Original Article
CD47 is associated with the up-regulation of the PD-1 oncogenic signaling pathway

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Abstract: Cluster of differentiation 47 (CD47) serves as an important negative indicator for phagocytic cells and has been reported to be overexpressed in multiple human tumor cells. Increasing evidence has suggested that CD47 overexpression may contribute to the immune escape of tumor cells by avoiding phagocytosis. However, it is currently unclear whether CD47 participates in the tumorigenesis of thyroid cancer (TC). The aim of this study was to explore the roles of CD47 in TC. In two TC cell lines, TPC-1 and K1, the CD47 expression was determined by Western blot analysis, qRT-PCR, and flow cytometry assays. The CD47 shRNA expression vector was applied to specifically decrease CD47 expression in TPC-1 and K1 cells, and the effects of CD47 knockdown on cell proliferation, apoptosis, cycle were evaluated by flow cytometry analysis. In addition, the effects of CD47 knockdown on the expression of proteins involved in the programmed death-1 (PD-1) signaling pathway were assessed by Western blot analysis. Our results indicated that when compared with normal human thyroid follicular epithelial Nthy-ori-3-1 cells, CD47 expression was significantly upregulated in both TPC-1 and K1 cells. In the functional assay, we revealed that the knockdown of CD47 inhibited cell growth and promoted cell apoptosis. Mechanistically, the PD-L1 signaling pathway was found to be activated in TPC-1 and K1 cells, and the knockdown of CD47 significantly suppressed the activation of this pathway. In conclusion, CD47 was highly expressed in TC cells, and may serve as an oncogenic molecule to promote TC progression by regulating PD-L1 signaling.

Keywords: Apoptosis, CD47, PD-L1, proliferation, thyroid cancer

Introduction

Worldwide, the incidence of thyroid cancer (TC), specifically papillary TC, has increased over the last decades [1, 2]. As one of the most common endocrine malignancies, TC accounts for over 90 percent of all newly identified endocrine cancers and roughly 60 percent of endocrine cancer-related deaths [3, 4]. According to a report of the American Cancer Society in 2017, in the United States, 56,870 new TC cases were estimated, including 2010 deaths that were related to TC [5]. The discrepancy between the total number of new TC cases and TC-related deaths reflected the relatively inactive character and outstanding long term survival rate of TC. However, TC poses a serious threat to human health and is a substantial economic burden to both families of TC patients [6, 7]. Despite the rapid development in medical technology, TC remains one of the most challenging diseases to treat [8]. Therefore, elucidating the

underlying pathogenesis of TC will significantly contribute to identifying novel effective measures to help treat TC patients.

Although the exact underlying molecular mechanisms of TC initiation and development remain largely unknown, recent evidence has revealed that several genetic alterations may be involved in the tumorigenesis of TC, including point mutations in multiple proto-oncogenes (NRAS, KRAS and HRAS), chromosomal rearrangements (NTRK1, RET/PTC and PPARG), and gene expression dysregulation [1]. For example, the Cbp/p300-interacting transactivator containing glutamic acid (E) and aspartic acid (D)-rich C-terminal domain 1 (CITED1) was identified as a novel potential TC-associated gene. CITED1 upregulation was found in TC tissue, which increases the risk of lymph node metastasis. Moreover, the knockdown of CITED1 significantly inhibits tumor growth of TC [9]. To the best of our knowledge, up to now, in only a few studies

have the roles of cluster of differentiation 47 (CD47) in TC been investigated. CD47, an important subtype of the immunoglobulin superfamily, is a ligand that recognizes the extracellular domain of signal regulatory protein α (SIRPα) [10, 11]. Under normal physiological circumstances, CD47 acts as a critical marker of macrophages that protects cells against phagocytosis [12, 13]. However, under pathological conditions, tumor cells can avoid the phagocytosis of infiltrating macrophages by activating the CD47 and SIRPα signaling pathways. In previous studies, the overexpression of CD47 has been reported in various human cancers, such as breast cancer, lung cancer, and oral cancer [10, 14, 15], thereby suggesting that CD47 may serve as a therapeutic target for tumor development.

In the present study, we aimed to investigate the effects of CD47 on the pathogenesis of TC. Our findings show that when compared with the normal Nthy-ori-3-1 human thyroid follicular epithelial cells, CD47 expression was significantly increased in two TC cell lines, TPC-1 and K1. In addition, the knockdown of CD47 in TC cells inhibited cell proliferation and promoted cell apoptosis. In addition, we revealed that the programmed death-1 (PD-1) signaling pathway may play a role in the effects of CD47 on TC.

Materials and methods

Tissue samples

TC pathological tissues were extracted from 11 patients with thyroid papillary carcinoma who were registered in the Ningbo No. 2 Hospital between July 2017 and November 2017. Healthy thyroid tissues were extracted from 7 patients with benign thyroid nodules who were registered concurrently in the Ningbo No. 2 Hospital.

Cell lines and culture

The Nthy-ori-3-1 normal human thyroid follicular epithelial cell line as well as TPC-1 and K1 TC cell lines were all purchased from the type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37°C, 5% CO_2 and 95% air in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml pen-

icillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

CD47 and PD-L1 shRNA construction and transfection

The CD47 (PD-L1) shRNA expression vector was applied to specifically decrease CD47-expression in TPC-1 and K1 cells. In brief, to generate recombinant plasmids, the shRNA construct targeting CD47was cloned into the lentiviral vector SMARTvector 2.0 (Dharmacon, Inc., Lafayette, CO, USA). The CD47 shRNA and PD-L1 shRNA were both designed and obtained from Sangon (Shanghai, China). The sequences were as follows: CD47 shRNA sequence: CCG GCC TGG TGA TTA CCC AGA GAT ACT CGA GTA TCT CTG GGT AAT CAC CAG GTT TTT. PD-L1 shRNA sequence: CCG GCG AAT TAC TGT GAA AGT CAA TCT CGA GAT TGA CTT TCA CAG TAA TTC GTT TTT G. For the transfection, the TC cells were seeded in 6-well plates at a density of 2×10^5 cells/well and cultured for 24 h. Then, the TC cells were transfected with the recombinant plasmid targeted CD47 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative real-time PCR assay

The total RNA of TPC-1, K1, and Nthy-ori-3-1 cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. Subsequently, 3 µg of RNA was reverse transcribed into cDNA using a BestarTM qPCR RT kit (#2220, DBI Bioscience, China), and a qRT-PCR assay was conducted using the ABI7500 with BestarTM qPCR MasterMix (#2043, DBI Bioscience, China). Primers were designed and obtained from Sangon (Shanghai, China), and were as follows: CD47 Forward, 5'-AGA AGG TGA AAC GAT CAT CGA GC-3', CD45 Reverse, 5'-CTC ATC CAT ACC ACC GGA TCT-3'; GAPDH: Forward, 5'-TGT TCG TCA TGG GTG TGA AC-3', GAPDH Reverse, 5'-ATG GCA TGG ACT GTG GTC AT-3'. CD47 mRNA expression was normalized to GAPDH expression.

Western blot analysis

Proteins were extracted from TPC-1, K1, and Nthy-ori-3-1 cells using a RIPA buffer (R0278, Sigma, St. Louis, MO, USA). After centrifugation
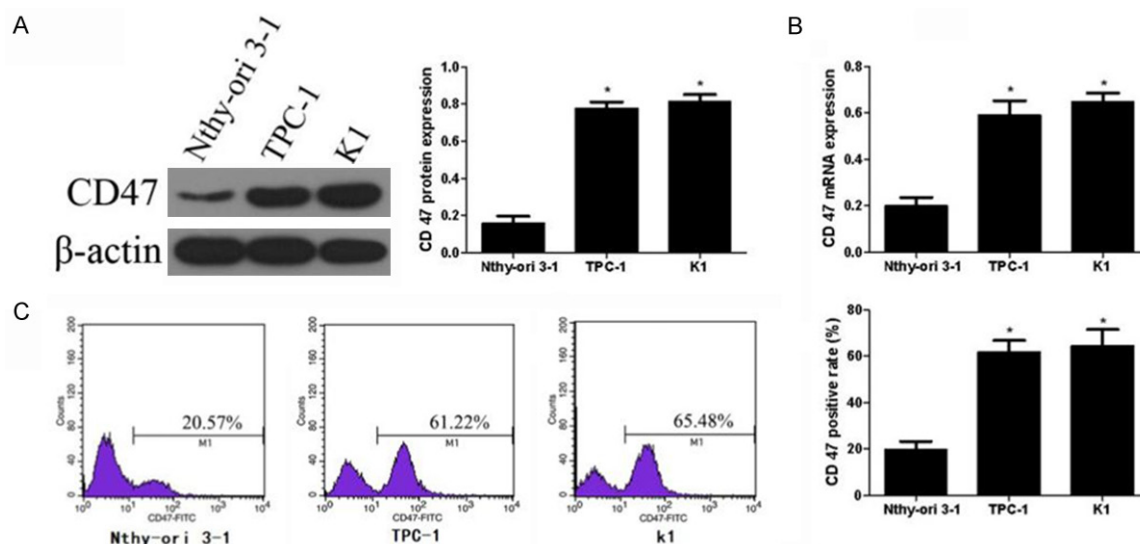
Figure 1. The expression of the CD47 protein and mRNA in thyroid cancer cell lines. A. The protein expression of CD47 in normal Nthy-ori-3-1 human thyroid follicular epithelial cells and TPC-1 and K1 thyroid cancer (TC) cell lines was evaluated by Western blot analysis. B. Relative mRNA expression of CD47 in Nthy-ori-3-1, TPC-1, and K1cells was determined by qRT-PCR analysis. C. Flow cytometry analysis was performed to identify CD47 positive Nthy-ori-3-1, TPC-1 ad K1 cells. *P < 0.05.

for 20 min at low temperature and at 12,000 rpm/min, the supernatants were collected, and the protein concentration was determined by a BCA kit (Pierce, Rockford, IL, USA). Next, proteins were separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% low fat dried milk for 2 h, then incubated overnight at 4°C with corresponding primary antibodies directed against CD47 (Rabbit, 1:2000, ab175388, Abcam); PD-L1 (Rabbit, 1:1000, ab213524, Abcam); AKT (Rabbit, 1:1000, ab38449, Abcam); p-AKT (1:500, ab38449, Abcam); SHP1 (Rabbit, 1:2000, ab124942, Abcam); p-SHP1 (1:1000, ab51171, Abcam); SHP2 (Rabbit, 1:2000, ab32083, Abcam); p-SHP2 (1:50000, ab62322, Abcam); ERK (Rabbit, 1:1000, ab17942, Abcam); and p-ERK (1:1000, ab131438, Abcam). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated donkey-anti-rabbit secondary antibody for 2 h at room temperature. Finally, the protein bands were visualized using an enhanced chemiluminescent reagent and quantified.

Co-immunoprecipitation

For co-immunoprecipitation purposes, the following antibodies were used: rabbit anti-CD47 (ab175388, Abcam), rabbit anti-PD-L1 (ab21-

3524, Abcam), goat anti-actin (ab6789, Abcam), Pierce protein A/G agarose (Cat. #20333; Thermo Scientific, Rockford, IL, USA). In brief, the agarose beads were washed with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and 10% SDS, then washed three times in PBS to remove SDS, and a binding solution was added. Then, a total of 4 µl (1:1500~1:2000) of antibody was added to the agarose beads, and the antibody was allowed to bind to the resin for 4 hours at 4°C. Subsequently, the agarose beads were washed three times with cold PBS to remove excess antibodies. Next, an overload of binding solution and 50 µg (for over loading conditions) or 20 µg (for standard conditions) of bait protein and prey protein were added to the agarose beads. After overnight incubation, the agarose beads were washed three times with cold PBS, and the 20 µl of 5% loading sample buffer (Life Technologies, Inc., Carlsbad, CA, USA) was added to each sample. Finally, SDS-PAGE and Western blot analyses were employed to evaluate the co-immunoprecipitated proteins.

Flow cytometry analysis

Cell apoptosis, the cell cycle, and the CD47 positive rate of treated TC cells were analyzed by flow cytometry analysis. In brief, treated TC cells were incubated with 20 nM docetaxel for
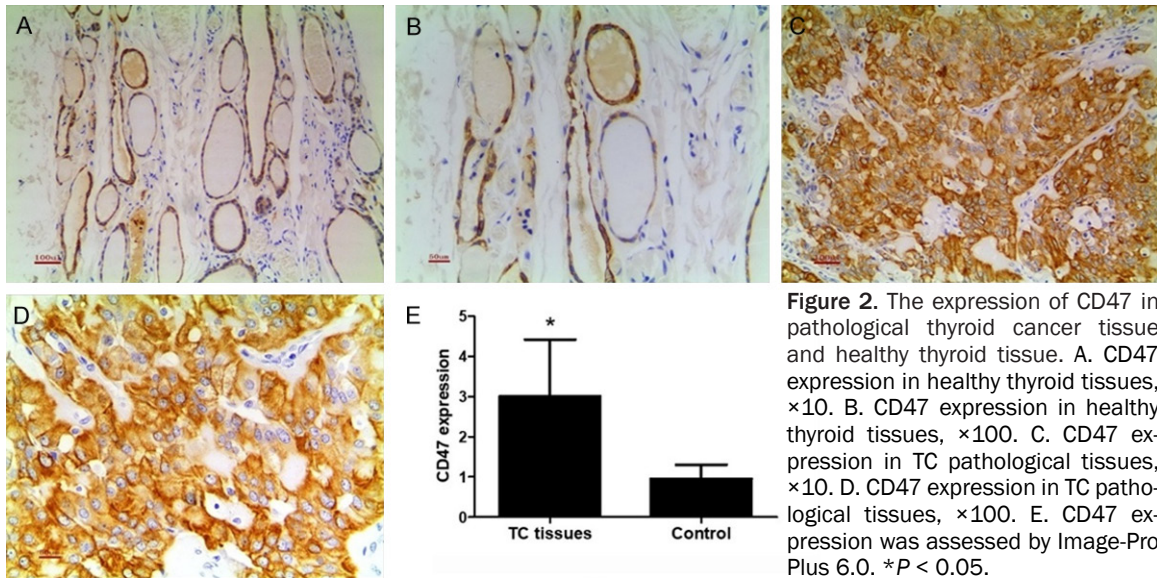
Figure 2. The expression of CD47 in pathological thyroid cancer tissue and healthy thyroid tissue. A. CD47 expression in healthy thyroid tissues, ×10. B. CD47 expression in healthy thyroid tissues, ×100. C. CD47 expression in TC pathological tissues, ×10. D. CD47 expression in TC pathological tissues, ×100. E. CD47 expression was assessed by Image-Pro Plus 6.0. *$P < 0.05$.

48 h and then harvested. For the cell cycle analysis, TC cells were washed with pre-chilled PBS, then fixed with pre-chilled 70% ethanol at -20°C for 2 h. Subsequently, the cells were resuspended and incubated in 500 μl PBS, containing RNase A (2 μg/ml) and PI (20 μg/ml) for 30 min. Flow cytometry analysis was conducted by a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ, USA). For the cell apoptosis analysis, treated TC cells were evaluated by the Annexin V-FITC Apoptosis Detection Kit (Oncogene Research Products, Boston, MA, USA) and analyzed by flow cytometry.

Statistical analysis

The Data were expressed as the mean ± SEM and compared using a Student's *t* test (for the comparison of 2 groups) or one-way ANOVA (when more than 2 groups were compared). P < 0.05 was considered significant.

Results

We used the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and searched and compared data derived from TC tissue and corresponding normal tissue and found that CD47 expression was significantly upregulated in TC tissue when compared to the control tissue. Moreover, to investigate the role of CD47 in TC, we first confirmed the upregulated CD47 expression by qRT-PCR and Western blot analysis. Our results showed that in the TC TPC-1 and K1 cells the expression of both the CD47

protein and mRNA was significantly higher when compared to the levels in the Nthy-ori-3-1 cells ($P < 0.05$, **Figure 1A** and **1B**). Next, flow cytometry analysis was performed to determine the percentage of CD47 positive cells in TPC-1, K1, and Nthy-ori-3-1 cells. Our results indicated that the percentage of CD47 positive cells was significantly increased in TPC-1 and K1 cells when compared with Nthy-ori-3-1 cells ($P < 0.05$, **Figure 1C**). In addition, we demonstrated that the CD47 expression in pathological TC tissues was higher when compared to the expression in healthy thyroid tissue ($P < 0.05$, **Figure 2**). Taken together, these findings suggest that the CD47 expression was upregulated in both the TC tissues and cell lines.

Both CD47 knockdown and PD-L1 knockdown in thyroid cancer cells significantly inhibited cell proliferation, and promoted cell apoptosis

To explore the underlying roles of CD47 and PD-L1 in TC, we silenced their expression by transfecting TPC-1 and K1 cells with either CD47 or PD-L1 shRNA. Flow cytometry analysis was employed to evaluate the effect of CD47/PD-L1 knockdown on cell apoptosis and the cell cycle phase of the TC cells. The silencing of CD47 in the TPC-1 and K1 cells resulted in a significant induction of cell apoptosis ($P < 0.05$, **Figure 3A**). In addition, cell cycle analysis showed that the percentage of cells in the G0/G1 phase was significantly higher, and the percentage of cells in the S and G2/M phases was
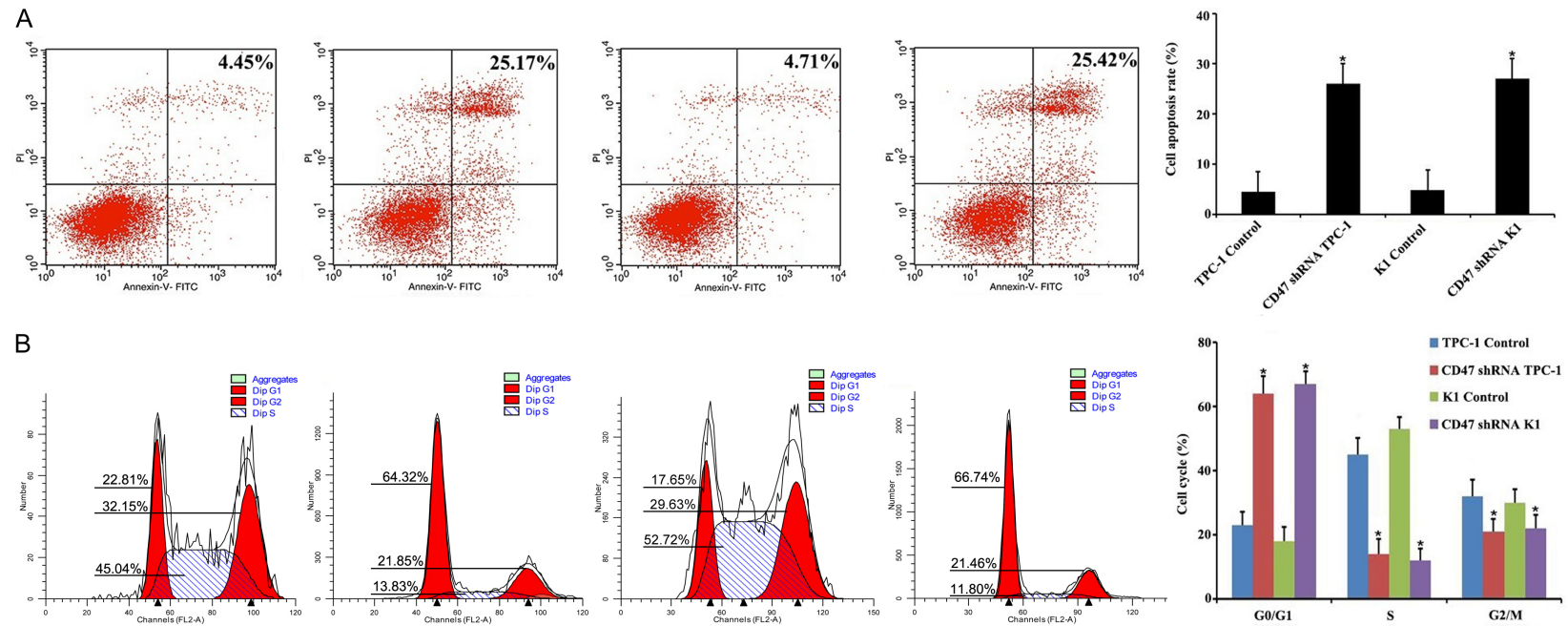
Figure 3. The effects of CD47 knockdown on cell apoptosis and cell cycle in thyroid cancer cells. A. The cell apoptosis of thyroid cancer (TC) cells using CD47 knockdown was evaluated by Annexin V/PI double staining, followed by flow cytometry analysis. B. The cell cycle of the CD47 knocked down TC cells was assessed by flow cytometry analysis. *P < 0.05.
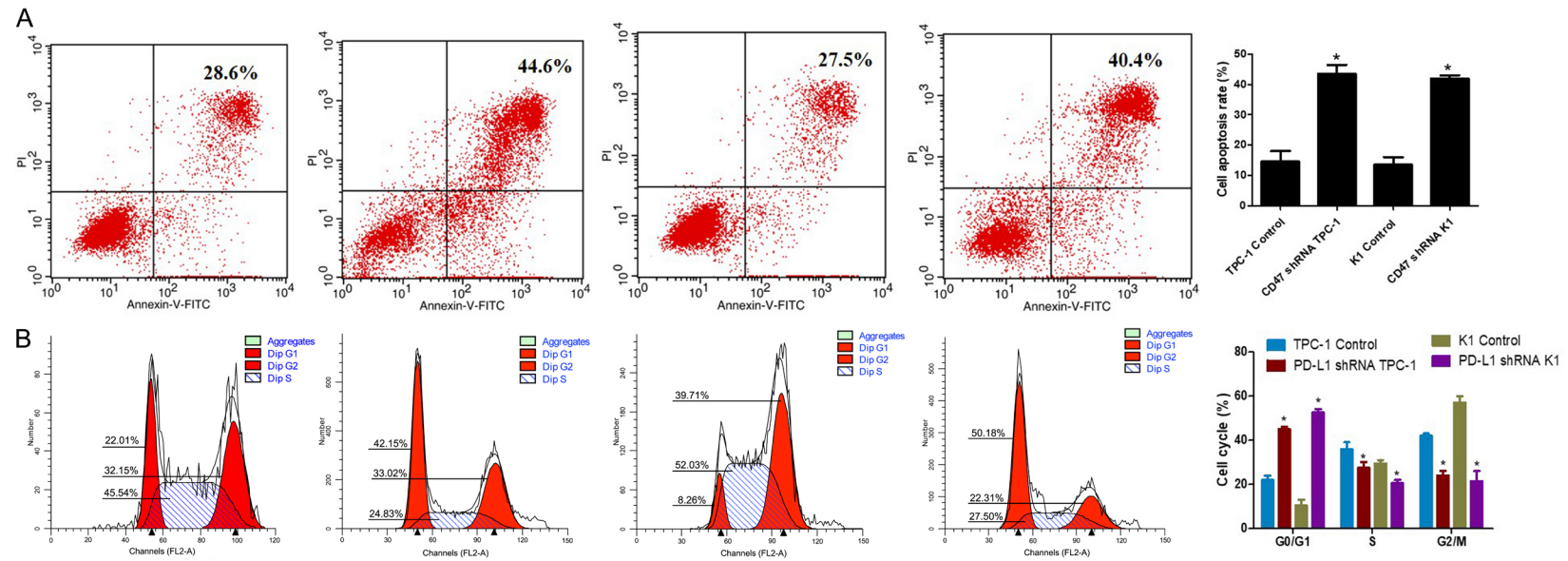
Figure 4. Effects of PD-L1 knockdown on cell apoptosis and cell cycle in thyroid cancer cells. A. The cell apoptosis of thyroid cancer (TC) cells using CD47 knockdown was evaluated by Annexin V/PI double staining, followed by a flow cytometry analysis. B. The cell cycle of TC cells that underwent CD47 knockdown was assessed by flow cytometry analysis. *P < 0.05.
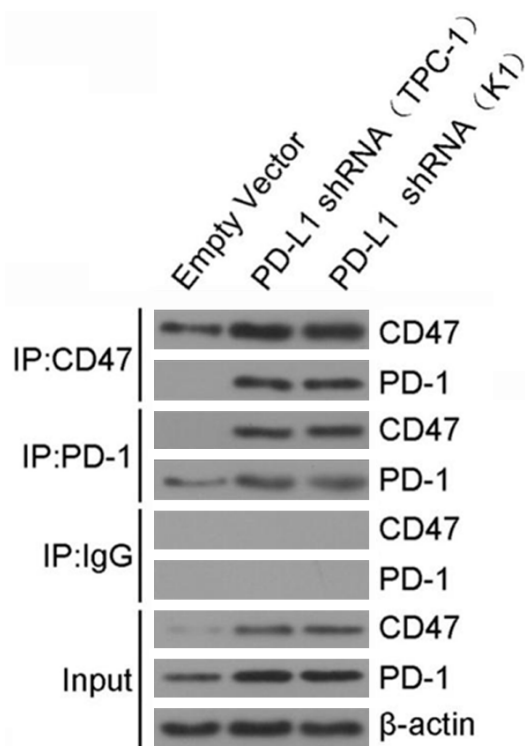
Figure 5. Co-immunoprecipitation for PD-L1 and CD47. PD-L1 and CD47 co-immunoprecipitated in the thyroid cells but not in the Nthy-ori-3-1 cells.

remarkably lower in the CD47-silenced TPC-1 and K1 cells when compared to the Nthy-ori-3-1 cells (*P* < 0.05, **Figure 3B**). Similarly, the silencing of PD-L1 in the TPC-1 and K1 cells resulted in an induction of cell apoptosis (*P* < 0.05, **Figure 4A**), and cell cycle stagnation in the G0/G1 phase (*P* < 0.05, **Figure 4B**). Thus, these findings indicated that CD47 silencing in TC cells suppressed cell proliferation and promoted apoptosis.

PD-L1 and CD47 co-immunoprecipitate in thyroid cancer cells but not in Nthy-ori-3-1 cells

To identify the interaction between CD47 and PD-L1, we employed a co-immunoprecipitation study to evaluate their co-expression in TPC-1, K1, and Nthy-ori-3-1 cells. Our findings showed that PD-L1 and CD47 co-precipitated in both TPC-1 and K1 cells, but not Nthy-ori-3-1 cells (**Figure 5**).

The PD-L1 signaling pathway was activated in thyroid cancer cells

Because CD47 function mainly focused on the mediation of the immune tolerance of T cells,

and PD-L1, involved in T cell depletion, is known as an important member of the immunoglobulin superfamily, we investigated whether PD-L1 was involved in the effects of CD47 on TC cells by evaluating the expression of PD-L1 related proteins by Western blot analysis. Results suggested that AKT protein expression was not significantly altered in the TPC-1 and K1 cells; however, the protein expression levels of p-AKT, small heterodimer partner 1 (SHP1), p-SHP1, SHP2, p-SHP2, extracellular signal-regulated kinase (ERK), p-ERK, and PDL-1 were significantly upregulated in TPC-1 and K1 cells when compared to Nthy-ori-3-1 cells (*P* < 0.05, **Figure 6**). Combined, these findings indicated that the PD-L1 signaling pathway was activated in TC cells.

The activated PD-L1 signaling pathway in thyroid cancer cells was abolished by CD47 silencing

Next, we confirmed involvement of the PD-L1 signaling pathway in CD47-silenced TPC-1 and K1 cells by measuring the CD47-associated protein expression via Western blots analysis. Lur findings showed that the protein expression levels of p-AKT, p-ERK, p-SHP1, p-SHP2, and PD-L1 were significantly downregulated in TPC-1 and K1 cells when compared with Nthy-ori-3-1 cells. However, no significant differences were observed in the protein expressions of AKT, ERK, SHP1, and SHP2 (*P* < 0.05, **Figure 7**). Combined, these results indicated that the activated PD-L1 signaling pathway in TC cells was reversed by silencing the CD47 expression.

Discussion

Worldwide, TC is considered one of the least morbid types of human cancers, with patients exhibiting better long-term survival than most other cancers [16]. Increasing evidence has not indicated a strong correlation between regional lymph node metastasis and overall survival in most cases [17, 18]. However, patients are still dying from TC-related causes. Therefore, it is of utmost importance to further investigate the pathogenesis of TC to identify novel effective therapeutic targets for the treatment of TC patients. In the present study, we demonstrated that CD47 may be involved in the tumorigenesis of TC. We observed that CD47 expression was significantly higher in
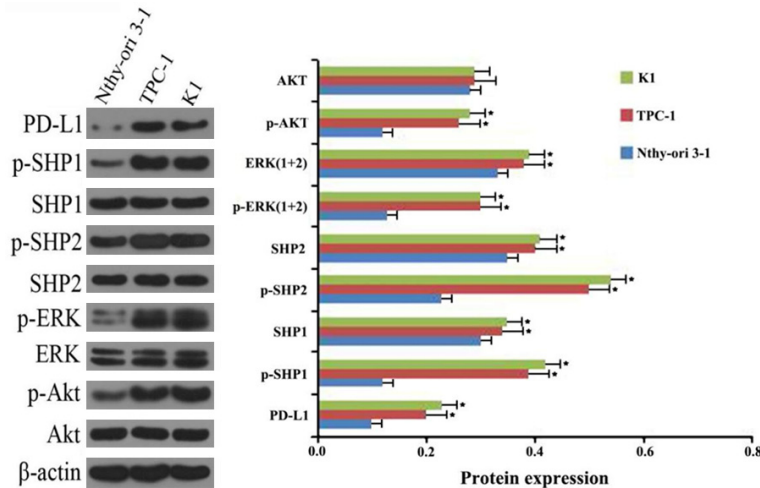
Figure 6. The expression of the PD-L1 signaling pathway-related proteins in the thyroid cancer cell lines. Protein expression levels of PD-L1, SHP-1, p-SHP-1, SHP-2, p-SHP-2, ERK, p-ERK, AKT, and p-AKT in thyroid cancer (TC) cells and Nthy-ori-3-1 cells were examined by Western blot analysis. *P < 0.05.
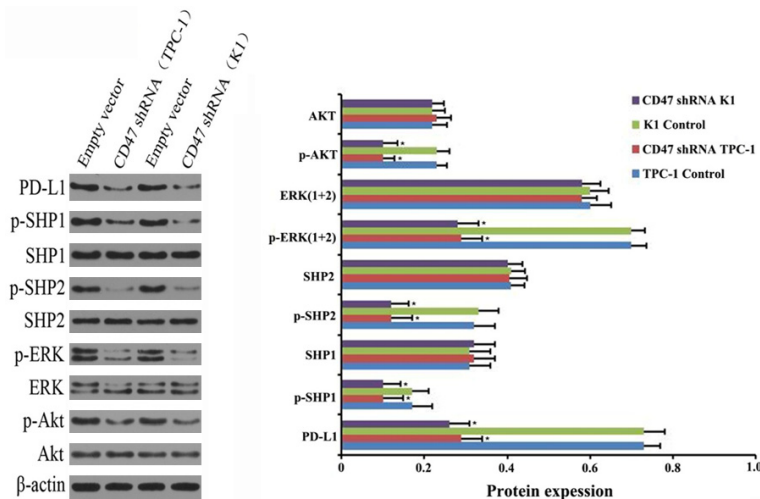

Figure 7. The effects of CD47 knockdown on the expression of PD-L1 signaling-related proteins in thyroid cancer cell lines. Protein expression levels of PD-L1, SHP-1, p-SHP-1, SHP-2, p-SHP-2, ERK, p-ERK, AKT, and p-AKT of thyroid cancer cells that underwent CD47 knockdown were evaluated by Western blot analysis. *P < 0.05.

The survival and development of cancer cells relies on multiple factors, including the escape from immune destruction, the enabling of replicative immortality, and resisting cell death [19]. Increasing evidence has suggested that various components of the immune system are involved in the immune surveillance of tumor cells [20]. Both the adaptive and innate immune system have been shown to play a critical role in anti-tumor immunity [21]. Macrophages are important components of the innate immune system and are involved in multiple functions, including phagocytosis [22]. Recently, it was demonstrated that tumor cells may escape from macrophage phagocytosis by expressing several anti-phagocytic markers, such as CD200 and CD47 [23, 24]. CD47 belongs to the immunoglobulin superfamily, is expressed in almost all cell types and has been implicated in multiple physiologic processes [25, 26]. In a previous study, it was demonstrated that CD47 could function as a suppressor of phagocytosis by binding to the SIRPα that was expressed on phagocytes [27]. Moreover, it was revealed that CD47 was widely expressed on many tumor cells, including those of leukemia, breast cancer, and bladder cancer, thereby implying that its role in regulating tumor progression was widespread [28-30]. When compared to normal counterpart tissues, the tumor tissue exhibited higher levels of CD47 expression. It was therefore suggested that CD47 overexpression in tumor cells may contribute to the evasion from phagocytosis. Moreover, it has previously been reported that hypoxia-inducible factor 1 (HIF-1) directly initiates the transcription of CD47 in breast cancer cells, and promotes the evasion of phagocyto-

TPC-1 and K1 TC cells when compared to Nthy-ori-3-1 cells. Subsequently, a functional analysis revealed that in CD47 silenced TC cells, cell proliferation was significantly inhibited, and cell apoptosis was markedly increased. In addition, the PD-L1 signaling pathway-related proteins were overexpressed in the TC cells, which were abolished by the CD47 silencing. Taken together, our findings suggest that CD47 promoted TC cell growth by activating PD-L1 signaling.

sis of cancer cells [14], which is similar to our findings in TC.

In general, PD-L1, a principal ligand of programmed death-1 (PD-1), acts as a critical "do not find me" marker to the adaptive immune system [31]. PD-L1 is widely expressed by cells in the tumor microenvironment, and acts as an inhibitor of T-cells by triggering suppressive signaling pathways in various types of cancer [32]. In addition, PD-L1 is overexpressed in diverse human tumors, and contributes to the initiation and development of tumor cells [32]. Therefore, we hypothesized that CD47 may exhibit its promoted effects on TC via PD-L1 signaling.

In conclusion, although additional *in vivo* studies are required to confirm the role of CD47 on TC, our results indicated that CD47 contributes to the immune escape of TC cells by activating PD-L1 signaling.

Disclosure of conflict of interest

None.

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