

Communication

# PTEN May Involve in Regulation of PD-L1 Expression in Triple Negative Breast Carcinoma

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**Abstract:** Triple negative breast carcinoma (TNBC) is a rapid progressive tumor and has a poor overall survival. Therefore, it is crucial to find out effective molecular targets and develop optimal therapeutic strategies for TNBC. In this study, immunohistochemical staining was used to detect expressions of programmed death-ligand 1 (PD-L1) and phosphatase and tensin homolog (PTEN) in 136 breast carcinomas including 50 TNBC. The effect of PTEN on regulation of PD-L1 expression was assessed in vitro in the PTEN knockdown TNBC cells. We found that PD-L1(SP142) positive rate in TNBC (48.0%) was significantly higher than non-TNBC (23.3%). PTEN negative rate was 42% in TNBC. The inverse correlation between PTEN and PD-L1(SP142) expression in TNBC was statistically significant ( $P < 0.05$ ). After PTEN knockdown, PD-L1 expression in TNBC cells increased significantly, and the expression level of AKT increased simultaneously. PTEN knockdown promoted cell proliferation, viability and G1/S switch of TNBC cells. These results suggested that PTEN may involve in regulation of PD-L1 expression, because PTEN loss can upregulate PD-L1 expression in TNBC. Antitumor immunity of PD-L1 could be enhanced in TNBC when targeting PTEN at the same time.

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## 1. Introduction

Triple negative breast carcinoma (TNBC) is characterized by lacking of expression of estrogen receptor (ER), progesterone receptor (PR) and gene amplification of human epidermal growth factor receptor type 2 (HER2), namely, it lacks corresponding therapeutic targets of endocrine and HER2[1]. Although TNBC constitutes merely 15% to 20% of all breast tumors, it is a heterogeneous tumor having six subtypes with more aggressive clinical behavior, high rate of relapse and distant metastasis than other types of breast cancer. The poor clinical outcomes occur even though using neoadjuvant chemotherapy options. Advanced patients with metastatic TNBC exhibit rapid progression and a median overall survival of less than 1 year[2]. Therefore, it is crucial to find out effective molecular targets, and develop optimal therapeutic strategies for patients with TNBC.

With the clinical application of checkpoint inhibitor - programmed death-ligand 1 (PD-L1) antibody, immunotherapy is gaining traction across many solid tumors, including breast cancer. PD-L1 expressed by tumor cells down regulates immune responses through binding to its two receptors programmed death-1 (PD-1) and B7.1. PD-1 is expressed on T cells following activation, which is sustained in chronic inflammation or malignancy. B7.1 is a molecule expressed on antigen presenting cells and activated T cells. PD-L1 binding to PD-1 and B7.1 on T cells and antigen presenting cells can mediate down-regulation of immune responses, including inhibition of T-cell activation and cytokine production, resulting in an immune evasion[3, 4]. Even though there

are abundant tumor-infiltrating immune cells, including lymphocytes and antigen presenting cells such as macrophages and dendritic cells in the lesion, TNBC still is a poor prognostic tumor, that makes it an example of immune evasion. Thus, targeting the PD-L1 pathway will reinvigorate tumor-specific T-cell immunity suppressed by PD-L1 in TNBC. Ghebeh *et al.*[5] reported that in 44 breast cancers including TNBC, 34% expressed PD-L1 in tumor cells and 41% in tumor-infiltrating lymphocytes. A recent phase III trial shows excellent synergy between T cell immune-checkpoint blockade therapy and cytotoxic chemotherapy in metastatic TNBC[6]. However, initial single-agent responses in PD-L1-positive TNBC are approximately 20% for both PD-1 and PD-L1 antibodies[7]. Treated with the PD-L1 antibody avelumab the patients with metastatic TNBC showed better outcomes with a 31% disease control rate[8]. These data suggest that immunotherapy using PD-L1 antibodies is an encouraging efficacy way for patients with TNBC.

Due to modest results of immunotherapy, PD-L1 regulation now becomes a research hotspot. Earlier study by Parsa *et al.*[9] demonstrated that expression of PD-L1 on glioblastomas is increased by the deletion or silencing of the phosphatase and tensin homolog (PTEN), which suggests PTEN may involve in regulation of PD-L1 expression. PTEN is a tumor suppressor gene that inhibits cell proliferation by inhibiting the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway. PTEN and PI3K mutations were detected in approximately 30% and 40% of primary breast cancers, respectively. In TNBC, the rate of PTEN loss was up to 82.1% as detected by immunohistochemistry[10]. Wang and colleagues examined PD-L1 and PTEN/miRNA in basal-like TNBC, and found PTEN-low/miRNA-low (hsa-miR-4324, hsa-miR-125b, hsa-miR-381, hsa-miR-145, or hsa-miR136) and PD-1 expression were poorest prognostications[11]. Interestingly, PTEN loss is also correlated with ER/PR negative and PI3K pathway activation in breast cancers[12].

Based on mention above, we detect PD-L1 expressions and PTEN status in TNBC, and explore their relationship in TNBC. Furthermore, we investigate PD-L1 expression levels and biologic behavior after PTEN knockdown in TNBC cells. Our results demonstrate that PTEN is involved in regulation of PD-L1 expression in TNBC.

## 2. Materials and methods

The study was approved by the hospital Research Ethics Committee. A total of 136 cases of surgery excision samples of breast carcinomas were collected from the archive of Department of Pathology, Kiang Wu Hospital in Macao from January 2010 to December 2020. All of patients are female. The age ranged from 21- to 91-year old (median age 55.6 years). The breast carcinomas were divided in two groups, non-TNBC and TNBC, defined by immunoreaction of ER, PR and *in situ* hybridization of HER2.

Immunohistochemistry was carried out by Benchmark XT automatic staining machine (Ventana, Tucson, AZ) with Ultra view detection system. PD-L1 was detected by using PD-L1(SP263) rabbit monoclonal antibody (Ventana, Tucson, AZ) and VENTANA anti-PD-L1(SP142) Rabbit Monoclonal Primary Antibody (Ventana, Tucson, Arizona). Positive and negative controls were set for each test. The staining result was interpreted according to Ventana's interpretation guidelines. For PD-L1(SP263) staining at the membrane of the cells or perinuclear dot-like staining were regarded as positive. According to the percentage of positive cells,  $\geq 1\%$  was considered as positive,  $< 1\%$  as negative. For PD-L1(SP142) only positive labeled tumor-infiltrating immune cells (IC) including lymphocytes, macrophages, dendritic cells, and granulocytes are evaluated, regardless any staining intensity in tumor cells. It was defined as positive when presence of discernible PD-L1 staining of any intensity in tumor-infiltrating immune cells covering  $> 1\%$  of tumor area occupied by tumor cells, associated intratumoral and contiguous peritumoral stroma. Otherwise,  $< 1\%$  was defined as negative.

PTEN protein was detected by using PTEN rabbit monoclonal antibody (Catalogue number: M362729, Dako, United States). Any staining of PTEN in cytoplasm or nuclei of tumor cells was considered as positive. Complete unstaining was defined as negative. Negative of PTEN protein implied mutation of the gene.

Human TNBC cell line MDA-MB-231 was purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin G, and 100 µg/ml streptomycin. Cells were maintained in incubator containing 5% CO<sub>2</sub> and humidified atmosphere at 37°C. The medium was exchanged every 2 days with fresh medium. The cells were subcultured when they reached 70-80% confluence to keep cells activity.

PTEN shRNA (shPTEN) lentiviral transfection particles (pLKO-UHRF1) and non-targeting shRNA lentiviral transfection particles (pLKO.1-puro Non-Target Control [SHC016V]) (shNC) were obtained from Sigma-Aldrich. Transfection was performed with  $2 \times 10^5$  293T cells per well in 6-well plates. Lentiviral particles (psPAX2, pMD2G) were added slowly in the medium, and then mixed well. After overnight (24 h) cultivation at 37°C, medium was changed to fresh medium with 2 µg/ml puromycin. Medium was replaced every third day with fresh puromycin-containing medium until stable clones were identified. PTEN knockdown was confirmed using Western blot analysis.

The effect of PTEN knockdown on cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay. Exponentially growing MDA-MB-231 cells were seeded at  $1 \times 10^3$  cells per well in 96-well culture plates overnight. From second to the seventh day after incubation, 10 µl of CCK-8 was added to each well and incubated for another 2 hrs to detect cell viability. Absorbance at 450 nm was detected with an automated microplate reader (BioTek, United States). Three replicates were set for detection each day. For the viability,  $1 \times 10^3$  cells per well in 6-cm dish were cultured for 7 days. Colony-forming units with cells numbers  $\geq 30$  were counted as cell colony forming efficiency.

To assess effect of PTEN knockdown on cell cycle, cells were harvested and suspended in PBS. For the cell cycle analysis, cells were fixed in 70% ethanol overnight at 4 °C. The fixed cells were washed with PBS and incubated with 500 µl PI working solution (propidium iodide: RNase A=9:1) in the dark for 30 min. The cell cycle distribution was assessed using a FACS can flow cytometer (Excitation=488 nm, Emission=620 nm).

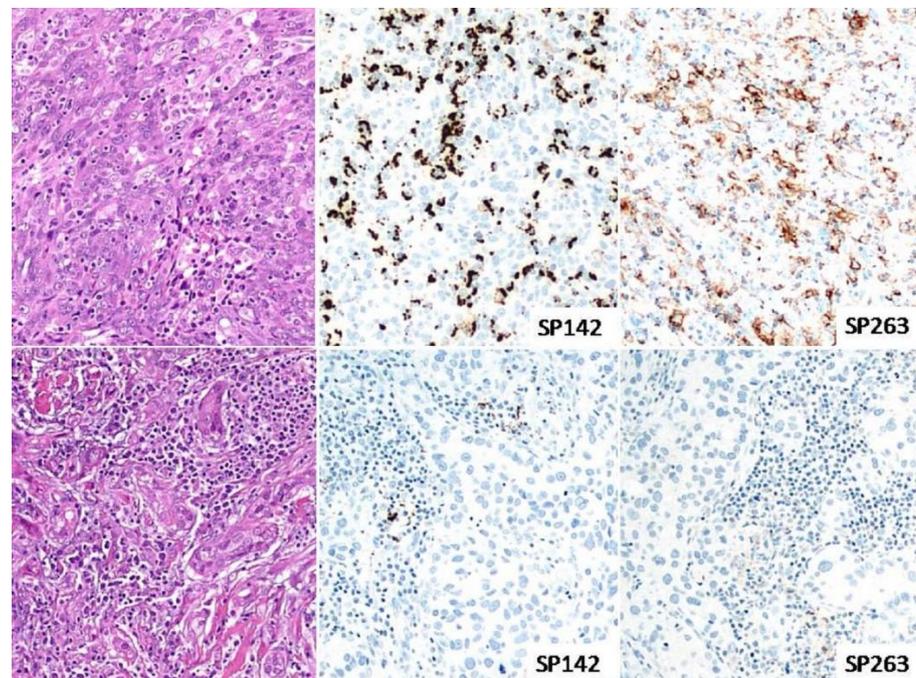
For the migration assays, cells were dissociated into single cell. A density of  $5 \times 10^4$  MDA-MB-231 cells were seeded onto the upper chambers (6.5 mm diameter, 8.0 µm pore size polycarbonate filters; Corning Incorporated, Corning, NY, United States) in a serum-free medium, and then allowed to migrate toward the lower transwell chamber filled with DMEM medium supplemented with 10% FBS. After 24 h, the filters with cells were fixed with methanol and stained with 0.1% crystal violet (2 mg/ml). The number of cells that migrated through the membrane was counted in five randomly selected fields under a light microscope.

Cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, boiled at 100 °C with 5×loading buffer and subjected to sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes (Pall Corporation, Port Washington, NY) and then nonspecific sites were blocked with 5% nonfat dry milk in phosphate-buffered saline for 1 hour at room temperature. The target protein were to be probed with anti-PTEN (1:1000, Catalogue number:22034-1-AP), pAKT (CST, United States), PD-L1 (Ventana, Tucson, AZ) and anti-GAPDH (Proteintech, China) overnight at 4 °C, and subsequently rinsed using Tris-buffered saline with Tween 20. The membranes were incubated with 1:1000 dilutions of mouse or rabbit secondary antibodies (Cell Signaling Technology). An imaging system (Uvitec, UK) was used to produce blot images, while band densities were analyzed with Odyssey 3.0 software using GAPDH as the standard.

Statistical analysis was performed using SPSS21.0 software (SPSS, Chicago, Illinois) for Windows. The chi-square test was used for comparison of quantitative differences between the groups. Student's t-test was performed in evaluation of qualitative differences between the groups. A *P* value of less than .05 was considered statistically significant.

### 3. Results

We used two antibodies, PD-L1(SP263) and PD-L1(SP142), to evaluate for the different expressive of PD-L1 in immune cells in non-TNBC and TNBC. Positive expression of PD-L1 was defined as  $\geq 1\%$  staining in immune cells in the tumor regardless positive proportion in tumor cells (Figure 1). We found that PD-L1(SP142) and PD-L1(SP263) positive rates in TNBC (48.0% and 54%) were significantly higher than in non-TNBC (23.3% and 30.3%) respectively ( $P=0.0029$  and  $P=0.0002$ ). Moreover, there was no statistical difference of positive rate between PD-L1(SP263) and PD-L1(SP142) in immune cells in TNBC ( $P>0.05$ ) (Table 1).



**Figure 1.** PD-L1(SP142) and PD-L1(SP263) immunoreactions. Up panel shows positive reaction (>50%) of both PD-L1 antibodies in a case of TNBC (immunoperoxidase; original magnification 200 $\times$ ), and >1% in another case (down panel) (immunoperoxidase; original magnification 200 $\times$ ).

**Table 1.** Comparison of PD-L1 in immune cells in TNBC and non-TNBC

	N	PD-L1(SP263)*		PD-L1(SP142)**	
		+	-	+	-
TNBC	50	27 (54.0%)	23 (46.0%)	24 (48.0%)	26 (52.0%)
Non-TNBC	86	20 (23.3%)	66 (76.7%)	20 (23.3%)	66 (76.7%)

\* $\chi^2= 13.2143$ ,  $P= 0.0002$ ; \*\* $\chi^2= 8.8453$ ,  $P= 0.0029$

Negative immunostaining of PTEN implied mutation of the gene. Out of 50 cases of TNBC, 21 (42.0%) were negative for PTEN staining, the rest 29 cases were positive (58.0%). In positive cases of PTEN, 38% cases were negative for PD-L1(SP142), while 20% PD-L1(SP142) positive cases expressed intact PTEN. On the other hand, in negative cases of PTEN, 28% cases were PD-L1(SP142) positive, 14% negative. Statistically, this inverse

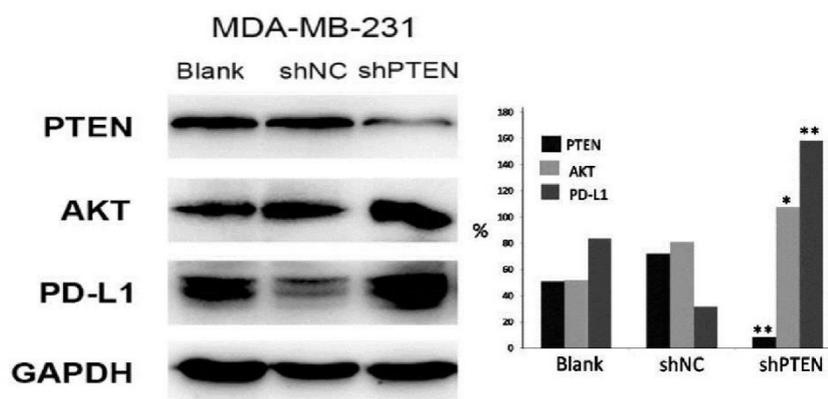
correlative difference between PTEN and PD-L1(SP142) was significant ( $P < 0.05$ ) (Table 2).

**Table 2.** Correlation of PTEN expression with PD-L1 in TNBC

	n	PTEN	
		+	-
PD-L1(SP142)	50	29 (58%)	21 (42%)
≥ 1% IC	29	10 (20%)	14 (28%)
< 1% IC	21	19 (38%)	7 (14%)

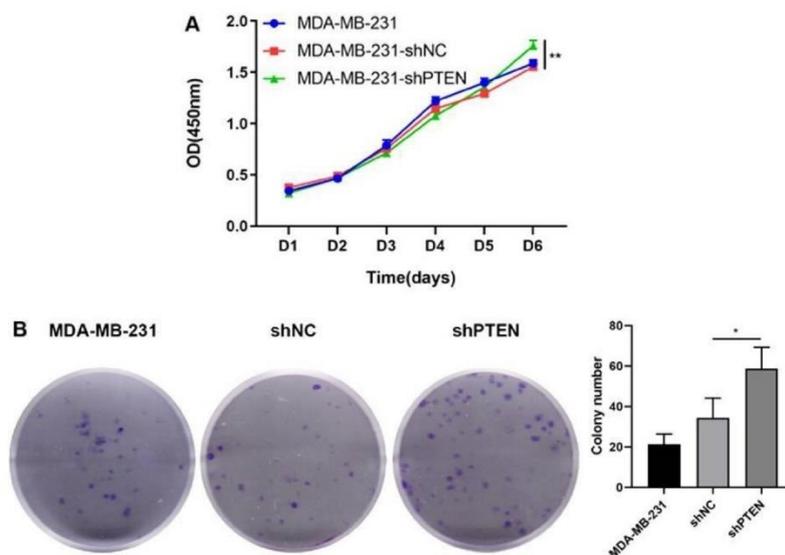
$$\chi^2 = 5.0545, P = 0.025$$

In the study, we explored the correlation of PTEN knockdown with PD-L1 expression. Firstly, we used a TNBC cell line MDA-MB-231 with intact PTEN expression to investigate the effect of PTEN knockdown on PD-L1 expression. After PTEN knockdown PD-L1 expression in MDA-MB-231 cells did significantly increase, the expression level of AKT, a key protein in PI3K/AKT/mTOR signal transduction, increased simultaneously (Figure 2).

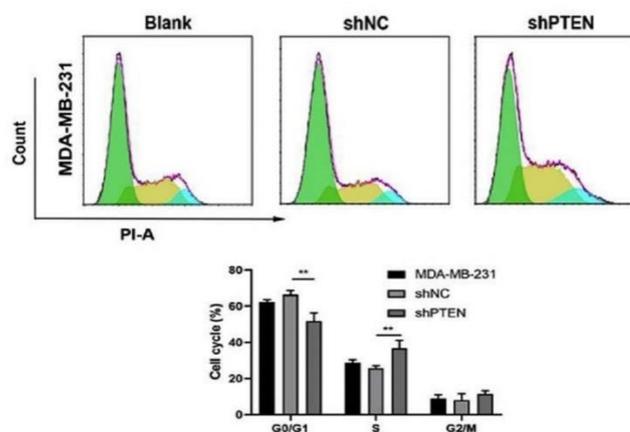


**Figure 2.** Effect of PTEN knockdown on PD-L1 and AKT expression (Western blot). After PTEN knockdown, PD-L1 and AKT expression increase significant in the shPTEN cells. GAPDH is as internal control. The columns in the figure represent comparison of PTEN, AKT and PD-L1 expressions in shPTEN with shNC. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

Subsequently, we detected the effect of PTEN knockdown on proliferation of TNBC cells. The growth curves revealed that MDA-MB-231 cells after PTEN knockdown sustained a more rapid proliferative trend than the controls. Cell colony formation assay of MDA-MB-231 cells indicated that colony forming efficiency was higher than the controls after PTEN knockdown (Figure 3). The result corroborates that PTEN is a tumor suppressor gene inhibiting cell proliferation. Similarly, proportion of MDA-MB-231 cells in S phase after PTEN knockdown (shPTEN) was significantly increased than the control (shNC) (Figure 4).



**Figure 3.** PTEN knockdown promoted the proliferation of TNBC cells in vitro. The results are shown as the mean  $\pm$  SD of three experiments. \* $P < 0.05$ , \*\* $P < 0.01$  compared with control.



**Figure 4.** PTEN knockdown promoted in vitro G1/S switch of cell cycles in TNBC MDA-MB-231 cells. The columns in the figure represent diverse phases and proportions of cell cycle. \*\*  $P < 0.01$ .

#### 4. Discussion

Even though abundant immune cells such as T-lymphocytes and macrophages exist within the tumor, TNBC still is one of most aggressive and worst prognostic breast carcinomas. Immune evasion should be the cause of clinical behavior of this tumor, because activation of the PD-1/PD-L1 pathway decreases proliferation and increases apoptosis of the intratumoral immune cells. Confirmed by Kim et al and our studies, PD-L1 was expressed in the intratumoral immune cells in almost half cases of TNBC[13]. By the way, there are two antibodies, PD-L1(SP142) and PD-L1(SP263), available for PD-L1 detection for tumor cells and intratumoral immune cells. Because PD-L1 expression in tumor cell was not associated with outcome of patients with TNBC[14], we evaluated expression of PD-L1 merely in intratumoral immune cells in this study. In addition, we found that positive rates of both antibodies had no statistically difference, suggesting both antibodies could be used in detection of PD-L1 expression in TNBC.

In further study, interestingly, we found that PD-L1(SP142) positive expression was mainly in PTEN mutated cases, while negative expression reversely mainly in PTEN intact cases, suggesting that PTEN may involve in regulation of PD-L1 expression in TNBC. If this hypothesis holds, PTEN knockdown should lead to significantly higher PD-L1 expression. Used PTEN shRNA to TNBC cell line (MDA-MB-157) carried wild-type PTEN and low levels of PD-L1 expression, Mittendorf *et al.*[15]found that PTEN knockdown could lead to significantly increase of cell-surface PD-L1 expression and PD-L1 mRNA. Even in theMDA-MB-231 cells expressed PTEN and PD-L1, PTEN knockdown could further increase cell surface PD-L1 expression of this cell line.

PTEN may involve in regulation of PD-L1 expression in TNBC by effect on PI3K/AKT/mTOR signaling, because the major genetic aberrations observed in TNBC occurred within the PI3K-Akt-mTOR (PAM) pathway[16]. Our results also showed that after PTEN knockdown the expression level of AKT and PD-L1increased significantly in TNBC cell line. These results imply that PD-L1 regulation by PTEN via PI3K signaling. Moreover, overexpression of AKT and PD-L1 by PTEN knockdown eventually increased cell proliferation and percentage of cells in S-phase.

In conclusion, the results of our study suggest a greater likelihood to enhanced anti-tumor immunity of PD-L1 when targeting PTEN in TNBC, because PTEN loss is commonly in this tumor.

**Author contributors:** K. C. W. and J. M. W. designed the study; K. C. W. collected the tumor specimens; K. I. C., H. T. V. and X. Y. Z. performed the immunostaining and in vitro experiments; K. C. W. and J. M. W. performed analysis and interpretation of data; J. M. W. wrote the manuscript.

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**Ethics statement:** This study was approved by the Research Ethics Committee of Kiang Wu Hospital, Macao

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

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