Effects of PD-1/PD-L1 on the malignant behavior of thyroid cancer cells through the TGF-β/Smad signaling pathway

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Abstract. Papillary thyroid cancer (PTC) is the most common type of malignant tumor in the endocrine system, with an incidence rate that is increasing year by year. In addition, the mortality rate of advanced PTC is also on the rise, posing a threat to human health. Programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) signaling has been reported to be abnormally expressed in PTC, while the possible effects and underlying mechanisms remain to be further explored. In the present study, the expression levels of PD-1 and PD-L1 were upregulated in clinical PTC tissues as well as TPC-1 and BCPAP cells as detected by reverse transcription-quantitative PCR and western blotting analysis. Notably, PD-1 expression in tissue samples may originate from both tumor cells and tumor-infiltrating immune cells, whereas PD-1 detected in cell lines solely represents tumor-intrinsic expression. Functionally, downregulation of PD-1 or PD-L1 notably inhibited the viability, proliferation and induced the apoptosis of TC cells, as measured by Cell Counting Kit-8, EdU, colony formation, flow cytometry and western blotting analysis. In addition, downregulation of PD-1 or PD-L1 markedly suppressed the migration, invasion and epithelial-mesenchymal transition progress of TC cells, as measured by wound healing and Transwell migration and invasion assays, as well as western blotting analysis. Furthermore, downregulation of PD-1 or PD-L1 inactivated the TGF-β/Smad signaling pathway. Exogenous TGF-β partially restored the effects of short hairpin (sh)-PD-1 or sh-PD-L1 on the viability, proliferation, apoptosis, migration, invasion and EMT progress of PTC cells. To conclude, the present study suggests PD-1/PD-L1 may represent potential therapeutic targets for the treatment of patients with PTC, though further studies across additional thyroid cancer subtypes are warranted.

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Introduction

Thyroid cancer (TC) is a common tumor of the endocrine system and its incidence rate has been rapidly increasing in recent years (1). The global incidence rate of TC stands at 6.7 per 100,000 individuals, with 190,000 new cases reported annually in China (2). TC primarily includes four pathological types, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), undifferentiated thyroid carcinoma and medullary thyroid carcinoma. Among these, PTC and FTC are referred to as differentiated thyroid carcinoma (DTC) (3). PTC is the most common type, accounting for 85-90% of the global total incidence rate (4). DTC (including PTC and FTC) generally exhibits a favorable prognosis, whereas poorly DTC (PDTC) and anaplastic thyroid carcinoma (ATC) are associated with progressively worse outcomes. Notably, programmed death-ligand 1 (PD-L1) expression and immune checkpoint activation differ notably across these subtypes, with ATC exhibiting the highest PD-L1 positivity rates (reported as 80-100% in ATC vs. 30-60% in PTC in previous studies) (5,6). This subtype-specific difference has notable implications for patient stratification and immunotherapy strategies. Due to the relatively indolent biological behavior of PTC, the 10-year survival rate for patients is >90% (7). Although the prognosis is favorable, 10-15% of patients with TC exhibit aggressive phenotypes such as local invasion, metastasis and drug resistance, resulting in a 5-year survival rate of 35% (8). Studies have reported that TC typically invades lymph node regions, leading to multifocal TC and increased lymph node metastasis rates, which markedly affect the selection of treatment options and prognosis (9,10). Currently, the main treatment modalities for TC are surgical resection, iodine-131 (I-131) therapy and thyroid stimulating hormone suppression therapy. The majority of patients experience notable improvement after undergoing these standardized treatments. However, studies have reported that 14.9% of patients still experience disease

persistence or recurrence and 7-23% of patients develop distant metastasis (11-13). Due to the high I-131 uptake in TC tumor lesions, surgical resection and I-131 therapy are often curative. However, ~30% of patients with TC with metastasis lose the ability to concentrate iodine, resulting in a lower survival rate and poor prognosis (14). Therefore, there is an urgent need to discover and evaluate new biomarkers and therapeutic targets for TC.

These subtype-specific differences in PD-L1 expression are thought to reflect underlying biological differences among TC subtypes: ATC and PDTC typically carry a higher burden of driver mutations (including tumor protein 53 and telomerase reverse transcriptase promoter mutations) and a more desmoplastic, inflammatory tumor microenvironment (TME) with denser immune-cell infiltration, both of which favor the upregulation of PD-L1 and the engagement of the programmed cell death protein-1 (PD-1)/PD-L1 checkpoint. By contrast, DTC (and in particular PTC) is generally associated with a more immunologically 'cold' microenvironment (sparse tumor-infiltrating lymphocytes and low PD-L1/tumor-mutational-burden) and comparatively lower PD-L1 positivity (15,16). Consequently, immune checkpoint activation is hypothesized to be most pronounced in ATC, intermediate in PDTC and least pronounced in DTC, which has direct implications for patient stratification and the design of immunotherapy trials across these subtypes.

The immune system serves a key role in recognizing and eliminating tumor cells, making immunotherapy an effective treatment for a number of types of cancer, such as melanoma, non-small cell lung cancer, renal cell carcinoma and Hodgkin's lymphoma (17). Studies have shown that the PD-1/PD-L1 pathway is key in maintaining immune tolerance and regulating immune responses. Upregulation of PD-1/PD-L1 can suppress immune responses, allowing tumor cells to evade immune surveillance (18,19). PD-1, also known as CD279, is a type I transmembrane glycoprotein encoded by the PDCD1 gene located on chromosome 2. It is primarily expressed by T and B lymphocytes, dendritic cells, activated natural killer cells and tumor-infiltrating lymphocytes (20). PD-L1 (also known as CD274, the ligand of PD-1) is a type I transmembrane glycoprotein with a relative molecular weight of 33,000. Its extracellular region consists of 290 amino acids and two protein domains, primarily expressed by cancer cells (21). Research has found that, compared with normal thyroid cells, PD-1 is more highly expressed in TC cells. Inhibiting PD-1 expression has been shown to notably suppress the phosphorylation of MEK1/2 and the levels of MAPK, as well as inhibiting the proliferation and migration of tumor cells (22). Furthermore, a study revealed that microRNA (miR)-199a-5p was downregulated in TC, while the expression of PD-L1 was positively associated with the elevated expression of tight junction protein 1 (23). Experimental overexpression of miR-199a-5p inhibited the expression of PD-L1 and tight junction protein 1, thereby suppressing the proliferation, metastasis and invasion abilities of TC cells (23). However, the exact effects and associated mechanisms of PD-1/PD-L1 on the tumorigenesis and metastasis of TC are still unclear.

Beyond its tumor-intrinsic functions, the PD-1/PD-L1 axis serves a key immunological role within the TME. PD-1 engagement by PD-L1 on tumor cells or antigen-presenting cells suppresses T-cell receptor signaling, leading to T-cell exhaustion, reduced cytotoxic activity and impaired anti-tumor immunity (24). In TC, tumor-associated macrophages (TAMs) are abundant in the TME and contribute to PD-L1 expression, immune suppression and disease progression (15). Furthermore, inflammatory cytokines such as IL-17 have been implicated in TC progression through the recruitment of myeloid-derived suppressor cells and the promotion of a pro-tumorigenic inflammatory milieu (25). Banerjee *et al* (24) demonstrated that cytotoxic T-cell activity and PD-1 immune checkpoint pathway activation are closely associated with clinical outcomes in papillary thyroid carcinoma and further showed that IL-17A is associated with disease progression in PTC (25). These findings highlight the complex interplay between PD-1/PD-L1 checkpoint signaling, IL-17-driven inflammation and TGF-β pathway activation in the thyroid tumor microenvironment. In addition to its canonical role on immune cells, accumulating evidence has indicated that PD-1 is expressed directly on a number of cancer cells, including melanoma, lung cancer and breast cancer cells, where it functions as an intrinsic oncogenic driver (26,27). In TC, Liotti *et al* (22) demonstrated that PD-1 is expressed on TC cells and promotes tumor growth through Src homology region 2 domain-containing phosphatase-2/Ras/MAPK signaling (20), providing a notable rationale for investigating tumor-intrinsic PD-1 functions.

TGF-β is a cytokine that serves a central role in regulating the cell cycle and participating in the processes of cell-cycle control, cell proliferation, differentiation, apoptosis, extracellular-matrix remodeling and epithelial-mesenchymal transition (28). Studies have shown that in TC cells, the expression of TGF-β is increased by 52-100% (29). TGF-β protein activates both Smad and non-Smad signaling pathways. The TGF-β/Smad signaling pathway is an important intracellular signaling pathway implicated in the regulation of biological behaviors such as tumor cell proliferation, differentiation, apoptosis, migration and invasion, angiogenesis and immune responses (30,31). Research has demonstrated that the activation of the TGF-β/Smad signaling pathway is associated with TC cell migration, lymph node metastasis, epithelial-mesenchymal transition and radioiodine/therapy resistance (32). A previous study showed that Foxp3 activates the phosphorylation of Smad3 by inducing the secretion of TGF-β1, thereby inhibiting the expression of sodium/iodine symporter (NIS) in TC (33). However, the role of the TGF-β/Smad signaling pathway in PD-1/PD-L1 regulation of PTC cells has not been extensively studied.

Therefore, the aim of the present study was to investigate the function and precise mechanisms of PD-1/PD-L1 in modulating tumorigenesis and metastasis of PTC partially through regulating TGF-β/Smad signaling pathway, indicating that PD-1/PD-L1 might represent potential therapeutic targets for the treatment of patients with PTC.

Materials and methods

Clinical samples. A total of 30 pairs of TC tissues and their corresponding adjacent non-cancerous thyroid tissues were obtained from patients who underwent surgical resection between February 2025 and December 2025 in the People's

Hospital of Ningxia Hui Autonomous Region (Yinchuan, China). The diagnosis of TC was histologically confirmed by two independent pathologists according to the World Health Organization classification criteria (34). All participants provided written informed consent and the present study protocol was approved by the ethics committee of People's Hospital of Ningxia Hui Autonomous Region (Yinchuan, China; approval no. 2025-NZR-291). Freshly collected tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C for subsequent protein and RNA analysis. Detailed clinicopathological characteristics of the 30 patients, including histological subtype, TNM stage, lymph node metastasis status, BRAF V600E mutation status (where available from clinical records), age and sex distribution, are presented in Table SI. The final cohort comprised 30 patients, including 11 males and 19 females (female:male, 1.7:1); 22 patients (73.3%) were aged <55 years and 8 patients (26.7%) were ≥55 years. The mean age of patients aged <55 years was 37.2 years (range, 21-54 years) and the mean age of patients aged ≥55 years was 61.3 years (range, 55-71 years) (Table SI). Inclusion criteria were: i) Histologically confirmed differentiated thyroid cancer after total or partial thyroidectomy at the People's Hospital of Ningxia Hui Autonomous Region between February and December 2025; ii) availability of complete clinicopathological records; and iii) written informed consent obtained from all participants. Exclusion criteria were: i) A history of another primary malignancy; ii) receipt of radioiodine (I-131), chemotherapy, external radiotherapy or targeted/anti-PD-1 therapy before surgery; and iii) incomplete clinical data.

Cell culture and transfection. Immortalized normal human thyroid follicular epithelial cells (Nthy-ori3-1) and TC cell lines (TPC-1 and BCPAP) were obtained from the American Type Culture Collection. Both cell lines were authenticated by short tandem repeat profiling within the past three years, with results matching the corresponding cell line databases. Both cell lines were routinely tested for *Mycoplasma* contamination using a PCR-based method, with negative results determined prior to experiments. Cells were cultured in DMEM containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2. As reported in the literature (35,36), BCPAP, although originally derived from PTC, is now regarded as a poorly differentiated/de-differentiated cell line. Throughout the present study, BCPAP is therefore described as a TC cell line rather than specifically a PTC cell line.

For short hairpin (sh)-RNA-mediated knockdown, three independent shRNA sequences targeting human PD-1 (https://www.ncbi.nlm.nih.gov/nuccore/NM_005018.3/) and PD-L1 (https://www.ncbi.nlm.nih.gov/nuccore/NM_014143.4) were designed using the RNAi Consortium design algorithm (http://portals.broadinstitute.org/gpp/public/) and synthesized by Shanghai GenePharma Co., Ltd. The most efficient shRNA sequence for each target was selected based on preliminary validation experiments. PD-1 and PD-L1 are distinct genes encoded on different chromosomes (PD-1: PDCD1 on chromosome 2q37.3; PD-L1: CD274 on chromosome 9p24.1). We hypothesize that the observed cross-knockdown effect, where sh-PD-L1 reduced PD-1 expression and sh-PD-1 reduced PD-L1 expression, may reflect reciprocal regulatory feedback between these two molecules at the transcriptional or post-transcriptional level, rather than direct sequence homology. shRNA-expressing lentiviral particles were prepared as concentrated, ready-to-use stocks supplied by Shanghai GenePharma Co., Ltd., and lentiviral particles were thus collected and concentrated by the manufacturer (titre: $1x10^8$ TU/ml) and stored at -80˚C until use. TPC-1 and BCPAP cells were transfected with 2 μg shRNA-expressing lentiviruses targeting PD-1 or PD-L1 at an MOI of 10 in 6-well culture plates, in the presence of polybrene (8 μg/ml) (MilliporeSigma). Cells were incubated at 37˚C in a 5% CO_2 humidified incubator for 12-16 h, after which the virus-containing medium was replaced with fresh complete medium. A non-targeting shRNA (sh-NC) was used as a negative transfection control (sequences provided in Table SII). Stable clones were selected using puromycin (2 μg/ml) for 7 days, after which positive stable clones were maintained in complete culture medium supplemented with puromycin (1 μg/ml). Knockdown efficiency was determined by reverse transcription-quantitative PCR (RT-qPCR) and western blotting analysis 48 h post-transfection.

Cell Counting Kit-8 (CCK-8) assay. TC cell viability after transfection was assessed using a CCK-8 assay (Beyotime Biotechnology). Cells with a density of $1x10^4$ were cultured in 96-well plates and after incubation for 0, 24, 48 and 72 h respectively, cells were incubated with 10 μl CCK-8 reagent for another 4 h at 37˚C. Subsequently, the absorbance was detected at the wavelength of 490 nm.

EdU assay. TC cell proliferation after transfection was assessed using an EdU assay (MilliporeSigma). Transfected TC cells ($4x10^4$/well) from different groups were inoculated into the 24-well plates for 48 h. After being fixed with 4% paraformaldehyde at room temperature for 15 min, goat serum (Beyotime Biotechnology) was used to block cells at room temperature for another 1 h. Furthermore, cells were incubated with the prepared EdU reaction mixture for 30 min at room temperature, protected from light, then washed twice with 3% BSA in PBS to remove unreacted dye. Cell nuclei were counterstained with DAPI (5 μg/ml) (MilliporeSigma) for 10 min at room temperature in the dark, then washed twice more with PBS before imaging.

Colony formation assay. TC cell colony formation ability after transfection was assessed using a colony formation assay. Transfected TC cells ($1x10^3$/well) from different groups were cultured in 6-well plates and the medium was replaced every 2-3 days for 2 weeks. Subsequently, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, then stained using crystal violet (1%) at room temperature for 20 min and imaged using a light microscope. Visible colonies with a diameter >1 mm (or containing >50 individual cells) were defined as positive scoring colonies. All colonies were counted manually by three independent investigators blinded to the group assignments, and the average count was calculated for statistical analysis.

Apoptosis analysis. TC cell apoptosis ability after transfection was assessed using a flow cytometry assay. Briefly, transfected TC cells after transfection were cultured in a 6-well plate at a density of $1x10^5$ cells/well and cultivated for 24 h. Afterwards,

cells were suspended in binding buffer and incubated at room temperature in the dark for 15 min. Annexin-V-FITC and propidium iodide were added to cell suspension for 15 min in the dark at room temperature according to the protocol of the manufacturer of the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). Next, stained cells were detected via the BD FACSVerse™ flow cytometer (BD Biosciences), and data were analyzed using FlowJo software version 10.8 (BD Biosciences).

Wound healing assay. Cell motility of TC cells after transfection was evaluated by a wound healing assay. Cells at a density of $1x10^5$/ml were seeded in a 6-well plate and carefully scratched using a yellow pipette tip. Subsequently, cellular debris was removed by washing with serum-free DMEM and cells were subsequently cultured in serum-free DMEM to minimize the confounding effect of proliferation on wound closure. Images of the crosses of each well were taken under a light microscope (Olympus IX73; Olympus Corporation) at 0 and 48 h, respectively. For quantification, the wound width was measured at three fixed positions along the scratch for each image. The wound closure rate was calculated as the difference between the average wound width at 0 h and the average wound width at 48 h, divided by the average wound width at 0 h, multiplied by 100 to get a percentage value.

Transwell assay. TC cell migration and invasion ability after transfection were evaluated by a Transwell assay. For the migration assay, PTC cells after transfection were trypsinized into a single cell suspension, washed 3 times in serum-free medium and then adjusted to a concentration of $2x10^5$/ml. The lower chambers of the 24-well Transwell system (Corning Costar) were filled with DMEM containing 10% FBS as a chemoattractant, while 200 μl of the prepared cell suspension (equal to $4x10^4$ total cells in serum-free DMEM) was added to the upper inserts without Matrigel coating. Following a 48-h incubation period at 37˚C, non-migratory cells remaining on the upper surface of the Transwell membrane were wiped off with a cotton swab. The migrated cells that attached to the lower surface of the membrane were fixed and stained with 0.1% crystal violet at room temperature for 15 min. The stained cells were observed and counted under a light microscope (Olympus CX33; Olympus Corporation). For Transwell invasion assays, Matrigel was pre-diluted with serum-free DMEM at a ratio of 1:3, then uniformly coated onto the polycarbonate membrane of the upper Transwell chamber and left to solidify for 1 h at 37˚C. After Matrigel polymerization, the same volume and number of cells (200 μl; $1x10^5$ total cells in serum-free DMEM) as the migration assay was seeded into the coated upper chamber. Lower chamber preparation, incubation, post-incubation processing and cell counting steps were identical to the migration assay protocol aforementioned. Five random fields of view were selected to count the number of migrated/invaded cells for each chamber using a light microscope, and results were averaged across three independent biological replicates.

RNA extraction and RT-qPCR analysis. Total RNA was extracted from human PTC tissues and cell lines using QIAzol Lysis Reagent (Qiagen GmbH). Subsequently, total RNA was reverse transcribed into cDNA using an RNeasy Plus Micro kit (Qiagen GmbH) according to the manufacturer's standard protocol, which was used as starting material for RT-qPCR, which was performed using SYBR Premix Ex Taq (TaKaRa Biotechnology Co. Ltd.) on the Step One System (Thermo Fisher Scientific, Inc.). Primer sequences used were designed as follows: PD-1 forward, 5'-CCGTGCCTGTGTTCTCTGTG-3', PD-1 reverse, 5'-GGTGGCATACTCCGTCTGCT-3', PD-L1 forward, 5'-GGCTGTTGAAGGACCAGCT C-3', PD-L1 reverse, 5'-CTTGTAGTCGGCACCACCATAG-3', GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3' and GAPDH reverse, 5'-AGGGGCCATCCACAGTCTTC-3'. Conditions for RT-qPCR were as follows: 95˚C for 10 min, 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All target gene transcripts were normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method as previously described by Livak and Schmittgen (2001) (37).

Western blotting analysis. Protein of TC tissues and cell lines was lysed in RIPA lysis buffer (Beyotime Biotechnology). After 12,000 x g centrifugation for 15 min at 4˚C, the total protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein (30 μg per lane) were separated on 10% gels using SDS-PAGE and then transferred onto a PVDF membrane for 2 h. Membranes were blocked with 5% non-fat skimmed milk in TBST (with Tween-20) buffer at room temperature for 1 h and incubated with the following primary antibodies: PD-1, PD-L1, NIS, Bax, Bcl-2, caspase-3, cleaved caspase-3, E-cadherin, N-cadherin, vimentin, TGF-β1, phosphorylated (p)-Smad2, Smad2, p-Smad3 and Smad3 at 4˚C overnight. After washing three times with TBST, secondary antibodies [Goat anti-Rabbit IgG (HRP) or Goat anti-Mouse IgG (HRP)] were added and incubated for 2 h at room temperature. An ECL detection system was used to evaluate the protein expression levels. GAPDH was used as the loading control. Detailed information regarding antibody suppliers, catalog numbers and dilution ratios is provided in Table SIII.

Immunohistochemistry (IHC). IHC staining was performed on 4-μm-thick 10% neutral buffered formalin-fixed, paraffin-embedded tissue sections (fixed at room temperature for 24 h). The same 30 pairs of TC and adjacent non-cancerous thyroid tissues used for RT-qPCR and western blotting were employed for IHC analysis; no additional tissue collection was required. Briefly, sections were deparaffinized in xylene and rehydrated through graded ethanol solutions. Antigen retrieval was performed by heating sections in citrate buffer (10 mM; pH 6.0) at 95˚C for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min.

Sections were then incubated with 5% normal goat serum (Beyotime Biotechnology) for 30 min. Primary antibodies against PD-1 (1:200; cat. no. ab137132; Abcam) and PD-L1 (1:200; cat. no. ab205921; Abcam) were applied and incubated overnight at 4˚C. After washing with PBS, sections were incubated with an HRP-conjugated secondary antibody (1:500; cat. no. ab205718; Abcam) for 30 min at room temperature. Signal was developed using DAB substrate (Dako; Agilent Technologies) for 3-5 min and counterstained with hematoxylin at room temperature for 2 min. Slides were dehydrated,

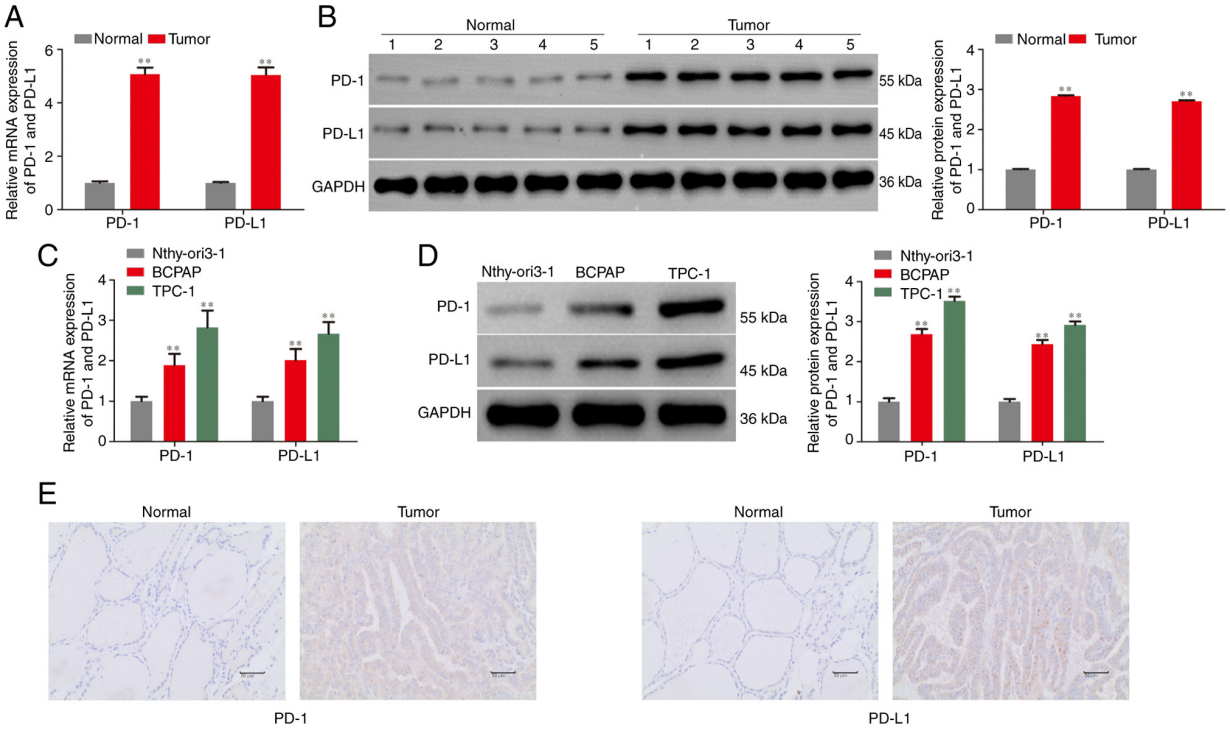

Figure 1. PD-1 and PD-L1 expression levels were upregulated in clinical PTC tissues and cell lines. (A) RT-qPCR and (B) western blotting were adopted to evaluate the expression levels of PD-1/PD-L1 in clinical PTC tissues. (C) RT-qPCR and (D) western blotting was adopted to evaluate the expression levels of PD-1/PD-L1 in thyroid cancer cells. (E) IHC analysis was adopted to evaluate the expression levels of PD-1/PD-L1 in clinical PTC tissues. **P<0.01 vs. Normal or Nthy-ori3-1 group. RT-qPCR, reverse transcription-quantitative PCR; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; PTC, papillary thyroid cancer.

cleared and mounted. IHC images were acquired using an Olympus BX53 light microscope (Olympus Corporation) at x200 and x400 magnification. PD-1 expression was evaluated as positive when membranous and/or cytoplasmic staining was observed in tumor-infiltrating lymphocytes (TILs) and/or tumor cells. PD-L1 expression was evaluated based on membranous staining in tumor cells.

Statistical analysis. All experiments were carried out in at least three independent biological replicates, each with three technical replicates and the data are presented as the mean ± SD analyzed by SPSS (version 22.0; IBM Corp). Paired Student's t-test or one-way ANOVA was used to measure statistical significance of differences between two groups or multiple groups, respectively. P<0.05 was considered to indicate a statistically significant difference. The Shapiro-Wilk test was used to assess normality before applying parametric tests; the Mann-Whitney U test or Kruskal-Wallis test was used when the normality assumption was violated. Following significant one-way ANOVA results, Tukey's HSD or Bonferroni post-hoc correction was applied for multiple comparisons. Associations between PD-1/PD-L1 expression and clinicopathological parameters in Table SIV were evaluated using Fisher's exact test/χ^2 test as appropriate.

Results

PD-1 and PD-L1 are upregulated in clinical PTC tissues and cell lines. To investigate the effects and possible mechanisms of PD-1/PD-L1 on the development and progression of PTC, firstly, RT-qPCR and western blotting were adopted to evaluate the expression levels of PD-1/PD-L1 in clinical PTC tissues. As shown in Fig. 1A and B, the expression of PD-1 and PD-L1 was significantly upregulated in PTC tissues compared with those in paracancerous tissues. Furthermore, the expression levels of PD-1 and PD-L1 in TPC-1 and BCPAP cells were also through with RT-qPCR and western blotting assays. As hypothesized, PD-1 and PD-L1 expression levels were significantly increased relative to those in immortalized normal thyroid follicular epithelial cells (Nthy-ori3-1), as demonstrated in Fig. 1C and D. Given that these were pure tumor cell populations cultured *in vitro*, the detected PD-1 expression represents tumor-intrinsic PD-1.

To evaluate the expression profiles of PD-1 and PD-L1 in the present patient cohort, IHC staining was performed on tissue sections. IHC staining for PD-1 was performed on TC tissues and matched adjacent non-tumorous tissues. In tumor tissues, PD-1 expression was predominantly detected in TILs, with strong positive staining observed. In addition, weak to moderate PD-1 expression was also noted in tumor cells themselves. By contrast, adjacent normal thyroid tissues exhibited primarily negative PD-1 staining in follicular epithelial cells, with only occasional PD-1-positive lymphocytes present. These results demonstrate that PD-1 is expressed in both TILs and tumor cells within the thyroid cancer microenvironment, while its expression is minimal in adjacent non-cancerous tissues. Correlation analyses between PD-1/PD-L1 expression levels and available clinicopathological parameters are presented in Table SIV. High and low expression groups for PD-1 and PD-L1 were defined based on the median $2^{-\Delta\Delta Cq}$ value

of RT-qPCR from all 30 PTC tissue samples; values above the median were classified as high expression, while those at or below the median were classified as low expression. Despite the data exhibiting a trend toward higher PD-L1 expression in tumors >2 cm (94.1%) compared to those ≤2 cm (61.5%), as well as in lymph node-positive (N1) patients (93.8%) compared to node-negative (N0) patients (64.3%), these differences did not reach statistical significance. Similarly, PD-1 expression showed no significant correlation with age, gender, TNM stage or BRAF mutation status. Overall, there were no statistically significant associations between PD-1/PD-L1 expression and the available clinicopathological parameters in the present study group. These data suggested that PD-1/PD-L1 might act as oncogenes in development and progression of PTC.

Downregulation of PD-1/PD-L1 inhibits the proliferation and induces the apoptosis of TC cells. To investigate the functional role of PD-1/PD-L1 in PTC development and progression, sh-NC, sh-PD-1 and sh-PD-L1 were transfected into TC cells, with the transfection efficiency being shown in Fig. 2A and B.

NIS exhibits an important role in TC progression (38). Previous studies have confirmed that loss of NIS function is a hallmark of TC dedifferentiation, which correlates with enhanced tumor invasiveness, distant metastasis and the development of radioactive iodine resistance, the main cause of mortality from advanced TC. Moreover, recent research has revealed that NIS expression is correlated with the tumor immune microenvironment and the expression of immune checkpoint molecules, including PD-1 and PD-L1, suggesting it may coordinate with immune remodeling to promote TC progression. Thus, western blotting was carried out to evaluate the effects of PD-1/PD-L1 on the expression of NIS. Fig. 2C shows that downregulation of PD-1/PD-L1 significantly promoted the expression of NIS. In addition, a CCK-8 assay was used to evaluate the effects of PD-1/PD-L1 on the viability of TC cells. As shown in Fig. 2D, downregulation of PD-1/PD-L1 significantly suppressed the viability of TC cells. An EdU assay was carried out to assess the effects of PD-1/PD-L1 on the proliferation of TC cells. As demonstrated in Fig. 2E, downregulation of PD-1/PD-L1 significantly inhibited the proliferation of TC cells. A colony formation assay was carried out to assess the effects of PD-1/PD-L1 on the clonogenic ability of TC cells and the Fig. 2F indicates that downregulation of PD-1/PD-L1 significantly suppressed the formation of TC cell colonies.

In addition, flow cytometry analysis was adopted to evaluate the effects of PD-1/PD-L1 on the apoptosis of PTC cells. Fig. 3A demonstrates that downregulation of PD-1/PD-L1 significantly promoted the apoptosis of TC cells. Furthermore, western blotting was performed to analyze the expression levels of proteins associated with apoptosis. As demonstrated in Fig. 3B, downregulation of PD-1/PD-L1 significantly reduced the protein expression level of Bcl-2, while promoting the expression of Bax and increasing the ratio of cleaved caspase-3/caspase-3 in TC cells. These findings indicate that downregulation of PD-1/PD-L1 inhibited proliferation and induced the apoptosis of TC cells.

Downregulation of PD-1/PD-L1 inhibits the migration and invasion of TC cells. Wound healing and Transwell assays were performed to analyze the effects of PD-1/PD-L1 on the migration and invasion of TC cells. As shown in Fig. 4A-C, downregulation of PD-1/PD-L1 significantly suppressed the migration and invasion of TC cells. In addition, western blotting was performed to analyze the expression levels of proteins associated with EMT progression. As demonstrated in Fig. 4D, downregulation of PD-1/PD-L1 significantly reduced the protein expression level of N-cadherin and Vimentin, while promoted the expression levels of E-cadherin in TC cells. These findings indicated that downregulation of PD-1/PD-L1 inhibited the migration and invasion of TC cells by suppressing EMT.

Downregulation of PD-1/PD-L1 suppresses PTC progression partially by regulating the TGF-β/Smad signaling pathway. TGF-β/Smad signaling pathways are key regulators of EMT, cell proliferation, apoptosis and extracellular matrix remodeling in cancer. During PTC progression, TGF-β/Smad exerts a key role by: i) Directly promoting EMT through transcriptional induction of Snail, Slug and Twist; ii) suppressing thyroid-specific genes including NIS through Smad-mediated transcriptional repression; iii) modulating the TME by regulating immune cell infiltration and cytokine production and; iv) cooperating with BRAF V600E signaling to drive TC dedifferentiation (39-42). Thus, western blot analysis was performed to evaluate the expression levels of TGF-β/Smad pathway. As shown in Fig. 5A, downregulation of PD-1/PD-L1 significantly inhibited the expression levels of TGF-β/Smad pathway. In addition, exogenous TGF-β (10 ng/ml) was used to treat TC cells with sh-PD-1 or sh-PD-L1 and western blotting was used to detect the TGF-β/Smad pathway. PD-L1 knockdown significantly suppresses basal and TGF-β-induced phosphorylation of Smad2 and Smad3 in TPC-1 cells, indicating that PD-L1 positively regulates the canonical TGF-β/Smad signaling pathway. Moreover, exogenous TGF-β partially rescues the suppression of p-Smad2/3 caused by PD-L1 silencing, suggesting a functional crosstalk between PD-L1 and TGF-β signaling in TC progression (Fig. 5B). CCK-8 assays showed that sh-PD-1 or sh-PD-L1 cells exhibited significantly reduced proliferation at 48 and 72 h compared with sh-NC (Fig. 6A). NIS was significantly downregulated upon PD-1 or PD-L1 knockdown (Fig. 6B). The result of CCK-8 assays was corroborated by EdU incorporation assays (Fig. 6C). Colony formation capacity was similarly suppressed (Fig. 6D), while apoptosis was increased (Fig. 6E). Importantly, all these phenotypic changes were significantly attenuated upon TGF-β supplementation.

PD-1 or PD-L1 deficiency significantly impaired wound closure (Fig. 7A), migration (Fig. 7B) and invasion (Fig. 7C). This coincided with a pronounced shift toward an epithelial state: E-cadherin was upregulated, whereas mesenchymal markers N-cadherin and Vimentin were downregulated (Fig. 7D). Moreover, TGF-β treatment reversed this molecular signature in sh-PD-L1 or sh-PD-1 cells. These findings indicated that downregulation of PD-1/PD-L1 suppressed TC progression partially through the TGF-β/Smad signaling pathway.

Discussion

PD-1 and PD-L1 were found to be significantly upregulated in PTC tissues and cell lines, with the present study

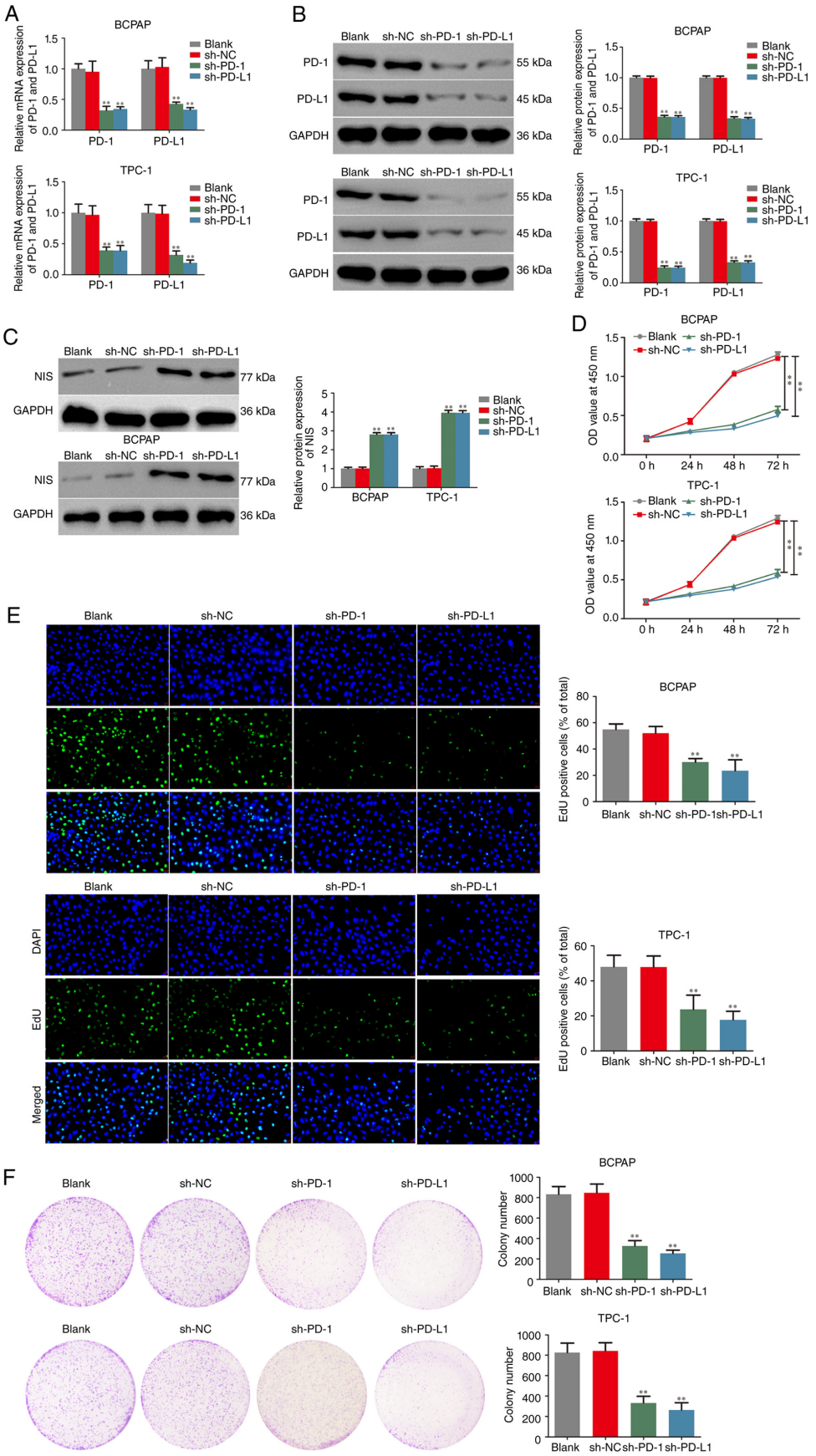

Figure 2. Downregulation of PD-1/PD-L1 inhibited the proliferation of TC cells. (A) RT-qPCR and (B) western blotting were used to assess the transfection efficiency. (C) Western blotting was carried out to evaluate the effects of PD-1/PD-L1 on the expression of NIS. (D) A Cell Counting Kit-8 assay was performed to evaluate the effects of PD-1/PD-L1 on the viability of TC cells. (E) An EdU assay was performed to evaluate the effects of PD-1/PD-L1 on the proliferation of TC cells (scale bar, 100 μm). (F) Colony formation assay was performed to evaluate the effects of PD-1/PD-L1 on the colony formation ability of TC cells. **P<0.01 vs. Blank group. RT-qPCR, reverse transcription-quantitative PCR; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TC, thyroid cancer; sh, short hairpin; NC, negative control; OD, optical density; NIS, sodium/iodine symporter.

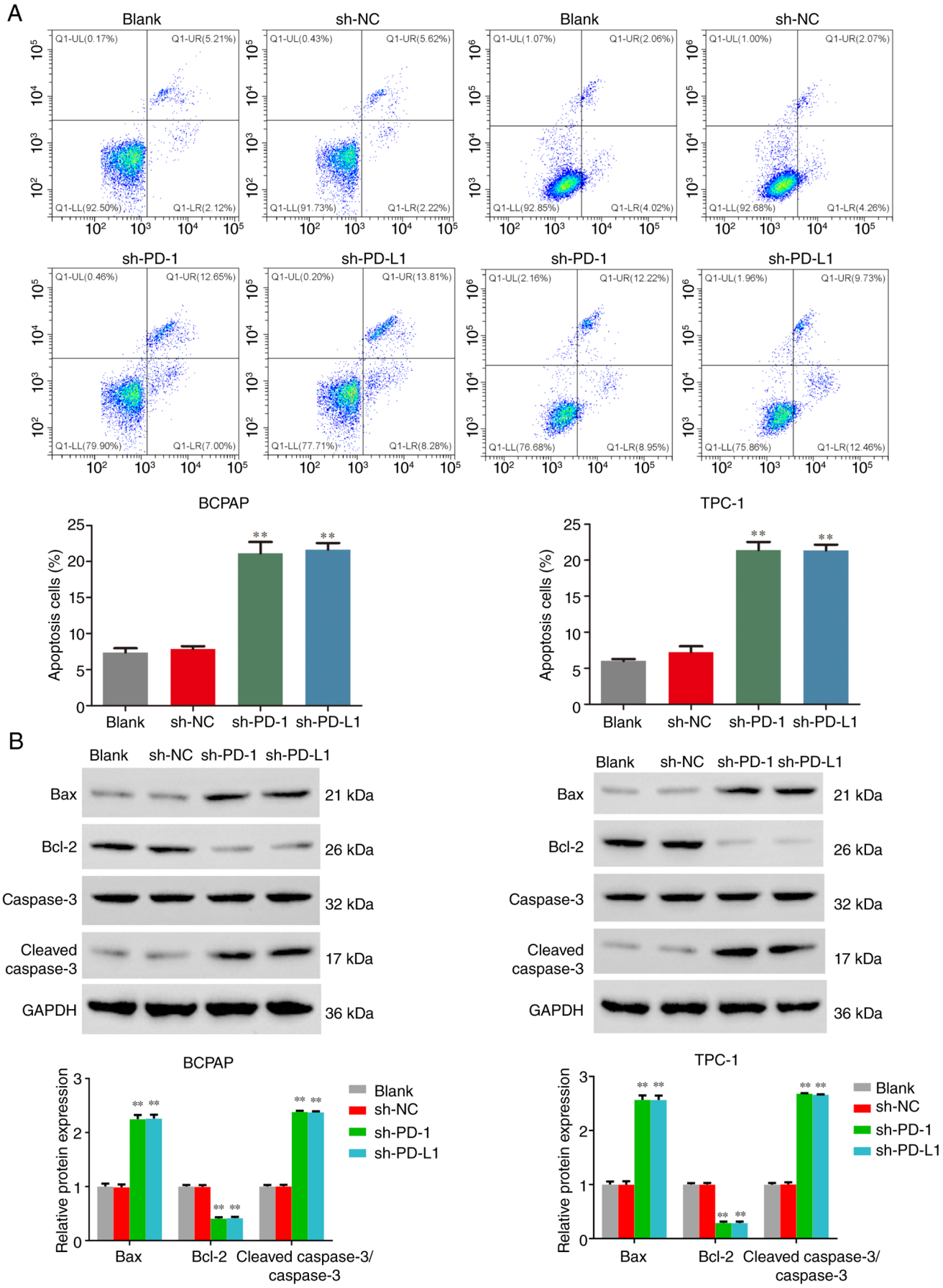

Figure 3. Downregulation of PD-1/PD-L1 induced the apoptosis of TC cells. (A) Flow cytometry analysis was adopted to evaluate the effects of PD-1/PD-L1 on the apoptosis of TC cells. (B) Western blotting was performed to evaluate the effects of PD-1/PD-L1 on the protein expression levels associated with the apoptosis of TC cells. **P<0.01 vs. the Blank group. RT-qPCR, reverse transcription-quantitative PCR; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TC, thyroid cancer; sh, short hairpin; NC, negative control.

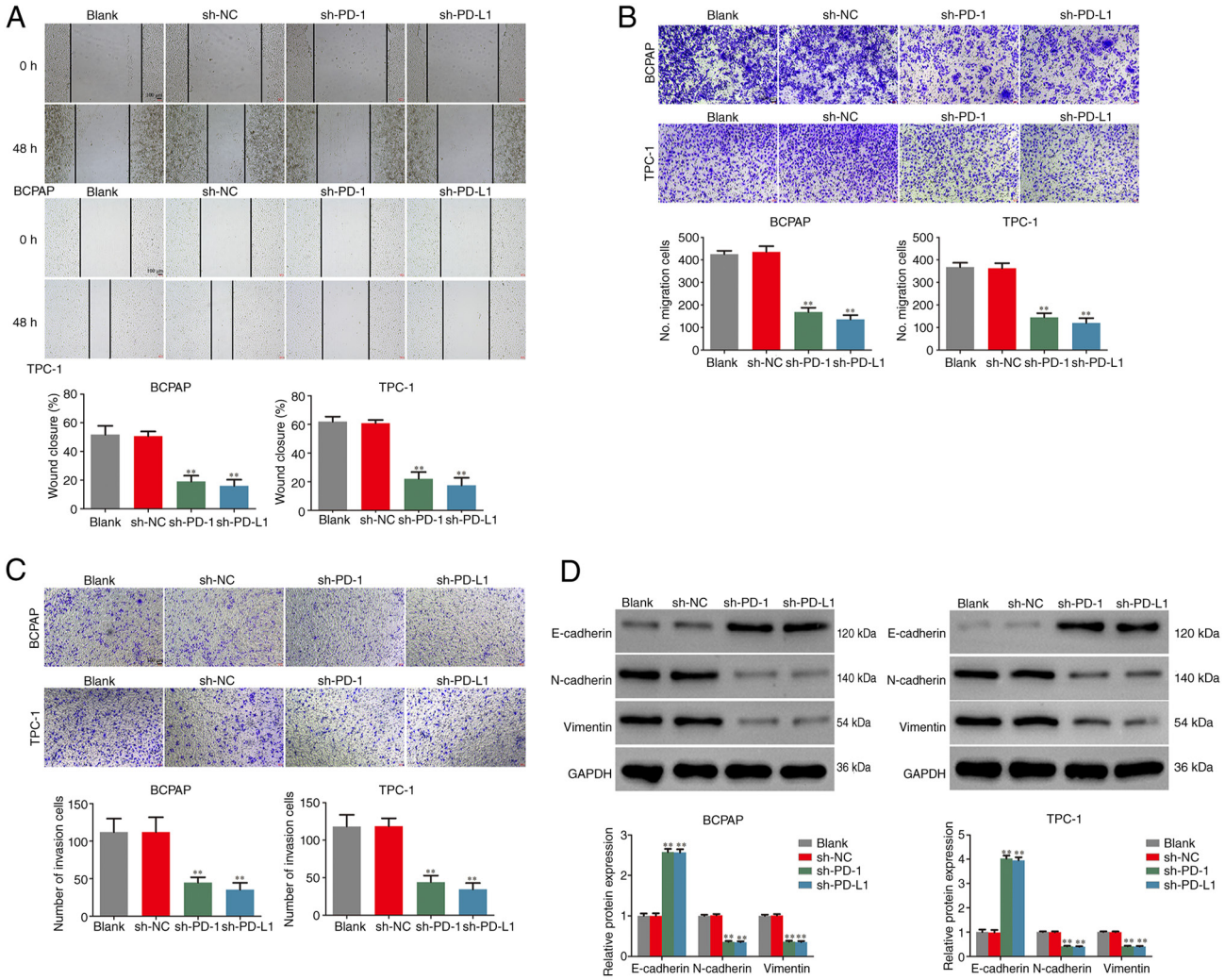

Figure 4. Downregulation of PD-1/PD-L1 inhibited the migration and invasion of TC cells. (A) Wound healing (scale bar, 100 μm), (B) Transwell migration (scale bar, 50 μm) and (C) invasion assays (scale bar, 50 μm) were performed to analyze the effects of PD-1/PD-L1 on the migration and invasion of TC cells. (D) Western blotting assay was performed to evaluate the effects of PD-1/PD-L1 on the protein expressions associated with EMT progress of TC cells. **P<0.01 vs. the Blank group. RT-qPCR, reverse transcription-quantitative PCR; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TC, thyroid cancer; EMT, epithelial-mesenchymal transition; sh, short hairpin; NC, negative control.

demonstrating their intrinsic carcinogenic effects through functional experiments. Knockdown of PD-1 or PD-L1 effectively inhibited the proliferation, colony formation, migration and invasion abilities of TC cells, while promoting apoptosis and upregulating the expression of the key differentiation/therapeutic target NIS. The mechanisms of action revealed that PD-1/PD-L1 achieved their pro-cancer effects by regulating the TGF-β/Smad signaling pathway. Intracellular PD-1/PD-L1 signaling activates transcription factors (such as STAT3 and NF-κB) that bind the TGFB1 promoter, upregulating TGF-β1 transcription. PD-1/PD-L1 signaling may also stabilize TGF-β1 mRNA or enhance its secretion. TGF-β1 then acts in an autocrine manner to activate canonical Smad2/3 signaling, as evidenced by increased Smad2/3 phosphorylation following PD-1/PD-L1 knockdown (43,44), specifically manifested as knockdown of PD-1/PD-L1 inhibiting TGF-β1 expression and Smad2/3 phosphorylation. A key rescue experiment further determined that exogenous TGF-β treatment could partially reverse the suppression of malignant phenotype caused by knockdown of PD-1/PD-L1, thus establishing the causal centrality of this signaling pathway. These results collectively constructed a clear evidence chain, indicating that the PD-1/PD-L1-TGF-β/Smad axis was a key intrinsic mechanism driving the malignant biological behavior of PTC.

The findings of the present study regarding the high expression of PD-1/PD-L1 and its intrinsic tumor functions were consistent with and complementary to a series of published articles, furthering our understanding of the role of this pathway in PTC. Previous studies have shown that PD-L1 expression was particularly prominent in TC, especially in highly aggressive subtypes such as anaplastic TC and was clearly associated with BRAF V600E mutations, disease progression and poor prognosis (5,6,15). With regard to PD-1 expression, different studies have reached varying conclusions on clinical relevance. For example, one study found that the positive rate of PD-1 in tumor cells or infiltrating lymphocytes in thyroid follicular carcinoma samples was 46.67% and its expression was associated with lower TNM staging and improved progression-free survival (45). Whilst the aforementioned study observed the basic high expression

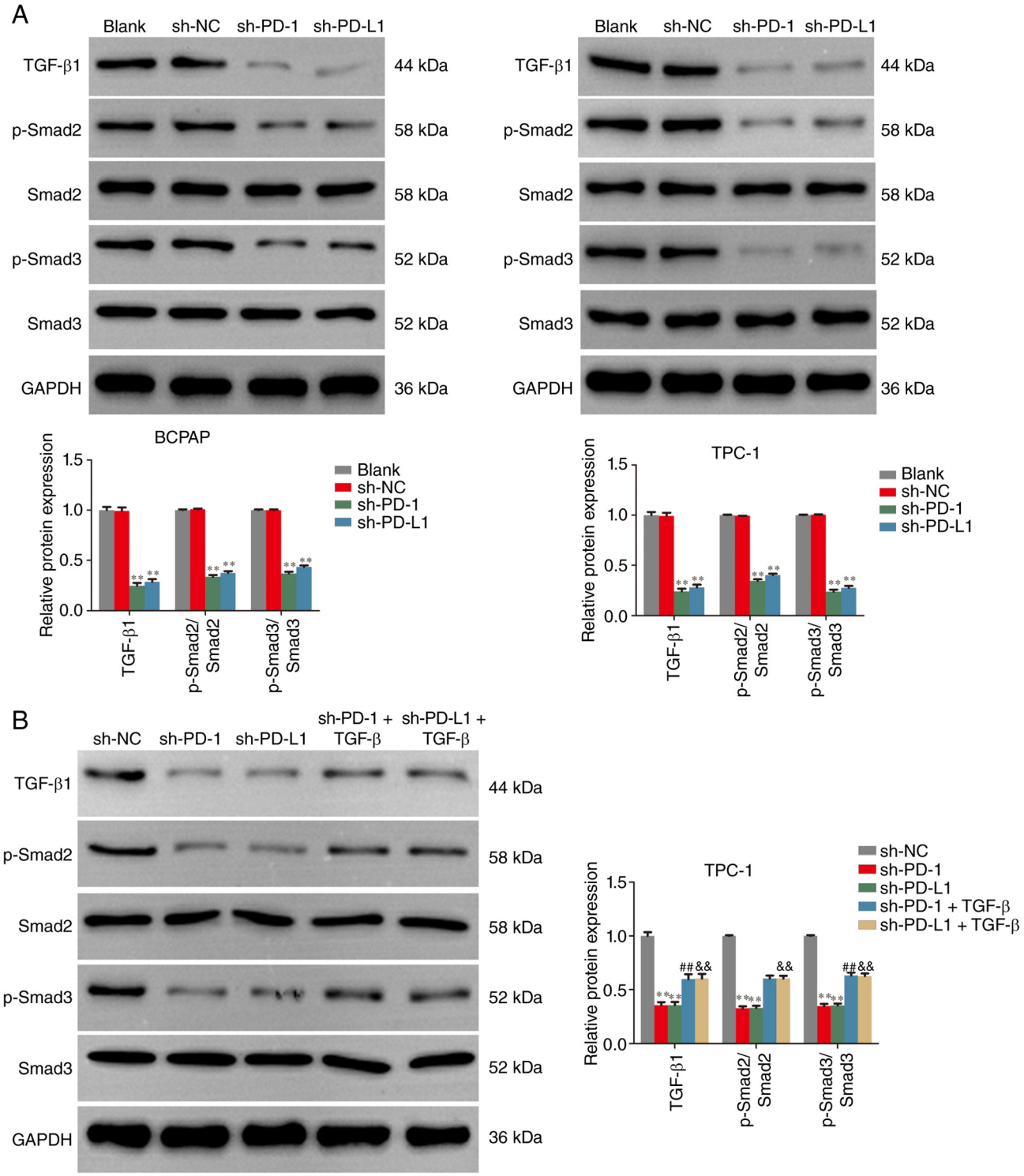

Figure 5. Downregulation of PD-1/PD-L1 inhibited the expression of TGF-β/Smad signaling pathway of TC cells. (A) Western blotting was performed to evaluate the effects of PD-1/PD-L1 on the expression levels of TGF-β/Smad signaling pathway of TC cells. (B) Western blotting was performed to evaluate the expression levels of TGF-β/Smad signaling pathway of TC cells. **P<0.01 vs. Blank or sh-NC group, $^{\#\#}$P<0.01 vs. sh-PD-1 group and $^{\&\&}$P<0.01 vs. PD-L1 group. PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TC, thyroid cancer; sh, short hairpin; NC, negative control; p-, phosphorylated.

of PD-1/PD-L1 in TC cell lines, it proposed at the molecular level that this axis participated in the direct intrinsic signaling mechanism of tumor cell EMT and proliferation-apoptosis through the TGF-β/Smad pathway, which was an important supplement to its intrinsic tumor functions (46). This not only demonstrated its potential value as prognostic biomarkers but also clarified the role as a driving force in tumor progression, providing a scientific hypothesis for further understanding the therapeutic efficacy differences among patients.

Beyond tumor-intrinsic mechanisms, the PD-1/PD-L1 axis exerts notable effects on the immune TME. PD-L1 expression on tumor cells engages PD-1 on tumor-infiltrating T lymphocytes, promoting T-cell exhaustion and functional impairment (24). This immune checkpoint mechanism

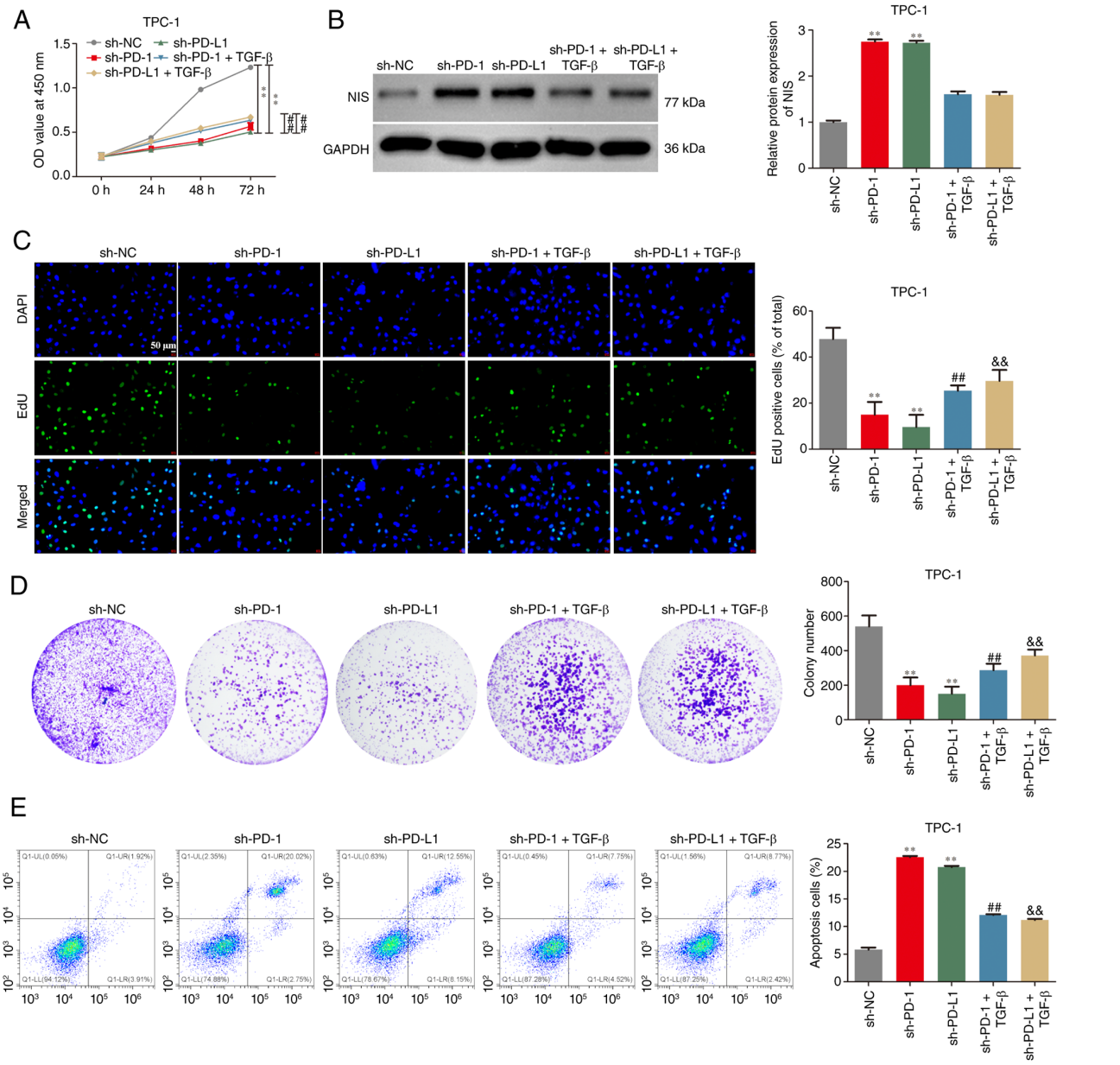

Figure 6. Downregulation of PD-1/PD-L1 inhibited the proliferation and induced the apoptosis of TC cells through regulating TGF-β/Smad signaling pathway. (A) A Cell Counting Kit-8 assay was performed to evaluate the viability of TC cells. (B) Western blotting was carried out to evaluate the expression of NIS. (C) An EdU assay was performed to evaluate the proliferation of TC cells (scale bar, 100 μm). (D) A colony formation assay was performed to evaluate the colony formation ability of TC cells. (E) Flow cytometry analysis was performed to evaluate the apoptosis of TC cells. **P<0.01 vs. Blank or sh-NC group, $^{\#\#}$P<0.01 vs. sh-PD-1 group and $^{\&\&}$P<0.01 vs. PD-L1 group. PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TC, thyroid cancer; sh, short hairpin; NC, negative control; NIS, sodium/iodine symporter; OD, optical density.

likely contributes to the immunosuppressive TME observed in aggressive thyroid cancers. TAMs, particularly the M2-polarized subtype, are abundant in thyroid cancer stroma and have been shown to express PD-L1, further amplifying immune evasion (15). In addition, IL-17A, a pro-inflammatory cytokine produced by Th17 cells, has been associated with disease progression in PTC (25). The canonical IL-17 signaling pathway is initiated by IL-17 binding to the IL-17RA/IL-17RC receptor complex, which recruits the adaptor protein Act1 and activates downstream NF-κB and MAPK signaling cascades, leading to the production of pro-inflammatory cytokines including IL-6, TNF-α and TGF-β. IL-17 has been shown to upregulate PD-L1 expression in non-small cell lung cancer, hepatocellular carcinoma, colorectal cancer and ovarian cancer through NF-κB-dependent mechanisms, and TGF-β serves as a downstream mediator of IL-17-driven inflammation. Thus, IL-17 signaling may potentiate the PD-1/PD-L1-TGF-β/Smad axis through both direct transcriptional regulation of PD-L1 and indirect amplification of TGF-β signaling within the tumor microenvironment. This crosstalk warrants further investigation in TC.

The interaction between IL-17 signaling and the TGF-β pathway is particularly relevant to the present findings, as IL-17 can potentiate TGF-β-mediated EMT and promote a pro-tumorigenic inflammatory microenvironment (25). Studies by Banerjee *et al* (24,25) have highlighted the clinical

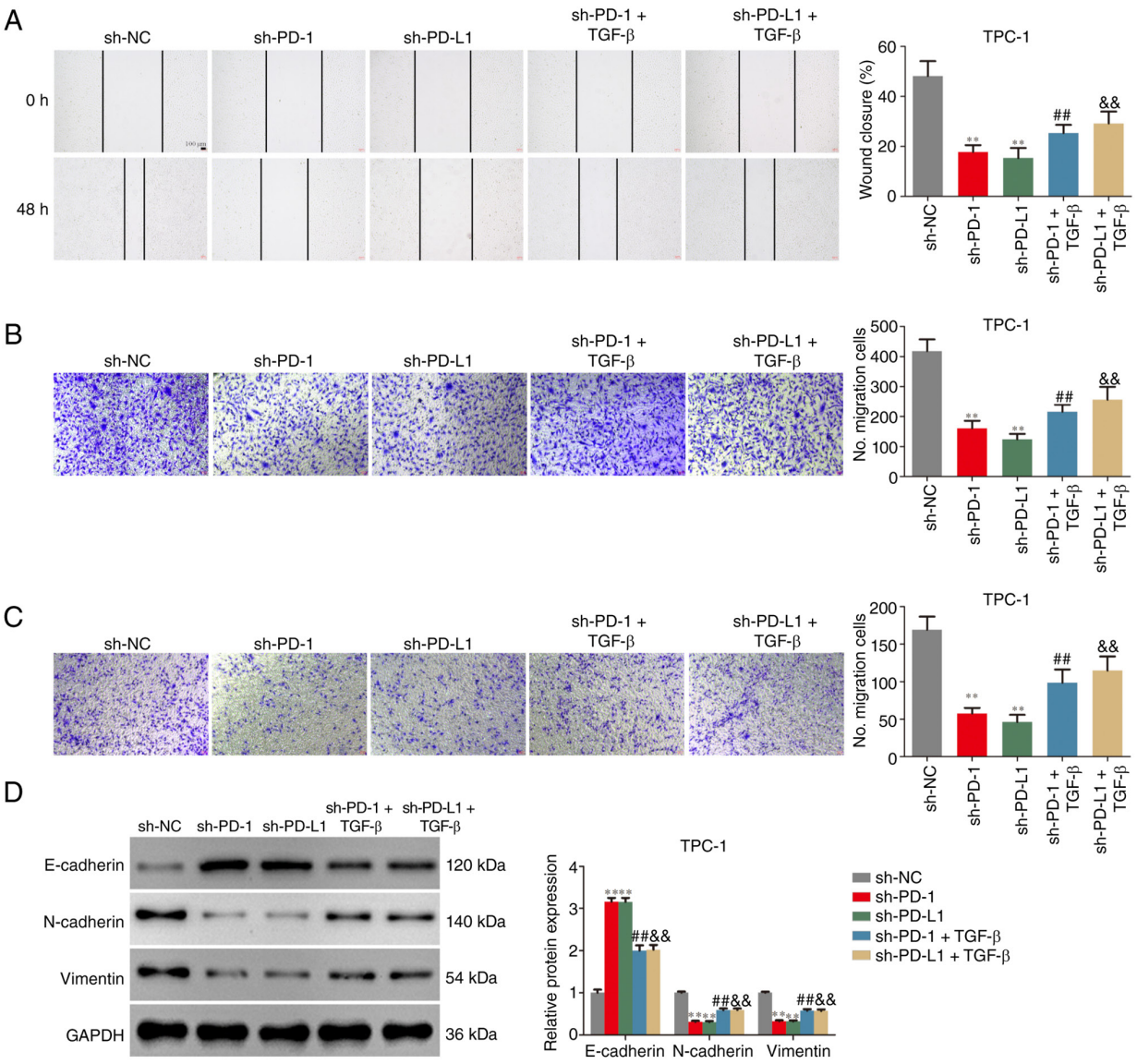

Figure 7. Downregulation of PD-1/PD-L1 inhibited the migration and invasion of TC cells regulating TGF-β/Smad signaling pathway. (A) Wound healing (scale bar, 100 μm), (B) Transwell migration (scale bar, 50 μm) and (C) invasion assays (scale bar, 50 μm) were performed to analyze the migration and invasion of TC cells. (D) Western blotting was performed to evaluate the expression levels associated with EMT progress of TC cells. **P<0.01 vs. Blank or sh-NC group, $^{\#\#}$P<0.01 vs. sh-PD-1 group and $^{\&\&}$P<0.01 vs. PD-L1 group. PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TC, Thyroid cancer; sh, short hairpin; NC, negative control; EMT, epithelial-mesenchymal transition.

importance of these interactions in PTC, including their association with higher tumor nodal stage, increased extrathyroidal invasion and elevated risk of lymph node metastasis, all clinical features linked to poor prognosis in patients with PTC. Future investigations examining the interplay between PD-1/PD-L1 signaling, IL-17-mediated inflammation, and TGF-β activation in the TME may provide further insights into the immune regulatory networks governing thyroid cancer progression.

It is important to reconcile the present findings with previous studies that have reported associations between PD-1 expression and favorable clinical outcomes in certain thyroid cancer contexts. For example, Ning *et al* (45) observed that PD-1 positivity in thyroid follicular carcinoma samples was associated with lower TNM staging and improved progression-free survival (40). This apparent discrepancy may reflect the context-dependent nature of PD-1 signaling. A number of possible explanations may therefore be hypothesized: First, PD-1 expression on tumor-infiltrating lymphocytes may serve as a marker of an active anti-tumor immune response, which would be associated with improved prognosis, whereas tumor-intrinsic PD-1 signaling (as investigated in the present study) may promote malignant behavior. Second, differences in detection methods (IHC vs. RT-qPCR/western blotting) and the specific antibodies used may capture different aspects of PD-1 expression, with IHC typically detecting cell-surface PD-1 protein on intact tumor tissue, while RT-qPCR measures total transcript levels and western blotting quantifies total cellular protein (including both intracellular and membrane-localized pools). Third, the clinical importance of PD-1 may differ across thyroid cancer subtypes and disease stages. The coexistence of these context-dependent roles underscores the complexity of PD-1/PD-L1 biology and highlights the need for integrated analyses that simultaneously evaluate both tumor-intrinsic and immune microenvironment-associated mechanisms.

The clinical importance of the present findings is further underscored by the observed upregulation of NIS expression following PD-1 or PD-L1 knockdown. NIS is the primary mediator of radioiodine uptake in thyroid cells and its downregulation is a hallmark of radioiodine-refractory thyroid cancer. TGF-β has been shown to repress NIS expression through Smad-dependent signaling (40-42) and the present data suggest that PD-1/PD-L1-mediated activation of the TGF-β/Smad pathway may contribute to NIS silencing and subsequent radioiodine resistance. These findings suggest that targeting the PD-1/PD-L1/TGF-β/Smad axis could restore radioiodine sensitivity in refractory patients, although this hypothesis requires rigorous preclinical and clinical evaluation.

At a mechanistic level, the present study was able to associate PD-1/PD-L1 with the TGF-β/Smad signaling pathway, providing a new molecular explanation framework that enhances the existing literature. Previous studies have primarily focused on the 'interaction' between PD-1/PD-L1 and immune function within the tumor microenvironment, revealing crosstalk with other cancer-promoting pathways such as MAPK and PI3K/Akt (47,48). The TGF-β/Smad pathway was selected based on the following rationale: i) TGF-β is the most consistently upregulated cytokine in TC and is associated with EMT and NIS repression; ii) BRAF V600E induces TGF-β secretion which suppresses NIS expression through Smad signaling; iii) the present preliminary data showed that PD-1/PD-L1 knockdown predominantly affected TGF-β/Smad signaling with minimal changes in MAPK or PI3K/Akt pathways and; iv) the role of PD-1/PD-L1 in TGF-β-driven tumor-intrinsic signaling was previously unknown in TC (47,48).

In addition, the TGF-β/Smad signaling pathway is an important intracellular signaling pathway involved in the malignant biological processes of tumor cells. Studies have shown that the activation of the TGF-β/Smad signaling pathway was associated with the migration of TC cells and lymph node metastasis. For example, REGγ mediated the upregulation of TGF-β by degrading Smad7, enhancing the dedifferentiation of TC cells. The absence of REGγ restored the expression of thyroid-specific genes, especially the expression of the NIS in TC cells, thereby enhancing the uptake of radioactive iodine (40). In TC, the BRAF V600E gene could induce the secretion of TGF-β, which in turn inhibited NIS expression (34). Furthermore, reduced NADPH oxidase 4 was found to be a key mediator of NIS downregulation in BRAF V600E-induced TC and this process was associated with the TGF-β/Smad signaling pathway (40). Therefore, inhibiting this signaling pathway may exert an anti-tumor effect on TC.

The novelty of the present study lies in: i) Demonstrating that PD-1 and PD-L1 function through the TGF-β/Smad pathway specifically in thyroid cancer cells, establishing a tumor-intrinsic mechanism beyond immune checkpoint function; ii) revealing that this signaling axis regulates NIS expression, which has direct clinical implications for radioiodine therapy and; iii) providing comprehensive functional evidence to associate intrinsic PD-1/PD-L1 signaling with the full spectrum of TC malignant behaviors (proliferation, apoptosis, migration, invasion, and EMT) through the TGF-β/Smad pathway.

Regarding the exclusion of other signaling pathways, the following additional considerations apply. For the MAPK pathway, although BRAF V600E is the most frequent oncogenic driver in PTC and signals principally through the MAPK/ERK cascade, the present preliminary screening showed that PD-1/PD-L1 knockdown produced negligible changes in ERK1/2 phosphorylation, indicating that the tumor-intrinsic effects observed in the present study were not primarily mediated by MAPK signaling. For the PI3K/Akt pathway: similarly, no consistent change in Akt phosphorylation was detected following PD-1/PD-L1 knockdown, and the PI3K/Akt axis is more commonly implicated in FTC and radioiodine-refractory disease rather than in the PTC-derived models used here. Collectively, the combination of the present preliminary pathway-screening data, the well-established association between TGF-β/Smad signaling and NIS repression/EMT in thyroid cancer (49,50) and the absence of changes in MAPK or PI3K/Akt activity supports the prioritization of the TGF-β/Smad pathway, although it should be acknowledged that parallel or compensatory signalling cannot be fully excluded on the basis of the present experiments (47,48).

Based on the present study and existing literature, it could be considered that strategies targeting the PD-1/PD-L1 pathway, especially combination therapies, hold potential in the treatment of TC. When exploring combination therapy strategies, additional studies have also provided support. For instance, basic experiments have found that simultaneous inhibition of IRE1α and PD-L1 in the endoplasmic reticulum stress pathway yields notable synergistic effects in inhibiting TC in both *in vitro* and *in vivo* models (51). Similarly, a further study has observed that in a mouse-derived TC model, the expression of TGF-βR2 promoted immune-associated adverse events in the liver induced by anti-PD-1/PD-L1 treatment (52). Analysis of the experiments carried out in the present study revealed that exogenous TGF-β treatment could partially reverse the malignant phenotype suppression caused by knockdown of PD-1/PD-L1. These findings collectively suggest that simultaneously targeting immune checkpoints and related signaling networks may be an effective approach to overcome monotherapy resistance and enhance therapeutic efficacy and that targeting TGF-β signaling may represent a potential combination for achieving synergistic effects. However, it must be emphasized that these combination strategies remain highly speculative at present and extensive preclinical validation in appropriate *in vivo* models, followed by carefully designed clinical trials, is needed before any clinical translation can be considered.

The present study exhibits certain limitations. The present research was primarily based on *in vitro* cell experiments and future verification in xenograft animal models (subcutaneous and orthotopic mouse models) is currently being planned, along with further exploration of whether there were differences in this mechanism among different TC subtypes. No dose response analysis or alternative pathway inhibitors were used to rule off target effects or other parallel pathways. In addition, PD-1/PD-L1 may also regulate other pathways besides TGF-β/Smad, which remain to be revealed using more comprehensive omics technologies. The lack of corresponding rescue experiments involving PD-1/PD-L1 overexpression limits the ability to definitively rule out potential off-target effects, although the consistency of

the present findings support the proposed mechanism. Finally, reviewing published literature revealed that the efficacy prediction of PD-1/PD-L1 not only depended on expression levels, but was also influenced by factors such as the inflammatory background of patients, epigenetic regulation and complex interactions with other signaling pathways. Future preclinical and clinical studies should aim to take these complex regulatory networks into account.

In summary, the present study provided experimental evidence for the direct carcinogenic role of the PD-1/PD-L1 axis in the progression of PTC and clarified the core molecular mechanisms through which it achieved this function by activating the TGF-β/Smad signaling pathway. This not only contributes to the understanding of the pathogenesis of PTC but also provides a theoretical foundation for future clinical exploration of more precise and effective treatment strategies, which is expected to bring new hope to patients with advanced and highly aggressive PTC who have failed to respond to traditional treatments. It is important to acknowledge that tumor-intrinsic and immune microenvironment-associated mechanisms likely coexist and interact in the regulation of PD-1/PD-L1 signaling in PTC and further studies are warranted to dissect their relative contributions and potential synergistic effects.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BZ is the guarantor of the integrity of the entire study and contributed to the study design and study concepts. XW and JW contributed to the literature research, data analysis and clinical studies. WC, JXW and XZ contributed to data acquisition and statistical analysis. All authors contributed to the manuscript editing and reviewing. All authors have read and approved the final version of the manuscript. BZ and XW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present experiments were approved by the Ethics Committee of the People's Hospital of Ningxia Hui Autonomous Region (Yinchuan, China; approval no. 2025-NZR-291). All procedures were performed under strict constraints of the Enhancing the Quality and Transparency of Health Research network guidelines, the relevant norms and regulations for clinical research and the ethics codes in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ma L, Xie Y, Ke S, Liu D, Wang L, Wang L and Liu L: Levothyroxine dose prediction post-thyroidectomy for differentiated thyroid carcinoma. Front Endocrinol (Lausanne) 16: 1727681, 2025.
2. Zhu Q, Liu J, Hu J and Zhang Y: The epidemiological landscape of thyroid cancer and estimates of overdiagnosis in China: A population-based study. Thyroid 35: 307-320, 2025.
3. Gao N, Han J, Sun X, Yu J, Huang J, Liu R, Tang YL and Nie C: Erianin induces apoptosis of thyroid cancer cells via TNF signaling pathway. Eur J Pharmacol 1006: 178167, 2025.
4. Li C, Li Q, Shi X, Han S, Song X, Li X and Zhuang X: Papillary thyroid microcarcinoma and papillary thyroid carcinoma: Clinical characteristics and stratification of treatment strategies. PLoS One 20: e0327423, 2025.
5. Liang L, Chen Z, Lei D, Mo C, Lan D, Ke J, Wang W, Yang Z, Guo X, Chen D, *et al*: APG-115 induces SLC7A11-mediated ferroptosis and upregulates PD-L1 expression in thyroid cancer. ACS Omega 10: 31099-31107, 2025.
6. Elyamany AM, Abdel Haleem RT, Soliman NA, Mahdi L, Ahmed HMA and Abdelwahab KH: Expression of long non-coding RNA-NEAT-1 in papillary thyroid carcinoma in relation to immunohistochemical expression of CD8 and PD-L1 as indicators of tumor immune microenvironment. Asian Pac J Cancer Prev 26: 3093-3101, 2025.
7. Xue Y, Zhuang Y and Chen S: Dynamic survival outcomes of the tall-cell variant of papillary thyroid carcinoma patients after surgery. Front Endocrinol (Lausanne) 16: 1517907, 2025.
8. Toloza FJK, Gubbi S and Klubo-Gwiezdzinska J: Trends in survival and factors associated with survival in primary thyroid lymphoma: A SEER-based analysis (1975-2021). Thyroid 35: 1074-1082, 2025.
9. Gu Y, Yu M, Deng J and Lai Y: Preoperative circulating tumor cells level is associated with lymph node metastasis in patients with unifocal papillary thyroid carcinoma. World J Surg Oncol 23: 47, 2025.
10. Shen P, Yang Z, Sun J, Wang Y, Qiu C, Wang Y, Ren Y, Liu S, Cai W, Lu H and Yao S: Explainable multimodal deep learning for predicting thyroid cancer lateral lymph node metastasis using ultrasound imaging. Nat Commun 16: 7052, 2025.
11. Liu Y, Huang S, Li X, Tian T and Huang R: Therapeutic efficacies of remnant ablation and radioiodine adjuvant therapy in differentiated thyroid cancer. Endocrine 87: 734-743, 2025.
12. Feng Y, Xiao A, Xing C, Dai Q, Liu X, Liu J and Feng L: Elevated thyroid-stimulating hormone levels, independent of Hashimoto's thyroiditis, increase thyroid cancer risk: Insights from genetic and clinical evidence. Endocrine 88: 175-184, 2025.
13. Pishdad R and Ahmadi S: Thyroid-stimulating hormone (TSH) suppression therapy in thyroid cancer: Balancing benefits and harms. Endocr Pract 32: 401-405, 2026.
14. He Z, Wang C, Liu C, Zhang K, Wang J, Wang X, Zhang Y and Chen L: Limited value of genetic profiling in guiding radioiodine therapy for metastatic differentiated thyroid cancer. Endocr Relat Cancer 32: e240298, 2025.
15. Ricarte-Filho JC, Caperton CO, Isaza A, Massoll NA and Franco AT: Macrophage abundance in oncocytic thyroid cancer is associated with PD-L1 expression. Endocr Oncol 5: e250026, 2025.
16. Piermattei A, Migliara G, Feraco A, Bruno C, Cioni L, Zhang Q, Padial-Urtueta B, Merenda E, Fadda G, Raffaelli M, *et al*: Evaluation of PD-L1, TERT promoter mutations, and BRAFV600E mutation in poorly differentiated, differentiated high grade thyroid carcinoma and anaplastic carcinoma of the thyroid: Our institutional experience. Virchows Arch 487: 605-618, 2025.
17. Forma A, Kłodnicka K, Pająk W, Flieger J, Teresińska B, Januszewski J and Baj J: Thyroid cancer: Epidemiology, classification, risk factors, diagnostic and prognostic markers, and current treatment strategies. Int J Mol Sci 26: 5173, 2025.

18. Li X, Gao S, Shan C, Zhang Q, Tan Y, Yu X and Yu J: Advances in PD-1/PD-L1 pathway inhibitors in the treatment of thyroid cancer: Mechanisms and clinical therapeutic perspectives. Front Immunol 16: 1643421, 2025.
19. Zhang J, Bu X, Wang H, Zhu Y, Geng Y, Nihira NT, Tan Y, Ci Y, Wu F, Dai X, *et al*: Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance. Nature 553: 91-95, 2018.
20. Wang Y, Ma X, Ma J, Li J, Lin Z, Gao W, Gong P and Dai P: Thyroid dysfunction as a predictor of PD- 1/PD-L1 inhibitor efficacy in advanced lung cancer. BMC Cancer 25: 791, 2025.
21. Yan X, Lv Q, Wu J, Fang J, Peng L and Zhao X: The efficacy and immune-mediated safety of PD-1/PD-L1 combined with neoadjuvant chemotherapy in triple-negative breast cancer: A meta-analysis. Front Oncol 15: 1635418, 2025.
22. Liotti F, Kumar N, Prevete N, Marotta M, Sorriento D, Ieranò C, Ronchi A, Marino FZ, Moretti S, Colella R, *et al*: PD-1 blockade delays tumor growth by inhibiting an intrinsic SHP2/Ras/MAPK signalling in thyroid cancer cells. J Exp Clin Cancer Res 40: 22, 2021.
23. Lin J, Qiu Y, Zheng X, Dai Y and Xu T: The miR-199a-5p/PD-L1 axis regulates cell proliferation, migration and invasion in follicular thyroid carcinoma. BMC Cancer 22: 756, 2022.
24. Banerjee S, Nahar U, Dahiya D, Mukherjee S, Dey P, Gupta R, Radotra B, Sachdeva N, Sood A, Bhadada SK and Bhansali A: Role of cytotoxic T cells and PD-1 immune checkpoint pathway in papillary thyroid carcinoma. Front Endocrinol (Lausanne) 13: 931647, 2022.
25. Banerjee S, Nahar U, Dahiya D, Gupta R, Mukherjee S, Sachdeva N, Sood A, Dey P, Radotra B and Bhansali A: IL-17 A correlates with disease progression in papillary thyroid carcinoma. Diagn Pathol 18: 93, 2023.
26. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, *et al*: Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nat Med 8: 793-800, 2002.
27. Cheng W, Kang K, Zhao A and Wu Y: Dual blockade immunotherapy targeting PD-1/PD-L1 and CTLA-4 in lung cancer. J Hematol Oncol 17: 54, 2024.
28. Han Y, Koehler VF, Nagarajah J, Schmohl KA, Stauss C, Schwenk N, Spellerberg R, Kitzberger C, Kumbrink J, Zach C, *et al*: TGF-β1-based restoration of sodium iodide symporter expression in radioiodine-refractory differentiated thyroid cancer via engineered MSCs. Mol Ther 33: 6130-6145, 2025.
29. Xiang J, Lv N, Yin S, Zhao T, Liu F, Cheng L, Liu F and Kuang J: TGF-β induces EMT in thyroid cancer cells by regulating transcription factors. Thyroid Res 18: 25, 2025.
30. Garcia-Rendueles AR, Rodrigues JS, Garcia-Rendueles MER, Suarez-Fariña M, Perez-Romero S, Barreiro F, Bernabeu I, Rodriguez-Garcia J, Fugazzola L, Sakai T, *et al*: Rewiring of the apoptotic TGF-β-SMAD/NFκB pathway through an oncogenic function of p27 in human papillary thyroid cancer. Oncogene 36: 652-666, 2017.
31. Zhao JJ, Hao S, Wang LL, Hu CY, Zhang S, Guo LJ, Zhang G, Gao B, Jiang Y, Tian WG and Luo DL: Long non-coding RNA ANRIL promotes the invasion and metastasis of thyroid cancer cells through TGF-β/Smad signaling pathway. Oncotarget 7: 57903-57918, 2016.
32. Lin Q, Qi Q, Hou S, Chen Z, Jiang N, Zhang L and Lin C: Activation of the TGF-β1/Smad signaling by KIF2C contributes to the malignant phenotype of thyroid carcinoma cells. Tissue Cell 73: 101655, 2021.
33. Ma S, Wang Q, Ma X, Wu L, Guo F, Ji H, Liu F, Zhao Y and Qin G: FoxP3 in papillary thyroid carcinoma induces NIS repression through activation of the TGF-β1/Smad signaling pathway. Tumour Biol 37: 989-998, 2016.
34. Jung CK, Bychkov A and Kakudo K: Update from the 2022 World Health Organization classification of thyroid tumors: A standardized diagnostic approach. Endocrinol Metab (Seoul) 37: 703-718, 2022.
35. van Staveren WCG, Solís DW, Delys L, Duprez L, Andry G, Franc B, Thomas G, Libert F, Dumont JE, Detours V and Maenhaut C: Human thyroid tumor cell lines derived from different tumor types present a common dedifferentiated phenotype. Cancer Res 67: 8113-8120, 2007.
36. Saiselet M, Floor S, Tarabichi M, Dom G, Hébrant A, van Staveren WC and Maenhaut C: Thyroid cancer cell lines: An overview. Front Endocrinol (Lausanne) 3: 133, 2012.
37. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
38. Shen DHY, Chan HP, Tsai FR, Chiu YL, Liang TJ, She Y, Liu AC, Yeh HY, Tsai KW and Li SC: MDM2 Knockdown reduces the oncogenic activities and enhances NIS protein abundance in papillary thyroid cancer. Cancer Genomics Proteomics 22: 444-457, 2025.
39. Bhatti MZ, Pan L, Wang T, Shi P and Li L: REGγ potentiates TGF-β/Smad signal dependent epithelial-mesenchymal transition in thyroid cancer cells. Cell Signal 64: 109412, 2019.
40. Jiao C, Li L, Zhang P, Zhang L, Li K, Fang R, Yuan L, Shi K, Pan L, Guo Q, *et al*: REGγ ablation impedes dedifferentiation of anaplastic thyroid carcinoma and accentuates radio-therapeutic response by regulating the Smad7-TGF-β pathway. Cell Death Differ 27: 497-508, 2020.
41. Riesco-Eizaguirre G, Rodríguez I, De la Vieja A, Costamagna E, Carrasco N, Nistal M and Santisteban P: The BRAFV600E oncogene induces transforming growth factor beta secretion leading to sodium iodide symporter repression and increased malignancy in thyroid cancer. Cancer Res 69: 8317-8325, 2009.
42. Azouzi N, Cailloux J, Cazarin JM, Knauf JA, Cracchiolo J, Al Ghuzlan A, Hartl D, Polak M, Carré A, El Mzibri M, *et al*: NADPH oxidase NOX4 is a critical mediator of $BRAF^{V600E}$-induced downregulation of the sodium/iodide symporter in papillary thyroid carcinomas. Antioxid Redox Signal 26: 864-877, 2017.
43. Yu H, Pardoll D and Jove R: STATs in cancer inflammation and immunity: A leading role for STAT3. Nat Rev Cancer 9: 798-809, 2009.
44. Lee KY, Ito K, Hayashi R, Jazrawi EPI, Barnes PJ and Adcock IM: NF-kappaB and activator protein 1 response elements and the role of histone modifications in IL-1beta-induced TGF-beta1 gene transcription. J Immunol 176: 603-615, 2006.
45. Ning Q, Liu J, Liu S, Zou Q, Li K and Li Z: TRx0237 induces apoptosis and enhances anti-PD-1 immunotherapeutic efficacy in anaplastic thyroid cancer. Int Immunopharmacol 155: 114610, 2025.
46. Eloy C, Santos J, Cameselle-Teijeiro J, Soares P and Sobrinho-Simões M: TGF-beta/Smad pathway and BRAF mutation play different roles in circumscribed and infiltrative papillary thyroid carcinoma. Virchows Arch 460: 587-600, 2012.
47. Bullock KK and Richmond A: Beyond anti-PD-1/PD-L1: Improving immune checkpoint inhibitor responses in triple-negative breast cancer. Cancers (Basel) 16: 2189, 2024.
48. Quan Z, Yang Y, Zheng H, Zhan Y, Luo J, Ning Y and Fan S: Clinical implications of the interaction between PD-1/PD-L1 and PI3K/AKT/mTOR pathway in progression and treatment of non-small cell lung cancer. J Cancer 13: 3434-3443, 2022.
49. Zhuang J, Huang B, Li J, Wang Y, Xu Y, Yu X, Shen H and Gao W: Upregulation of FLT1 activates the TGF-β1/Smad3 pathway to promote iodide resistance in thyroid cancer. Cancer Treat Res Commun 45: 100991, 2025.
50. Yan Z, Yangyanqiu W, Shuwen H, Jing M, Haihong L, Gong C, Yin J, Qing Z and Weili G: Downregulation of Rap1GAP expression activates the TGF-β/Smad3 pathway to inhibit the expression of sodium/iodine transporter in papillary thyroid carcinoma cells. Biomed Res Int 2021: 6840642, 2021.
51. Yao X, Tu Y, Xu Y, Guo Y, Yao F and Zhang X: Endoplasmic reticulum stress-induced exosomal miR-27a-3p promotes immune escape in breast cancer via regulating PD-L1 expression in macrophages. J Cell Mol Med 24: 9560-9573, 2020.
52. Qiu X, Zhu J, Sun Y, Fan K, Yang DR, Li G, Yang G and Chang C: TR4 nuclear receptor increases prostate cancer invasion via decreasing the miR-373-3p expression to alter TGFβR2/p-Smad3 signals. Oncotarget 6: 15397-15409, 2015.