higher GRK6 predicted poor disease-free survival (DFS) in PTC patients ($P=0.002$). Furthermore, Cox regression analysis confirmed that GRK6 was an independent prognostic factor for a higher recurrence risk of PTCs ($P=0.047$). MTT assay and Transwell assay demonstrated that GRK6 overexpression can significantly enhance tumor proliferation and invasion, which was consistent with clinical findings.

Conclusions: Our data show the oncogenic effects of GRK6 in promoting PTC progression.

MeSH Keywords: **Cell Proliferation • Multiple Endocrine Neoplasia Type 2a • Prognosis**

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Background

Thyroid cancer is the most common endocrine malignancy, and more than 80% is papillary thyroid cancer (PTC) [1]. The overall survival for most PTC patients is satisfactory, but its general mortality is still higher than other endocrine neoplasms. Surgical resection is the most effective therapy for PTCs, ranging from thyroid lobectomy to a total thyroidectomy [2]. However, even after surgical treatment, local metastases and recurrence are observed in many patients [3,4]. Classification of these high-risk patients is helpful for clinical decision-making, such as resection extent, postoperative treatment, and prognosis prediction. At present, the most clinically applicable predictor for PTC recurrence is the status of regional lymph nodes [5], but increasing attention is being focussed on investigating novel biomarkers at the gene or protein level, which will not only help predict prognosis, but also direct therapy development.

G-protein-coupled receptor kinases (GRKs) are kinases that predominantly phosphorylate G protein-coupled receptors (GPCRs) [6]. Upon ligand stimulation, GRKs quickly phosphorylate GPCRs, leading to receptor desensitization and internalization [7]. From the aspect of physiology, GRKs exert critical regulatory functions in neurodevelopment [8], cardiovascular efficiency [9], and immunoreaction [10]. Accumulating evidence suggests that GRKs also participate in malignancy development [11,12]. Specifically, the function of GRK6 was shown to be suppressed in medulloblastoma by Src signaling, leading to enhanced tumor cell migration [13]. In addition, GRK6-knockout mice showed a higher risk of lung cancer metastasis [14], indicating the role of GRK6 in suppressing tumor progression. However, another study reported that GRK6 was highly expressed in hepatocellular carcinoma and was correlated with poor prognosis [15]. Therefore, GRK6 may play distinct roles in different tumor types.

The present study is the first to investigate the expression profile of GRK6 in PTCs. We found that GRK6 was up-regulated in PTCs, which was correlated with tumor size and lymph node metastasis. Also, high expression of GRK6 can independently indicate a high risk of PTC recurrence, as reflected by univariate and multivariate analyses. In addition, cellular results showed that GRK6 overexpression can directly enhance cell proliferation and invasion, whereby its siRNA silencing inhibits tumor progression. Our data reveal the role of GRK6 in predicting clinical outcomes and its potential as a novel drug target for PTCs.

Material and Methods

Patients and samples

A total of 108 PTC patients diagnosed and surgically treated in Weihai Hospital of Traditional Chinese Medicine from

June 2009 to June 2016 were enrolled in this study. All the patients were confirmed as having PTC by pathologically examination [16]. None of the patients had received chemotherapy or radiotherapy before surgery. All the patients underwent therapeutic total or near-total thyroidectomy. This study was approved by the Ethics Committee of Weihai Hospital of Traditional Chinese Medicine, and written informed consents were obtained from all patients. Clinical information was retrieved for all patients (Table 1). Fresh-frozen PTC tissues and adjacent tissues were obtained from our hospital and stored in liquid nitrogen until use.

RT-qPCR

Total RNA was isolated from 23 pairs of PTC tissues and adjacent thyroid tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After quantified by Nanodrop, 1 µg of total RNA was reverse-transcribed into cDNA using the SuperScript cDNA system (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was conducted to test the mRNA level of GRK6 using SYBR Green PCR master mix (Applied Biosystems) with the following primers: sense, 5'-TTTGGCTGATGGTCTGTTC-3'; antisense, 5'-CGCTGCAGTCCACAGCAATC-3' [17]. Gene expression levels were evaluated by normalization against the housekeeping gene *GAPDH* (sense, 5'-TGCACCACCAACTGCTTAGC-3'; antisense, 5'-GGCATGGACTGTGGTCATGAG-3') using the $2^{-\Delta\Delta CT}$ method [18]. Data are presented as mean \pm SD from 3 independent experiments.

Immunohistochemistry (IHC)

Paraffin sections (4 µm) of the PTC tissues were deparaffinized and dehydrated with graded ethanol. After blocking the endogenous peroxidase with 3.0% H₂O₂, slides were incubated with citric acid buffer to retrieve antigen by heating in 120°C for 15 min. The slides were then blocked with 5% BSA for 1 h and incubated with primary rabbit polyclonal anti-GRK6 antibody (C20, Santa Cruz Biotechnology) overnight at 4°C. After that, slides were washed with PBS and further incubated with biotinylated second antibody for 15 min at room temperature. Finally, the slides were stained using the VECTASTAIN Elite ABC HRP kit and DAB reagents. For negative control, PBS was used instead of primary antibody.

IHC evaluation

We asked 2 independent pathologists blinded to this study to help us perform the IHC evaluation. The expression was calculated based on both the percent of positive cells and staining intensity, as described by others [19]. Briefly, the positivity percentage was scored as 0 (0–10%), 1 (11–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The staining intensity was scored as 0 (no immunoreactivity), 1 (weakly stained), 2 (moderate stained), and 3 (strong stained). The final IHC score (IHCS) was

Table 1. Characteristics of the PTC patients and associations with GRK6 expression level.

Variable	Cases (n=108)	GRK6 expression		P value
		Low (n=61)	High (n=47)	
Age (years)				0.108
≤46 yrs	48	23	25	
>46 yrs	60	38	22	
Gender				0.448
Female	86	47	39	
Male	22	14	8	
Tumor size				0.045*
≤2.0 cm	71	45	26	
>2.0 cm	37	16	21	
ETE				0.527
Negative	56	30	26	
Positive	52	31	21	
TCI				0.118
Negative	73	45	28	
Positive	35	16	19	
LN metastasis				0.233
Negative	84	50	34	
Positive	24	11	13	
TNM stage				0.001*
I-II	75	50	25	
III-IV	33	11	22	

ETE – extrathyroidal extension; TCI – thyroid capsular invasion; LN – lymph node. * P<0.05.

weighted by multiplying the intensity score with percentage score (range, 0–12). Patients were classified into a GRK6 low expression group (IHCS ≥6) and a high expression group (IHCS <6) according to the IHCS.

Cell culture and transfection

The human PTC cell line, TPC-1, was cultured in DMEM supplemented with 10% FBS in a 5% CO₂ atmosphere at 37°C. For the transfection, pRK5-GRK6 plasmid was purchased from Addgene (#32693) and GRK6-siRNA was synthesized by Santa Cruz Biotechnology (AACAGUAGGUUUGUAGUGAGC, position 724-744). Both overexpression and silencing were conducted using Fugene-6 (Roche) in OptiMEM (Invitrogen) for 48 h. The transfection efficiency was confirmed by Western blot.

Western blot

For protein expression analysis, transfected cells were homogenized in radioimmune precipitation assay (RIPA) buffer

at 4°C for 30 min. After centrifugation at 13 000 rpm for 20 min, the protein concentration in supernatant was tested using a Pierce BCA kit (Thermo Fisher Scientific). Approximately 20 µg of total proteins were resolved on 12% SDS-PAGE, blotted to PVDF membrane, and probed with primary antibodies, followed by incubating with HRP-conjugated secondary antibodies, and visualized by adding ECL reagent.

Proliferation assay

The cell viability assay was achieved using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, transfected TPC-1 cells were seeded at 4×10³ cells/well in 96-well plates in triplicate and cultured at 37°C. At designated time points (1, 2, 3, and 4 days), 10 µl of Cell Counting Kit-8 reagent was added into the well and incubated for another 3 h. The absorbance of each well was measured at 450 nm under a Bio-Rad microplate reader. Each experiment was performed 3 times.

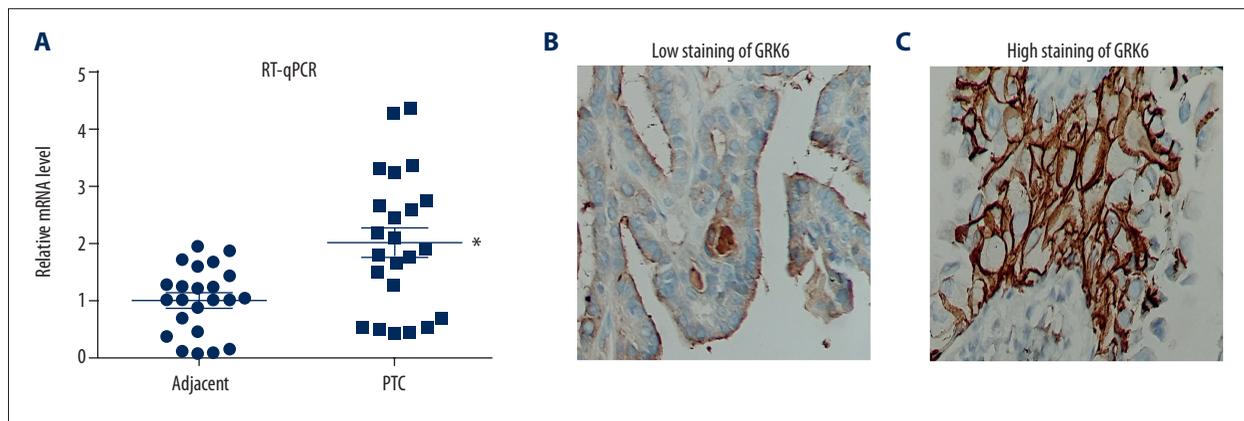


Figure 1. GRK6 was up-regulated in PTCs. (A) RT-qPCR results from 23 matched PTCs and adjacent thyroid tissues showed an elevated RNA level of GRK6 in PTCs ($P=0.004$). IHC images showed representative low expression (B) and high expression (C) of GRK6 protein in PTC cells, especially in the cytoplasm.

Invasion assay

After being coated with Matrigel (BD Biosciences, San Jose, CA), transfected TPC-1 cells were seeded into the upper chamber of the Transwell apparatus (8.0 μm , Corning, NY, USA) at a density of 5×10^4 cells/chamber (200 μl serum-free medium). Another 800 μl of medium supplemented with 20% serum was added to the lower chamber to induce cell invasion. After we had cultured the cells at 37°C for 36 h, the cells on the upper side of the membrane was gently removed with cotton swabs. Invaded cells on the lower side of the membrane were fixed and stained with 0.5% crystal violet. Cells were counted under an inverted light microscope (Olympus) in 5 randomly selected visual fields. The experiment was performed 3 times independently.

Statistical analyses

Data are presented as mean \pm SD, and all statistical analyses were performed using SPSS software (version 16.0; Chicago, IL, USA). Correlations between GRK6 expression and patients' clinical parameters were calculated by the Pearson chi-square test. Univariate survival analysis was performed by Kaplan-Meier method and tested with the log-rank test. Cox regression modelling was used to investigate the independent effect of prognostic factors. For the cellular results, the difference was compared by Student's t -test. $P < 0.05$ was considered statistically significant.

Results

Patients information

This study included 108 PTC patients with a mean age of 49.7 years (range, 20–76 years). As shown in Table 1, most of the

patients were female (86/108, 79.63%). Seventy-one patients (71/108, 65.74%) had a tumor diameter smaller than 2.0 cm, while the other 37 patients (37/108, 34.26%) had a tumor diameter larger than 2.0 cm. Fifty-six patients (56/108, 51.85%) were diagnosed without extrathyroidal extension (ETE), and the other 52 patients (52/108, 48.15%) had positive ETE. The status of thyroid capsular invasion (ETI) was also evaluated; about one-third of patients (35/108, 32.41%) had positive ETI. Most of the patients (84/108, 77.78%) had negative regional lymph nodes. Seventy-five (75/108, 69.44%) patients were classified as TNM stage I/II, and the other 33 (33/108, 30.56%) had TNM stage III/IV. The 5-year disease-free survival rate of the entire cohort was 84.22%.

GRK6 expression pattern and its associations with clinicopathological characteristics

We first assessed the transcription level of *GRK6* by RT-qPCR in 23 pairs of PTC and adjacent thyroid tissues. In 17 patients (17/23, 73.91%), *GRK6*-mRNA showed a higher level in PTC tissues than in adjacent normal tissues. The mean *GRK6*-mRNA level was 2.7 times higher in PTC tissues (Figure 1A), and the protein expression of *GRK6* was predominantly identified in the cytoplasm of tumor cells (Figure 1B, 1C).

According to the staining percentage and staining intensity, we classified patients into low-*GRK6* and high-*GRK6* groups (Table 1). By chi-square testing, we found that *GRK6* was positively correlated with the tumor size ($P=0.045$) and TNM stage ($P=0.001$). No significant association was observed between *GRK6* expression and other parameters.

GRK6 is a novel biomarker indicating high recurrence risk

Since higher *GRK6* was observed more frequently in PTCs with larger tumor size and advanced TNM stages, we suspected

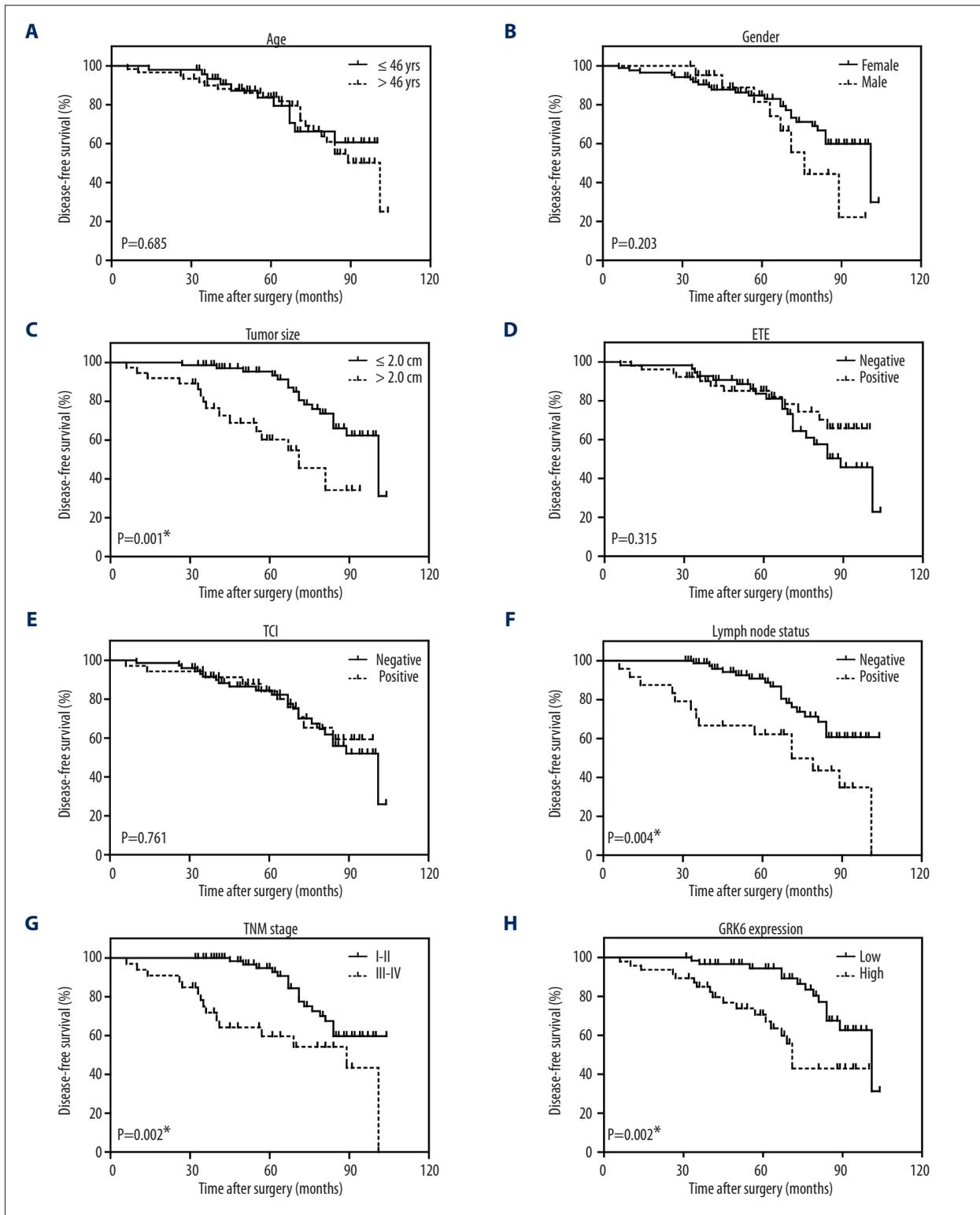


Figure 2. Kaplan-Meier survival analyses of the disease-free survival of PTC patients. All statistical significance was tested by log-rank test. * P<0.05.

Table 2. Univariate analysis for the disease-free survival of PTC patients.

Variable	Cases (n=158)	Disease-free survival		P value
		Mean ±SD (months)	5-year (%)	
Age (years)				0.685
≤46 yrs	48	83.3±4.2	83.6%	
>46 yrs	60	82.7±3.6	84.0%	
Gender				0.203
Female	86	84.9±3.2	84.7%	
Male	22	76.5±5.1	81.5%	
Tumor size				<0.001*
≤2.0 cm	71	90.6±2.6	95.3%	
>2.0 cm	37	65.6±5.3	60.2%	
ETE				0.315
Negative	56	82.1±3.6	83.7%	
Positive	52	84.6±3.9	85.1%	
TCI				0.761
Negative	73	83.6±3.3	84.5%	
Positive	35	82.2±4.6	84.0%	
LN metastasis				0.004*
Negative	84	89.4±2.8	90.8%	
Positive	24	67.0±7.3	62.2%	
TNM stage				0.002*
I-II	75	90.6±2.6	94.8%	
III-IV	33	69.9±6.5	59.6%	
GRK6 expression				0.002*
Low	61	91.8±2.7	94.4%	
High	47	71.7±4.8	70.6%	

ETE – extrathyroidal extension; TCI – thyroid capsular invasion; LN – lymph node. * P<0.05.

it may help predict patients' clinical outcomes. We therefore analyzed the prognostic factors for disease-free survival (DFS) in PTCs using Kaplan-Meier method (Figure 2, Table 2). A larger tumor size (P<0.001), positive lymph node metastasis (P=0.004), and advanced TNM stage (P=0.002) were shown as unfavorable factors affecting DFS. Importantly, patients with low GRK6 expression showed a better prognosis than those with high GRK6 expression (DFS 91.8±2.7 months vs. 71.7±4.8 months, P=0.002).

Furthermore, we analyzed tumor size, lymph node metastasis, TNM stage, and GRK6 expression in a Cox regression multivariate analysis model to explore the independent hazard effect of each factor (Table 3). Although lymph node metastasis showed no statistical significance, tumor size (HR 3.146,

95% CI 1.362–7.267, P=0.007), TNM stage (HR 2.423, 95% CI 1.147–5.117, P=0.020), and GRK6 expression (HR 2.132, 95% CI 1.009–4.507, P=0.047) were all independently correlated with disease recurrence.

GRK6 directly promotes PTC cell proliferation and invasion

We performed cellular studies to investigate the underlying mechanisms of GRK6 in prompting PTC progression. We transfected TPC-1 cells with GRK6 plasmid or GRK6 siRNA (Figure 3A, 3B). After we confirmed the transfection efficiency by Western blot, the cells were tested by MTT assay to evaluate cell viability, which showed a significantly enhanced proliferation pattern in GRK6-overexpressing cells. In contrast, cells transfected with GRK6 siRNA exhibited decreased cell viability (Figure 3C). We

Table 3. Multivariate analysis for the disease-free survival of PTC patients.

Variable	Hazard ratio	95% CI	P value
Tumor size (vs. ≤ 2.0 cm)	3.146	1.362–7.267	0.007*
LN metastasis (vs. negative)	1.455	0.668–3.172	0.345
TNM stage (vs. I–II)	2.423	1.147–5.117	0.020*
GRK6 expression (vs. low)	2.132	1.009–4.507	0.047*

LN – lymph node; CI – confidence interval. * $P < 0.05$.

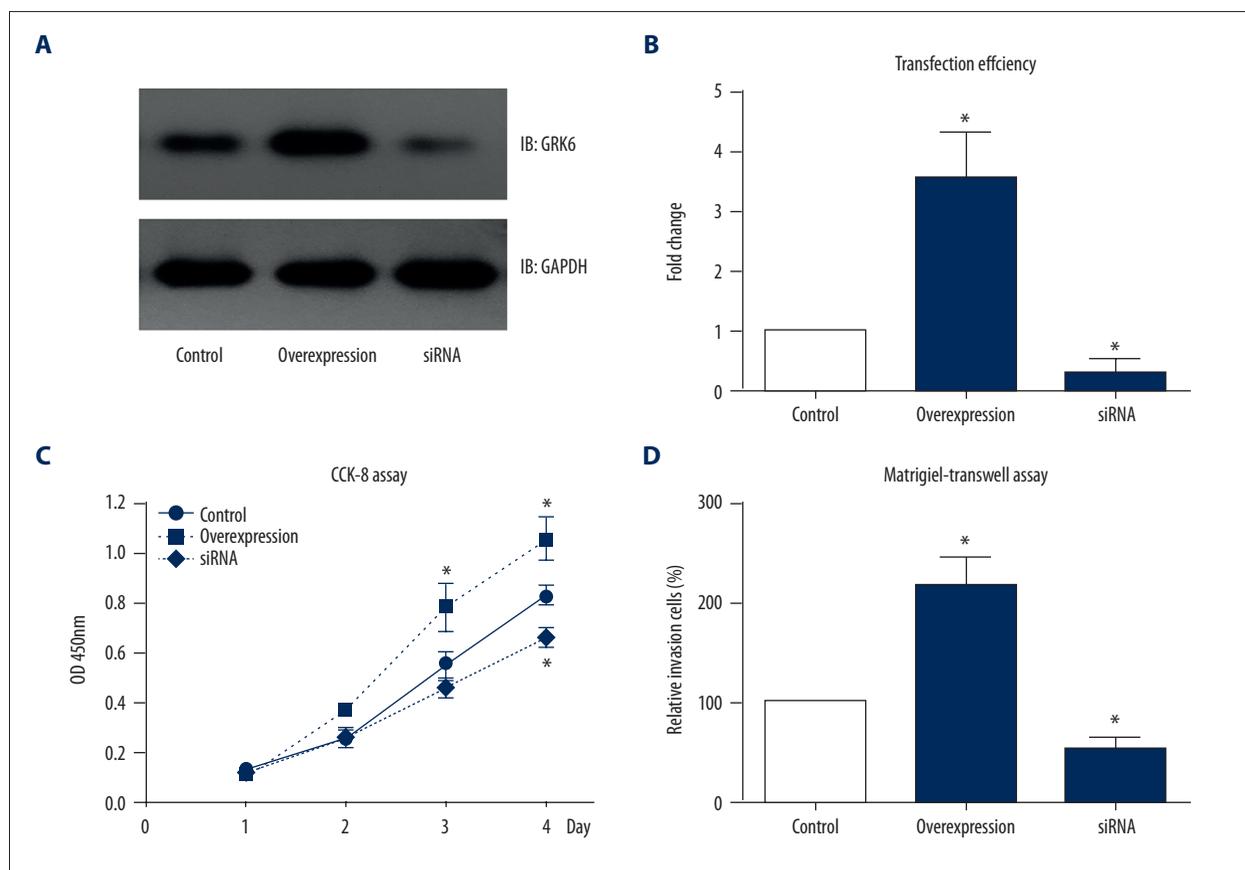


Figure 3. GRK6 enhanced PTC cell proliferation and invasion. (A) GRK6-plasmids and GRK6-siRNA were transfected into TPC-1 cells using Fugene6 transcription reagent, and immunoblotting was performed 48 h after transfection. (B) Semi-quantification of the transfection efficiency compared to the protein level in the control group. (C) Cell viability was tested using a CCK-8 kit, which revealed a positive regulatory role of GRK6 in the proliferation of TPC-1 cells. (D) Similarly, transfected cells were subjected to a Transwell assay pre-coated with Matrigel. Data analysis showed that GRK6-overexpression enhanced cell invasion, while GRK6-siRNA attenuated cell invasion. * $P < 0.05$ compared to control group.

also assessed the invasion of TPC-1 cells by use of Matrigel-Transwell strategy. As expected, GRK6 can positively regulate the invasion process of PTC tumor cells (Figure 3D). Cells with overexpressed GRK6 showed approximately 2.3 times more invasion cells than in the control group, while cells silencing GRK6 showed a 40% decrease in invasion capacity.

Discussion

Post-translational modification is critical in regulating protein stability and functions, which is catalyzed by various enzymes. Accumulating results show the nonnegligible roles of modification enzymes in tumor progression, such as the ubiquitinases [20], deubiquitinases [21], kinases [22], and phosphatases [23]. GRKs were first reported to catalyze the phosphorylation

and desensitization of GPCRs. Nevertheless, recent studies identified its GPCR-independent roles in regulating cellular functions [24]. For example, GRK2, GRK5, and GRK6 can mediate TNF α -induced NF- κ B signaling via direct phosphorylation of I κ B α [25,26]. Moreover, GRKs were reported to be involved in tumor development and progression. GRK5 can functionally regulate well-known cancer-related proteins such as Wnt and tumor suppressor p53 [27]. Another example is the upregulation of GRK5 in glioblastoma stem cells, indicating its significance in promoting tumor proliferation [12].

Here, we identified the expression pattern of GRK6 in PTCs for the first time. According to our data, GRK6 was up-regulated in PTCs, and was significantly correlated with tumor size and tumor stage. In addition, univariate and multivariate analyses of disease-free survival suggested that high GRK6 level was an unfavorable factor indicating a higher disease recurrence risk. In other words, GRK6 showed tumor-promoting roles in PTCs. However, it seems that GRK6 showed completely different functions in medulloblastoma, in which it can suppress tumor proliferation [13]. In our opinion, the distinct roles of GRK6 can be explained by its subcellular localization and catalyzed substrates. In medulloblastoma, GRK6 may function through phosphorylating and desensitizing the CXCR4 receptor, which was previously reported to promote medulloblastoma [28].

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Conclusions

In summary, our data provide the first evidence that GRK6 expression is up-regulated in PTCs. We also explored the clinical value of GRK6 in PTC patients, such as assessing disease progression and predicting recurrence risk. We showed that GRK6 can directly promote PTC cell proliferation and invasion, revealing its potential in therapeutic development.

Conflict of interest

None.

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