

Original Article

Silencing of DJ-1 reduces proliferation, invasion, and migration of papillary thyroid cancer cells in vitro, probably by increase of PTEN expression

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Abstract: Aims: To explore the function of DJ-1 on cell proliferation, migration, and invasion in human papillary thyroid carcinoma (PTC) cells. Materials and Methods: DJ-1 was knocked out by siRNA in K1 and TPC-1 cells and the efficiency of siRNA was examined by qRT-PCR and western blot. Cell proliferation, cell cycle, migration, and invasion were measured by CCK-8 assay, flow cytometry, colony formation assay and trans-well assay, respectively. Results: K1 and TPC-1 cells that were transfected with siRNA of DJ-1 had significantly lower expression levels of DJ-1 mRNA and protein. Down-regulation of DJ-1 significantly suppressed the cell proliferation, migration, and invasion. siRNA-mediated knock-down of DJ-1 increased the number of cells in the G0/G1 phase but reduced it in the S phase, while the G2/M phase was not affected. Moreover, the expression level of PTEN (Phosphatase and Tensin Homolog, PTEN) was found up-regulated in DJ-1-null cells. Conclusions: This work suggested that DJ-1 implicated in cell proliferation, migration, and invasion of papillary thyroid cancer cells, possibly by the DJ-1/PTEN/PI3K/Akt signal pathway.

Keywords: Papillary thyroid carcinoma, DJ-1, phosphatase, tensin homolog

Introduction

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy, accounting for 80%-85% of all thyroid cancers in women and children. It mainly originates from the thyroid follicle cells and presents as a palpable thyroid mass or nodule [1, 2]. Although the majority of the PTCs have a good prognosis after treatment (surgical resection, radio-/chemotherapy) mortality rate less than 10%, the incidence and metastases of PTC are increasing. Patients who suffer from recurrences and metastases have poor prognosis due to the lack of therapeutic options [3, 4]. Understanding the molecular pathogenesis of PTC and identification of novel diagnostic and therapeutic targets can therefore provide alternative solutions to improve the diagnosis, therapy, and prevention of this disease.

DJ-1 is as an oncogene with significant transforming activity that transforms mouse NIH3T3 cells together with ras [5]. Mutations of DJ-1

were found to be involved in autosomal early-onset Parkinson's disease [6]. Previous studies revealed that DJ-1 was also involved in multiple biological processes, including oxidative stress [7], androgen receptor and p53 signaling [8], apoptosis induced by TRAIL [9], and fertilization [10]. It was reported that DJ-1 regulated different downstream effectors, such as PTEN, IKK or NF- κ B, which then influenced human tumorigenesis, invasion, and metastasis [11]. Dysregulation of DJ-1 was found in various cancers, including breast cancer [12], lung cancer [13], pancreatic cancer [14], bladder cancer [15] and prostate cancer [16]. Although DJ-1 was highly expressed in thyroid carcinoma, where it protected cancer cells from TRAIL-mediated apoptosis [17], the effect of DJ-1 on cell proliferation and invasion in PTC has not yet been investigated.

In this study, the role of DJ-1 in PTC tumorigenesis was investigated. The influence of DJ-1 on cell proliferation, invasion and migration were examined in K1 and TPC-1 cells by siRNA-medi-

ated knock-down of DJ-1. The expression level of PTEN, phosphorylation of Akt, and activity of NF- κ B in the DJ-1 knocked-down cells were determined. The results suggested that knock-down of DJ-1 inhibited the proliferation, invasion, and migration of K1 and TPC-1 cells, potentially by the PTEN/PI3K/Akt pathway, indicating that DJ-1 was associated with the oncogenesis of PTC.

Materials and methods

Cell culture and siRNA transfection

K1 and TPC-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. siRNA of DJ-1 (Gene ID: 11315) and the negative control scramble siRNA were transfected into cells using Lipofectamine™ RNAiMAX. The target sequences for siRNA were: si-DJ-1-1, GG-GATTAAGGTCACCGTTGCA; si-DJ-1-2, AGGCG-CGGCTGCAGTCTTTAA; si-DJ-1-3, GTAGCCGTG-ATGTAATGATT.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

RNAiso Plus (Takara, Dalian, China) was used to extract total RNA from cells following the manufacturer's instructions. For the detection of DJ-1 and PTEN mRNA expression, PrimeScript RT reagent kit (Takara, Dalian, China) was used for synthesizing the first strand of cDNA. The mRNA levels of DJ-1 and PTEN were quantified using SYBR® Premix Ex Taq™ II (Takara, Dalian, China). β -actin was used as the internal control. The sequences of primers (from 5' to 3') are listed below: DJ-1: Forward: TTGTAGGCTG-AGAAATCTCTGTG; reverse: ATCCATTCTCACTGT-GTTTCG; PTEN: Forward: GGATTGACGGCTCC-TCTAC; reverse: 5'-GGATTGACGGCTCCTCTAC-3'; β -actin: forward: CCCTAAGGCCAACCGRG-AA; reverse: ACGACCAGAGGCATACAGGA.

Western blot

Proteins were extracted by RIPA (Beyotime, China), quantitated by BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and examined by western blot. 20 μ g samples were separated by SDS-PAGE and transferred onto PVDF membrane. Bands were incubated with primary antibodies of DJ-1 (ab18257, Abcam), PTEN (ab31-392, Abcam), Akt (9272, Cell Signaling Techno-

logy), phospho-Akt (9271, Cell Signaling Technology), ERK or β -actin (ab8226, Abcam) at 4°C overnight, and then with the corresponding HRP-conjugated secondary antibody at room temperature for 45 min. The blots were developed with ECL substrate.

Cell growth assay

Cell growth was examined using Cell Counting Kit-8 (CCK-8) assay. Cells transfected with siRNA or si-NC were seeded in 96-well plates. CCK-8 reagent was added at indicated times and was incubated along with the cells for 1 hour, after which the absorbance was measured at a wavelength of 450 nm and growth curves were plotted. Each sample was assessed in sextuplicate at each time point.

Colony formation assay

Cells were seeded into 6-well plates at a seeding density of 1×10^3 cells/well and were then transfected with siRNA or si-NC. Cells were cultured for 14 days and were then fixed with methanol and stained with crystal violet. Colonies (> 0.1 mm in diameter) were counted under a light microscope upon washing.

Flow cytometric analysis

Cells were washed with cold PBS and fixed with cold ethanol (75%). The fixed cells were incubated with propidium iodide staining buffer (0.5% Triton X-100, 250 μ g/mL RNase A, 50 μ g/mL propidium iodide in PBS) for 30 min and were then analyzed by flow cytometry with the DNA content calculated.

Migration assay

Cells transfected with siRNA were seeded (200 μ L, 1×10^6 cells/ml) into the upper chambers of the transwells. After 24 hours of incubation, cells that migrated into the lower chamber were stained with Giemsa. The stained cells were counted under a microscope. Experiments were performed in triplicate.

Invasion assay

Cells transfected with siRNA were seeded (200 μ L, 1×10^6 cells/ml) into the upper chambers of the transwell coated with Matrigel. After 24 hours of incubation, L1 cells that stayed on the lower side were stained with Giemsa. The

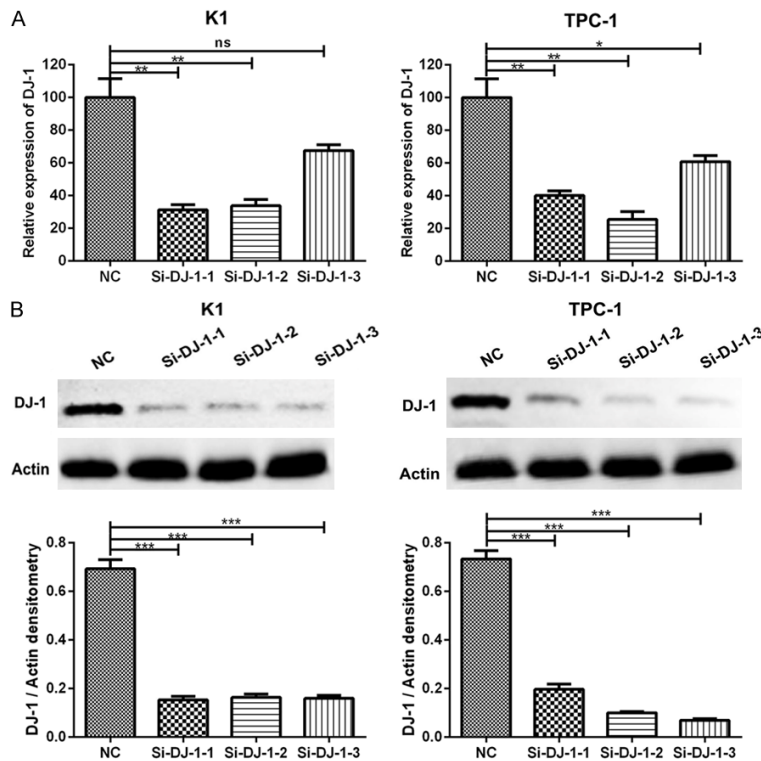


Figure 1. DJ-1 was knocked down by transfection with siRNA. A. Relative mRNA expression levels of DJ-1 were determined by qRT-PCR. Transfection with si-DJ-1-1, si-DJ-1-2 and si-DJ-1-3 reduced the expression levels of DJ-1 to $31.3 \pm 3.14\%$, $33.8 \pm 3.88\%$ and $67.53 \pm 5.32\%$ respectively. B. Relative protein expression levels of DJ-1 were determined by western blot; actin was used as internal reference. Representative images of western blot were shown, bands were quantitated by densitometry and normalized against actin. **, $P < 0.01$; ns, no statistically significant difference, compared to the control group (NC) transfected with scramble siRNA.

Expression levels of mRNA and protein of DJ-1 were measured by qRT-PCR and western blot. Results showed that two siRNAs, Si-DJ-1-1 and Si-DJ-1-2, significantly decreased the expression levels of DJ-1, while the third one (Si-DJ-1-3) slightly decreased it. Two days after transfection, mRNA of DJ-1 significantly decreased in cells transfected with Si-DJ-1-1 or Si-DJ-1-2 (decreased to $31.3 \pm 3.14\%$ and $33.8 \pm 3.88\%$, respectively) compared with that in the cells transfected with scramble siRNA (NC). In accordance with the changes in the level of mRNA, protein expression of DJ-1 also decreased in cells transfected with Si-DJ-1-1 or Si-DJ-1-2 (decreased to $23.8 \pm 5.6\%$ and $18.7 \pm 3.4\%$) compared with that in the NC (**Figure 1A** and **1B**). The results indicated that DJ-1 in K1 cells could be knocked down by both Si-DJ-1-1 and Si-DJ-1-2, which were hence used in the following experiments.

stained cells were counted under a microscope. Experiments were performed in triplicate.

Statistical analysis

The data were analyzed by SPSS (version 19.0; SPSS Science, Chicago, IL). Experiments were repeated at least for three times in duplicate and all data were reported as mean \pm SEM. Data were examined using Student's unpaired *t* test or one-way ANOVA (ANOVA). $P < 0.05$ was considered significant.

Results

Expression of DJ-1 was reduced after transfection with siRNA molecules in K1 cells

To determine the function of DJ-1 in PTC cells, DJ-1 was knocked down by transfection with siRNA. Three siRNAs targeting different sequences of DJ-1 were transfected into K1 cells.

Knock-down of DJ-1 suppressed cell proliferation

To assess the effect of DJ-1 on the cell proliferation, cell growth was analyzed by the CCK-8 assay and colony formation assay. In K1 and TPC-1 cells, transfection with siRNA significantly decelerated cell growth. Growth curves generated from the CCK-8 assay showed that 60 hours after transfection with Si-DJ-1-1 or Si-DJ-1-2, cell proliferation was significantly inhibited compared with that of NC (**Figure 2A**). The effect was confirmed by the colony formation assay. The cells transfected with the two siRNAs had fewer and smaller colonies than those formed by the cells transfected with scrambled siRNA, indicating knock-down of DJ-1 by siRNA inhibited cell proliferation (**Figure 2B**).

Since cell proliferation was mainly controlled during the G1 phase of the cell cycle, the effect of DJ-1 knock-down on cell cycle distribution

DJ-1 effect on papillary thyroid carcinoma

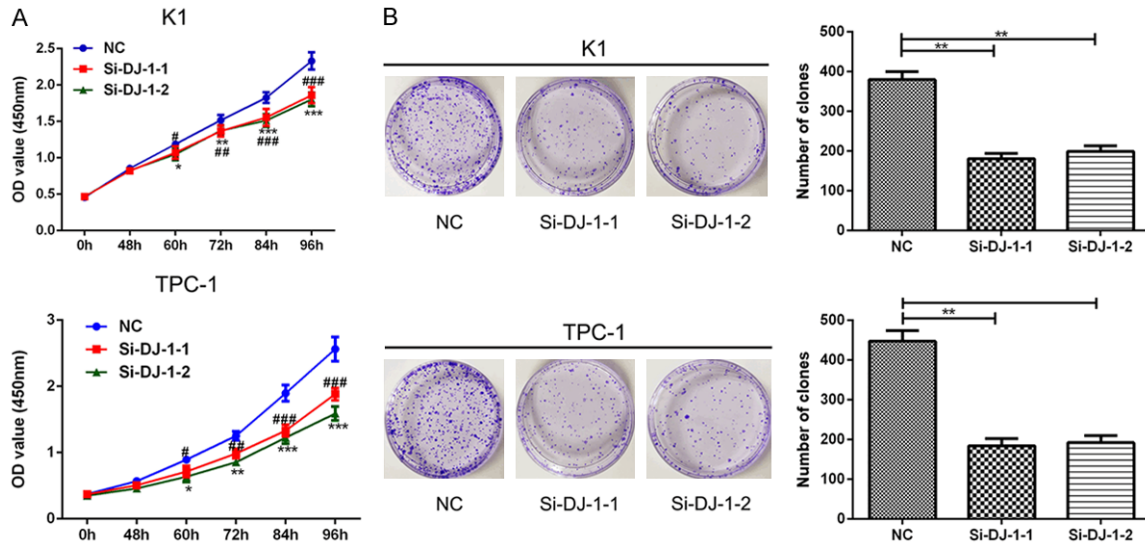


Figure 2. Knock-down of DJ-1 in K-1 and TPC-1 cells suppressed cell proliferation. A. Growth curves were analyzed by the CCK-8 assay; OD₄₅₀ was measured at 0, 48, 60, 72, 84, 96 hours. B. Colony formation was performed in cells transfected with Si-DJ-1-1, Si-DJ-1-2 and scramble-siRNA. Representative images of colony formation are shown and average number of colonies which were > 0.1 mm in diameter were counted. Transfection with siRNA significantly decreased the average number of colonies. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared to NC (scramble).

was further determined by flow cytometry. The results showed that siRNA mediated knock-down of DJ-1 increased the number of cells in G0/G1 phase and reduced that in S phase compared with the NC, while the amount of the cells in the G2/M phase was nearly unchanged (**Figure 3A** and **3B**). The results indicated knock-down of DJ-1 in K1 and TPC-1 cells delayed the transition from G1 to S phase. In conclusion, the results showed that knock-down of DJ-1 suppressed cell proliferation and delayed the G1 to S phase transition.

Knock-down of DJ-1 suppressed cell migration and invasion

Since migration and invasiveness are two pathophysiologic features of PTC, it was worth investigating whether knock-down of DJ-1 was associated with migration and invasiveness of K1 and TPC-1 cells. *In vitro* Transwell assay showed that knock-down of DJ-1 significantly decreased cell migration. The number of K1 cells transfected with Si-DJ-1-1 or Si-DJ-1-2 that migrated to the undersurface was much fewer than that of those transfected with scrambled siRNA (**Figure 4A** upper). In transwell matrix penetration assay, the number of K1 cells transfected with Si-DJ-1-1 or Si-DJ-1-2 penetrated was fewer than that of the NC (**Figure 4A**

lower). Similar assay results were found with TPC-1 cells (**Figure 4B**). The results indicated that knockdown of DJ-1 had an inhibitory effect on migration and invasion of K1 and TPC-1 cells.

Silencing of DJ1 reduces proliferation, invasion, and migration of PTC cells probably by increase of PTEN expression

Considering DJ-1 is a well-known regulator of the tumor suppressor PTEN [18], it was assumed that DJ-1 suppressed cell proliferation, migration, and invasion by regulating PTEN. To examine this assumption, the expression levels of PTEN in DJ-1 knocked-down cells were determined by qRT-PCR and western blot. The relative mRNA expression levels of PTEN in cells transfected with Si-DJ-1-1 or Si-DJ-1-2 were greatly upregulated compared with those in the NC (**Figure 5A** top). Consistently, the protein levels of PTEN were also significantly upregulated (**Figure 5A** middle and bottom). The mechanism for PTEN regulating proliferation, invasion, and migration of PTC cells was then studied. Since the PI3K/AKT pathway was modulated by PTEN and played an important role in cancer cell migration and invasion, it was of value to investigate whether they were also involved in PTC. Phosphorylation of Akt

DJ-1 effect on papillary thyroid carcinoma

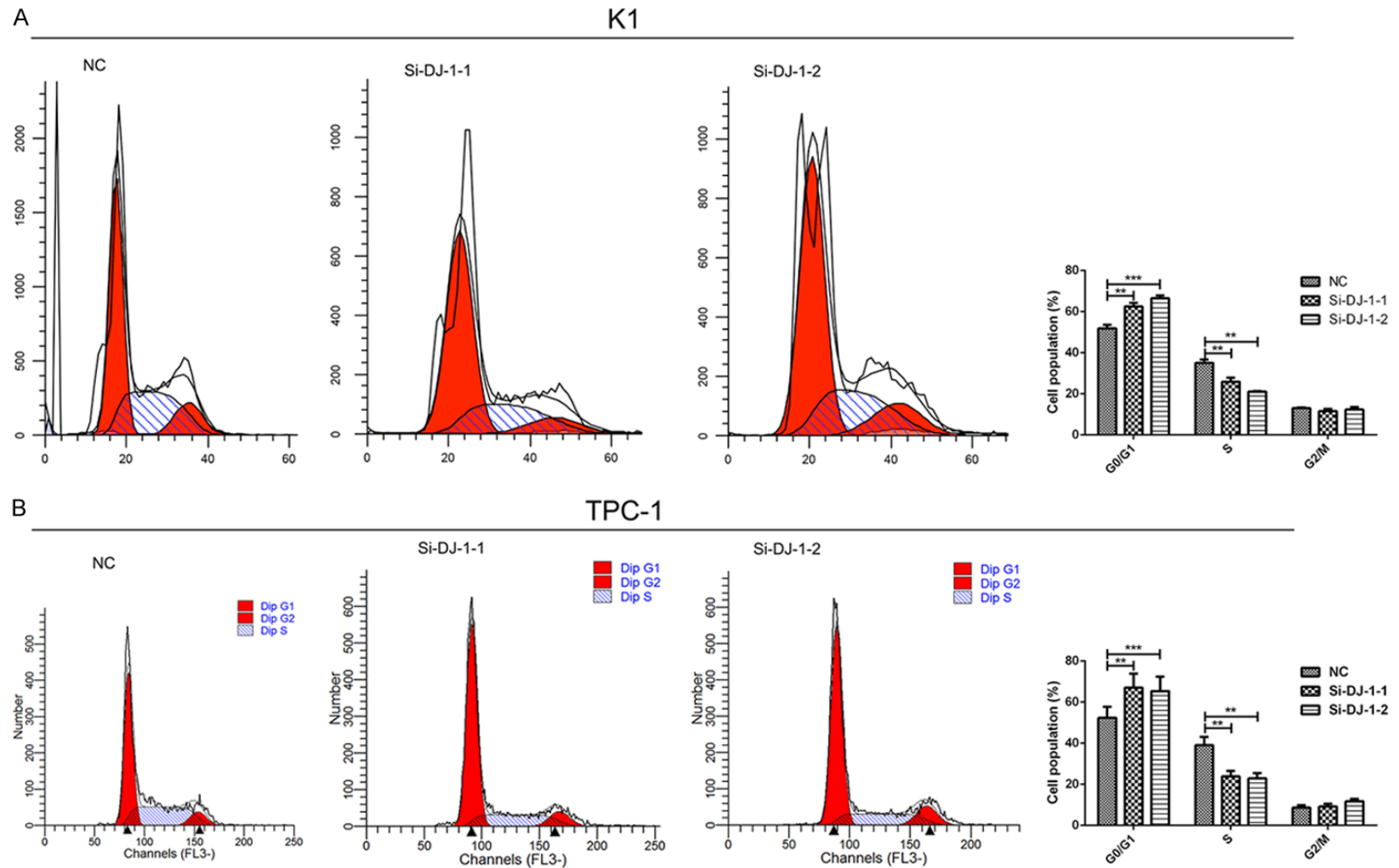


Figure 3. Knock-down of DJ-1 in K-1 and TPC1 cells altered cell cycle. A and B. Representative histograms of DNA content analysis using flow cytometry of cells transfected with Si-DJ-1-1, Si-DJ-1-2 and scramble-siRNA are shown. The percentage of the cell population of each cell subset (G0/G1, S and G2/M) is shown. **, $P < 0.01$; ***, $P < 0.001$, compared to NC.

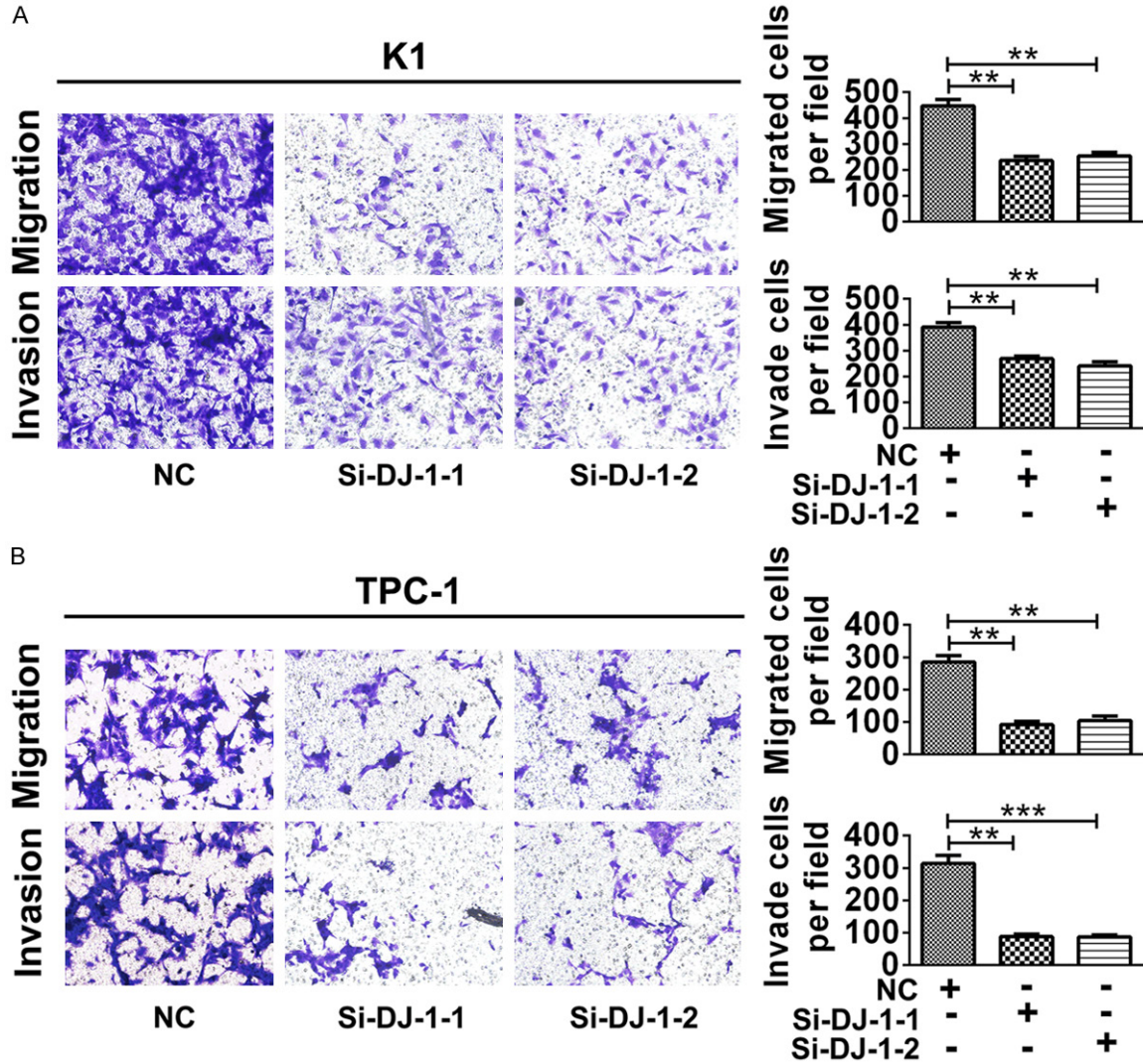


Figure 4. Knock-down of DJ-1 in K-1 and TPC-1 cells suppressed cell migration and invasion. Representative photomicrographs (200 × magnification) of cells transfected with Si-DJ-1-1, Si-DJ-1-2 and scramble-siRNA on transwell filters were shown. The number of migrated and invaded cells were counted in each group. **, P < 0.01, compared to NC.

was thus measured. The results showed that expression levels of phosphorylation of Akt decreased in DJ-1 knocked-down cells, whereas total expression levels of Akt had no obvious change (Figure 5A middle and bottom). The fact that the decrease in the phosphorylation of Akt was consistent with the upregulation of PTEN indicated that inhibition of proliferation, invasion, and migration of PTC cells by silencing of DJ-1 was potentially through the PTEN/PI3K/AKT pathway. Previous studies showed that expression of PTEN affected NF-κB by PI3K/Akt pathway, so the activity of NF-κB (p65) in the cells transfected with siRNA of DJ-1 was hence

studied. The expression levels of NF-κB (p65) decreased in the nuclei of DJ-1 knocked-down cells compared with those in the NC (Figure 5B). The results indicated that increasing PTEN by knocking down DJ-1 decreased the activity of NF-κB (p65).

Discussion

PTC is the most common subtype of thyroid cancer. Although the majority of PTC patients are well prognosed, 7% to 23% of them are still suffering from distant metastases and over 65% may develop radioactive iodine-refractory. The survival rate of these PTC patients within

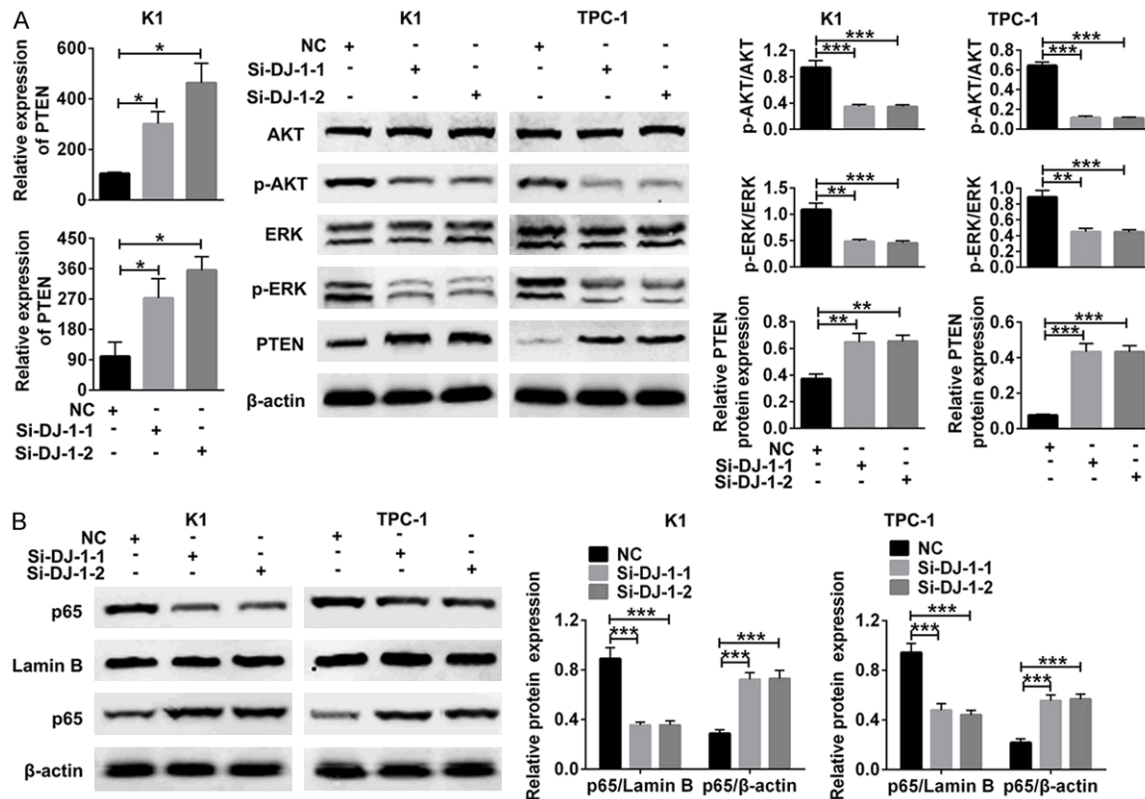


Figure 5. PTEN was up-regulated in DJ-1 knocked-down cells. A. Relative mRNA expression levels of PTEN were determined by qRT-PCR (top). Phosphorylation and total expression levels of Akt, ERK and expression levels of PTEN were determined by western blot (middle). Quantitative analysis of the gray density of western blotting bands (bottom). B. Relative expression levels of p65 in nucleus (upper) and cytoplasm (lower) were determined by western blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with NC.

10-year was much less ($< 10\%$) compared with that of the PTC patients with good prognosis (90%) [19, 20]. Recent development of innovative therapy strategies for radioactive iodine-refractory that employed protein kinase inhibitors (PKIs), re-differentiating drugs or nanodevices showed promising possibilities due to a better understanding of genetic and epigenetic alterations of PTC [19, 29]. Hyperactivation of RAS/BRAF/MAPK or PI3K/Akt pathway was frequently found in differentiated thyroid cancer [29]. This work reported that knock-down of DJ-1 could inhibit the proliferation, invasion and migration of PTC cells probably by the PTEN/PI3K/Akt pathway.

DJ-1 is involved in various cellular processes and also functions as an oncogene [21]. Over-expression of DJ-1 has been found in various cancers. DJ-1 not only decreases the sensitivity of tumor cells to chemotherapeutic drugs, but also is involved in tumorigenesis and metastasis

by accelerating the cell proliferation, migration, and invasion [22]. Proteomics analysis of lung adenocarcinoma cell lines showed that expression levels of DJ-1 corresponded to the grade of metastasis; cell line with more advanced metastasis (e.g. Anip973) had higher DJ-1 expression than the cell line with lower metastasis (e.g. AGZY83-a) [23]. Moreover, the expression levels of DJ-1 were related to tumor stages of colorectal cancer: higher DJ-1 expression was observed in the advanced-grade (Stage III and IV) tumors than in the lower-grade (Stage I and II) ones [24]. Esophageal squamous cell carcinoma (ESCC) with highly distant metastasis had a significantly higher level of nuclear DJ-1 expression than the primary ESCC [25]. The studies suggested cancers with high expression level of DJ-1 exhibited more aggressive clinical behaviors. The present work also showed that knock-down of DJ-1 in K1 and TPC-1 cells suppressed cell proliferation, migration, and invasion, suggesting the recurrence

and metastasis of PTC might be related to the expression of DJ-1 in PTC.

Cell proliferation, migration, and invasion can be regulated by various signal pathways, including the PTEN/PI3K/AKT signal pathway, which is hyper-activated in the majority of cancers. PTEN affected cell proliferation, apoptosis, cell cycle, invasion, and metabolism by negatively regulating Akt through de-phosphorylation of PIP3 [26]. Loss of function of PTEN led to increased cell proliferation, reduced cell death, and tumor development [27]. DJ-1 was also a negative regulator of PTEN; over-expression of DJ-1 suppressed the expression of PTEN and increased the cell proliferation and migration [18]. DJ-1 was assumed to be involved in the oncogenesis by PTEN/PI3K/AKT signal pathway; the expression level of PTEN and phosphorylation of Akt in DJ-1 knock-down cells was therefore determined. It was found that expression level of PTEN was up-regulated and phosphorylation of Akt down-regulated. Furthermore, up-regulation of PTEN could induce cell cycle arrest in the G1 phase by decreasing Cyclin D1 [28], consistent with the results in this work. In conclusion, the results indicated the DJ-1/PTEN/PI3K/Akt/signaling pathway might play important roles in modulation of proliferation, migration, and invasion of K1 and TPC-1 cells, further influencing the tumorigenesis and metastases of PTC. It was also found that activity of NF- κ B decreased in the DJ-1 knocked-down cells. NF- κ B was not only a well-known target of DJ-1, but also a core central pathway interacting with multiple signaling pathways including the PI3K/Akt pathway [30, 31]. The results suggested that the decrease in the activity of NF- κ B in the DJ-1 knock-down cells was possibly by the DJ-1/PTEN/PI3K/Akt/signaling pathway.

BRAF^(V600E) mutation which results in hyper activating of RAS/BRAF/MAPK signaling is the most frequent genetic alteration in PTC. BRAF^(V600E) mutation is not only associated with greater tumor size, extra-thyroid invasion and cervical lymph nodal metastases, but also is associated with a lower expression of sodium iodide symporter (NIS), which contributes to diminished iodide-concentrating capacity. Phosphorylation of ERK1/2 was examined and found decreased in the DJ-1 knocked-down cells (**Figure 5A** middle and bottom). However,

the BRAF^(V600E) mutation might not be involved in the DJ-1 knock-down induced decrease in the phosphorylation of ERK1/2, as TPC-1 and K1 cells carried wild type BRAF and BRAF^(V600E) mutation, respectively [34].

In summary, we evaluated an inhibitory effect of silencing DJ-1 on cell proliferation, migration, and invasion in PTC cells. In addition, DJ-1 knock-down increased the expression of PTEN and decreased the phosphorylation of AKT. The findings suggested that the DJ-1/PTEN/PI3K/Akt/signaling pathway played a crucial role in the oncogenesis of PTC. It was also found that knock-down of DJ-1 decreased the activity of NF- κ B and phosphorylation of ERK1/2, further inhibiting the cell proliferation, migration and invasion. The mechanism for the inhibition of cell proliferation, migration and invasion by DJ-1 knock-down should be further investigated.

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Disclosure of conflict of interest

None.

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