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ORIGINAL RESEARCH

Positive correlation between programmed death ligand-1 and p53 in triple-negative breast cancer

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Purpose: Tumors with high mutation load tend to have a stronger immune response in some tumors. The correlation between expression of programmed death ligand-1 (PD-L1), a biomarker of immune response in tumors, and p53, accepted as the most frequently mutated gene in many cancers, in triple-negative breast cancer (TNBC) has not been fully investigated in cancer patients.

Materials and methods: 132 cases of TNBC and 32 cases of non-TNBC paraffin-embedded tissue sections were selected to detect the expression of PD-L1 and p53 by immunohistochemistry, and results were correlated with clinical data and survival outcomes. The staining of PD-L1 in tumor cells (TCs) and tumor-associated immune cells (TAICs) was assessed separately.

Results: Strong positive correlations were observed between expression of p53 and PD-L1 both in TCs (r=0.338, P=0.000) and TAICs (r=0.186, P=0.033). The same positive correlation was found in the expression of PD-L1 in TCs and TAICs (r=0.764, P=0.000). Like p53 (P=0.024), positive rate of PD-L1 in TCs was significantly higher in TNBC than in non-TNBC (P=0.02). PD-L1 and p53 in TCs staining were significantly associated with histological grade, tumor size and Ki67 index (P<0.05). PD-L1 in TCs staining was also associated with lymphatic metastasis status (P=0.000). However, PD-L1 in TAICs was only related to histological grade in statistically (P=0.012). Kaplan–Meier survival analysis showed that positive groups of p53, PD-L1 in TCs and TAICs had a worse overall survival and a worse progression-free survival as compared with the negative groups, but marginal significance was found only in overall survival of PD-L1 in TCs and TAICs, and progression-free survival of PD-L1 in TAICs (P=0.074, 0.097, 0.068, respectively).

Conclusion: Our findings suggest that positive correlation between p53 and PD-L1 in TNBC and the higher expression rates are closely correlated with some key prognostic factors and worse survival outcomes. These findings would lay the foundation for further study on the relationship of p53 and PD-L1 and the combination of mutated p53 inhibitors and PD-1/PD-L1 antibodies in TNBC.

Keywords: p53, programmed death ligand-1, PD-L1, immunohistochemistry, IHC, tumor cells, TCs, tumor-associated immune cells, TAICs, triple-negative breast cancer, TNBC

Introduction

Programmed death ligand-1 (PD-L1) is a biomarker for response to anti-PD-1/PD-L1 therapy and proved over-expressed on the surface of various tumor cells (TCs). PD-L1 is capable to bind with PD-1 on activated T-cells to inhibit the proliferation and killing effect of T lymphocytes and to induce the apoptosis of T cells. Killing of tumor cells by the immune system was inhibited as a result.[1] The aim of Anti-PD-1/PD-L1 therapy is to inhibit or weaken the relationship between PD-1 and PD-L1.

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Triple-negative breast cancer (TNBC), the most immunogenic subtype of breast carcinoma, accounted for 15–20% of total breast cancers and 25% of deaths resulted from breast cancers, is characterized by lacking of estrogen receptor, progesterone receptor, and human epidermal growth factor-2 (HER2) expression. TNBC is usually presenting in premenopausal women, larger in size, higher grade and more aggressive biologically.[2–4] Re-activating anti-tumor immunity can eliminate partial tumor cells makes TNBC suitable for immune checkpoint blockade therapy, especially for anti-PD-1/PD-L1 therapy.[5] However, clinic trials suggested that only 10–20% of TNBC patients have a partial response to anti-PD-L1 or anti-PD-1 therapy.[6] Therefore, it is of great significance to understand the difference in the molecular level of PD-L1 in TNBC and the correlation with its clinical features.

p53 gene (also known as tp53) is accepted as the most frequently mutated tumor suppressor gene in human malignancy. p53, functioning toward the regulation of important cellular activities including cell cycle, senescence, and apoptosis in carcinogenesis,[7] is mutated in 80% of TNBC. Moreover, the rate is clearly higher than in luminal A (12%), luminal B(29%), and HER2-amplified (72%) subtypes.[8,9] Research has shown that p53 is able to communicate to the adaptive immune system and control the cytotoxic T-lymphocyte (CTL) response to cancer cells. An decreased CTL response due to p53 mutations could reduce response rates to immunotherapeutic drugs in cancers.[10] High mutation load tends to cause stronger immune responses[11] and elevated PD-L1 expression.[12] In cervical cancer, PD-L1 levels can be increased by miR-18a via targeting SOX6 to activate the Wnt/β-catenin pathway and inactivate p53 signaling.[13] Similarly in lung cancer, p53 can suppress PD-L1 expression via miR-34a.[14] However, there is no research about the connection between PD-L1 and p53 in TNBC.

In this study, immunohistochemistry (IHC) was used to detect the protein level of PD-L1 and p53 in TNBC tissue sections. The relationship with clinicopathological factors was systematically validated. For the first time, correlation of the two elements was preliminarily studied in TNBC.

Materials and methods

Patients

A total of 132 female samples of TNBC between June 2013 and November 2017 were obtained from the Department of Pathology of Chongqing Medical University. In addition, 32 cases of non-TNBC at the same time were chosen and used as controls (Figure 1). Cases, which were clearly diagnosed with TNBC or non-TNBC by IHC or fluorescence in situ hybridization (FISH) by the Department of Pathology were included. Patients with any radiotherapy, chemotherapy or endocrine therapy before surgery were excluded. Among the TNBC samples, 15 cases were ductal carcinoma in situ (DCIS), and 117 cases were invasive ductal carcinoma. Median age was 47 and ranged from 20 to 86. Of all the samples, 19 cases were grade I, 33 cases were grade II and 80 cases were grade III. The study protocol was approved by the Human Ethical Committee of Chongqing Medical University. Written informed consent was obtained from each patient, and the experiments were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

IHC

Formalin-fixed, paraffin-embedded tissue sections were prepared for IHC. Xylene and a series of ethanol solutions were used to deparaffinize and rehydrate the sections. EDTA (pH 8.0) and sodium citrate (pH 6.0) were performed to epitope retrieved, respectively, for PD-L1 and p53 by microwave. Endogenous peroxidase was blocked

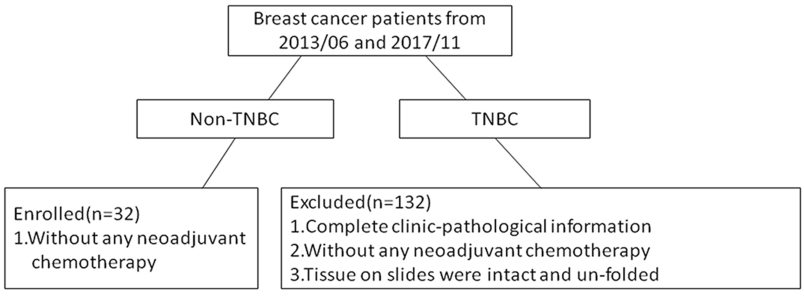

Figure 1 Flow diagram of the study cohort.
Abbreviation: TNBC, triple-negative breast cancer.

Table 1 Expression of PD-L1 and p53 in TNBC and non-TNBC groups

	n	PD-L1 in TCs		PD-L1 in TAICs		p53 in TCs	
		+(n%)	−(n%)	+(n%)	−(n%)	+(n%)	−(n%)
Non-TNBC	32	5 (15.6)	27 (84.4)	9 (28.1)	23 (71.9)	15 (46.9)	17 (53.1)
TNBC	132	49 (37.1)	83 (62.9)	53 (40.2)	79 (59.8)	90 (68.2)	42 (31.8)
χ^2 score		5.389		1.584		5.076	
P-value		0.02		0.208		0.024	

Abbreviations: PD-L1, programmed death ligand-1; TNBC, triple-negative breast cancer; non-TNBC, non-triple-negative breast cancer; TCs, tumor cells; TAICs, tumor-associated immune cells.

in 3% hydrogen peroxide for 15 mins at room temperature. Then, the sections were incubated with rabbit anti-PD-L1 monoclonal antibody (#13684, 1:100 dilution; Cell Signaling Technology, Danvers, MA, USA) and anti-p53 monoclonal antibody (#86630, 1:200 dilution; Cell Signaling Technology) respectively overnight at 4°C. These antibodies were detected using a biotinylated secondary antibody (PV-9000; zhongshan Jinqiao, Beijing, China) labeled with streptavidin-horseradish peroxidase and a DAB staining kit (ZLI-9018; zhongshan Jinqiao). Finally, the sections were counterstained by hematoxylin, then dehydrated and mounted.

Evaluation of immunostaining

PD-L1 and p53 protein levels were evaluated by microscopic examination of the stained tissue slides by two pathologists, Chenglong Wang and Youde Cao, who were blinded to the patient characteristics and finally reached a consensus through discussion. PD-L1 in TCs and tumor-associated immune cells (TAICs) were evaluated separately. Results of the staining were assessed by the intensity of staining and the proportion of positive cells. The staining intensity of PD-L1 and p53 was classified as 0, 1, 2, and 3(A) representing negative, weak, moderate and strong, respectively. The proportion of positive cells ranged from 0 to 100(B). The H score (H=A × B, range 0–300) was used to analyze the correlation between PD-L1 and p53. The positive staining of PD-L1 was defined as any discernible DAB positivity localized in TCs or TAICs regardless of the proportion of staining.[15] Positive p53 was defined as the positively stained tumor cells not <10% regardless of the proportion of staining.[16]

Statistical analysis

SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and statistical drawing was performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between PD-L1 and p53 in TNBC and non-TNBC and the various clinical factors in TNBC were determined using χ^2 test. The correlation between PD-L1 and p53 expression was detected by Spearman's rank correlation analysis according to the immunohistochemical results. Survival outcomes were analyzed by Kaplan–Meier method and compared using the log-rank test. All tests were bilateral, and $P<0.05$ was considered as statistically significant.

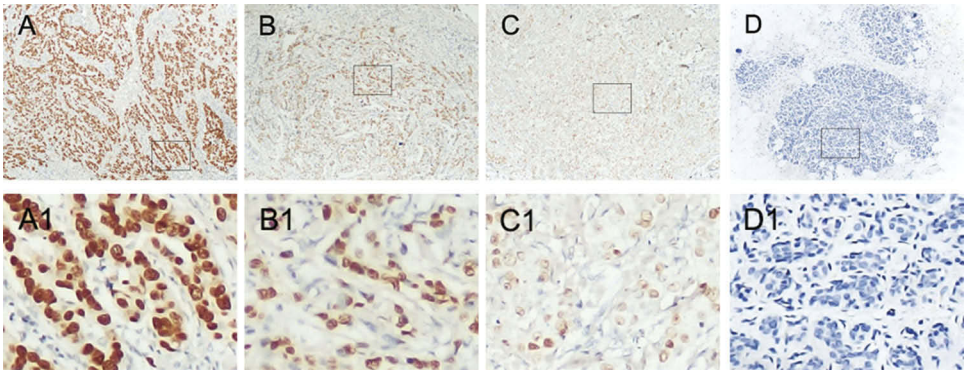

Figure 2 Different expression intensities of p53 in TNBC.
Notes: (**A and A1**): strong positive expression of p53 in TCs. (**B and B1**): moderate positive expression of p53 in TCs. (**C and C1**): weak positive expression of p53 in TCs. (**D and D1**): negative expression of p53 in TCs (magnification ×100, x400).
Abbreviations: TNBC, triple-negative breast cancer; TCs, tumor cells.

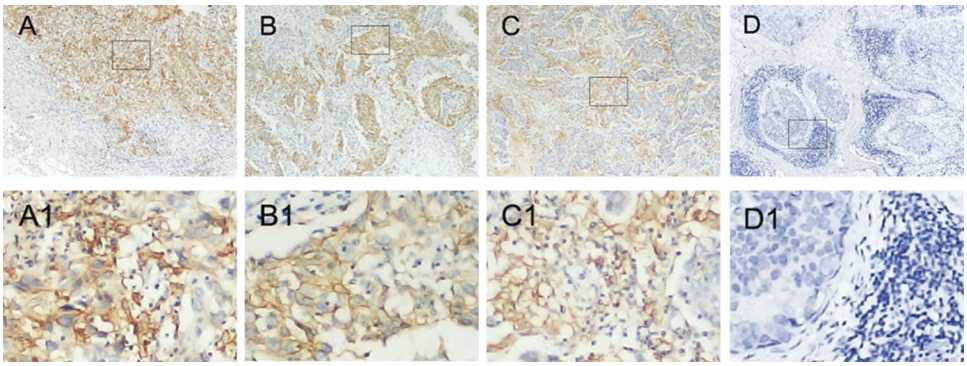

Figure 3 Different expression patterns of PD-L1 in TNBC.
Notes: (**A and A1**): positive expression of PD-L1 both in TCs and TAICs. (**B and B1**): positive expression of PD-L1 in TCs, but not in TAICs. (**C and C1**): positive expression of PD-L1 in TAICs, but not in TCs. (**D and D1**): negative expression of PD-L1 both in TCs and TAICs (magnification ×100; ×400).
Abbreviations: PD-L1, programmed death ligand-1; TNBC, triple-negative breast cancer; TCs, tumor cells; TAICs, tumor-associated immune cells.

Results

Expression of PD-L1 and p53 in TNBC and non-TNBC

PD-L1 expression mainly locates in the cytoplasm and cell membrane of TCs and TAICs. The positive rates of PD-L1 in TCs and TAICs in non-TNBC were 15.6% (5/32) and 28.1% (9/32), and the rates in TNBC were 37.1% (49/132) and 40.2% (53/132), respectively. Rates of PD-L1 in TCs and TAICs of TNBC were higher than in non-TNBC, but the difference was statistically significant only in TCs (P=0.02), not in TAICs (P=0.21). The positive rate of p53 in TNBC was 68.2% (90/132), which was significantly higher than 46.9% (15/32) in non-TNBC in statistically (P=0.02) (Table 1, Figures 2 & 3).

The relationship of PD-L1 and p53 with clinicopathological factors in TNBC

The expressions of PD-L1 and p53 in breast cancer cells were correlated with histological grade, tumor size and Ki67 index, and the differences were statistically significant (P<0.05). The expression of PD-L1 in TCs was also significantly correlated with lymph node metastasis status (P<0.05), but p53 was not (P>0.05). Both expressions of PD-L1 and p53 were not related to patients' age, menopausal status, or vascular invasion (P>0.05). The expressions of PD-L1 in TAICs were only correlated with the histological grade significantly (P<0.05). It was not related to patient's age, menopausal status, tumor size, vascular invasion, lymph node metastasis or Ki67 index (P>0.05) (Table 2).

Table 2 Relationship with clinicopathological factors

Factors	n	PD-L1 in TCs	χ^2 score	*P*-value	PD-L1 in TAICs	χ^2 score	p53 in TCs	χ^2 score	*P*-value
		+ n (%)			+ n (%)		+ n (%)		
Number of cases	132	49 (37.1)			53 (40.2)		90 (68.2)		
Age (years)			0.006	0.938		0.030		0.096	0.757
≤50	76	28 (36.8)			31 (40.1)		51 (67.1)		
>50	56	21 (37.5)			22 (39.3)		39 (69.6)		
Menopausal status			0.475	0.491		2.805		0.701	0.403
Presence	73	29 (39.7)			34 (46.6)		52 (71.2)		
Absence	59	20 (33.9)			19 (32.2)		38 (64.4)		
Histological grade			9.372	0.002		6.248		4.352	0.037
I–II	52	11 (21.1)			14 (26.9)		30 (57.7)		
III	80	38 (47.5)			39 (48.8)		60 (75.0)		
Maximum diameter of tumor (cm)			6.108	0.013		2.542		8.839	0.003
<2	32	6 (18.8)			9 (28.1)		15 (46.9)		
≥2	100	43 (43.0)			44 (44.0)		75 (75.0)		
Lymphatic metastasis			59.43	0.000		1.690		0.059	0.809
Presence	39	34 (87.2)			19 (48.7)		26 (66.7)		
Absence	93	15 (16.1)			34 (36.6)		64 (68.8)		
Vascular invasion			1.127	0.288		0.122		0.958	0.328
Presence	6	1 (16.7)			2 (33.3)		3 (50.0)		
Absence	126	48 (38.1)			51 (40.5)		87 (69.0)		
Ki-67 index			4.440	0.035		0.413		4.934	0.026
≤20%	26	5 (19.2)			9 (34.6)		13 (50.0)		
>20%	106	44 (41.5)			44 (41.5)		77 (72.6)		

Abbreviations: PD-L1, programmed death ligand-1; TCs, tumor cells; TAICs, tumor-associated immune cells.

Table 3 H score of PD-L1 and p53

Groups	H score			
	H<0.01	0.01≤H<1	1≤H<2	H≥2
PD-L1 in TCs	83	32	11	6
PD-L1 in TAICs	79	44	9	0
p53	42	68	11	11

Abbreviations: PD-L1, programmed death ligand-1; TCs, tumor cells; TAICs, tumor-associated immune cells.

Correlation between PD-L1 and p53 expression in TNBC

According to the H score (Table 3) obtained from IHC, Spearman rank correlation analysis showed that there were significant positive correlations with each other among PD-L1 in TCs[a], PD-L1 in TAICs[b] and p53 in TCs[c] (r^{ac}=0.338, P^{ac}=0.000; r^{bc}=0.186, P^{bc}=0.033; r^{ab}=0.764, P^{ab}=0.000) (Table 4).

Survival outcomes analysis in TNBC

Kaplan–Meier survival curve analysis showed that P53 positive group, PD-L1 positive in TCs and in TAICs groups had a worse overall survival and a worse progression-free survival as compared with the negative groups (Figure 4). Although no significance was found, the differences of overall survival of PD-L1 in TCs and TAICs, and progression-free survival of PD-L1 in TAICs reached marginal significance (P=0.074, 0.097, 0.068, respectively).

Discussion

Recent studies have shown that PD-L1 is up-regulated in various malignant tumors and is associated with poor prognosis.[17–19] In consistent with previous studies, our research showed that PD-L1 was highly expressed in TNBC than in non-TNBC, and the positive rate of PD-L1 in TNBC was significantly higher than in non-TNBC. Recently the anti-PD-L1 monoclonal antibody has shown excellent efficacy in TNBC,[20] which suggests that PD-L1 has potential value as a prognostic biomarker of TNBC. Meanwhile, TNBC, as a subtype with high immunogenicity in breast cancer, immune infiltrates have been shown to influence response to therapy and prognosis in TNBC.[21] In our research, we evaluated the expression of PD-L1 in TAICs alone, high expression was found and significantly correlated with tumor grade, which is a key prognostic factor of cancers. Furthermore, the expression of PD-L1 in TAICs was considered to be a predictive biomarker for anti-PD-L1 antibody MPDL3280A in the previous study.[22]

Table 4 Correlation between PD-L1 and p53

Groups		n	PD-L1 in TCs		r score	P-value	PD-L1 in TAICs		r score	P-value
			+(n%)	−(n%)			+(n%)	−(n%)		
p53					0.338	0.000			0.186	0.033
	+	90	41 (45.6)	49 (54.4)			42 (46.7)	48 (53.3)		
	−	42	8 (19.0)	34 (81.0)			11 (26.2)	31 (73.8)		
PD-L1 in TAICs					0.764	0.000				
	+	53	44 (93.0)	9 (17.0)						
	−	79	5 (6.3)	74 (93.7)						

Abbreviations: PD-L1, programmed death ligand-1; TCs, tumor cells; TAICs, tumor-associated immune cells.

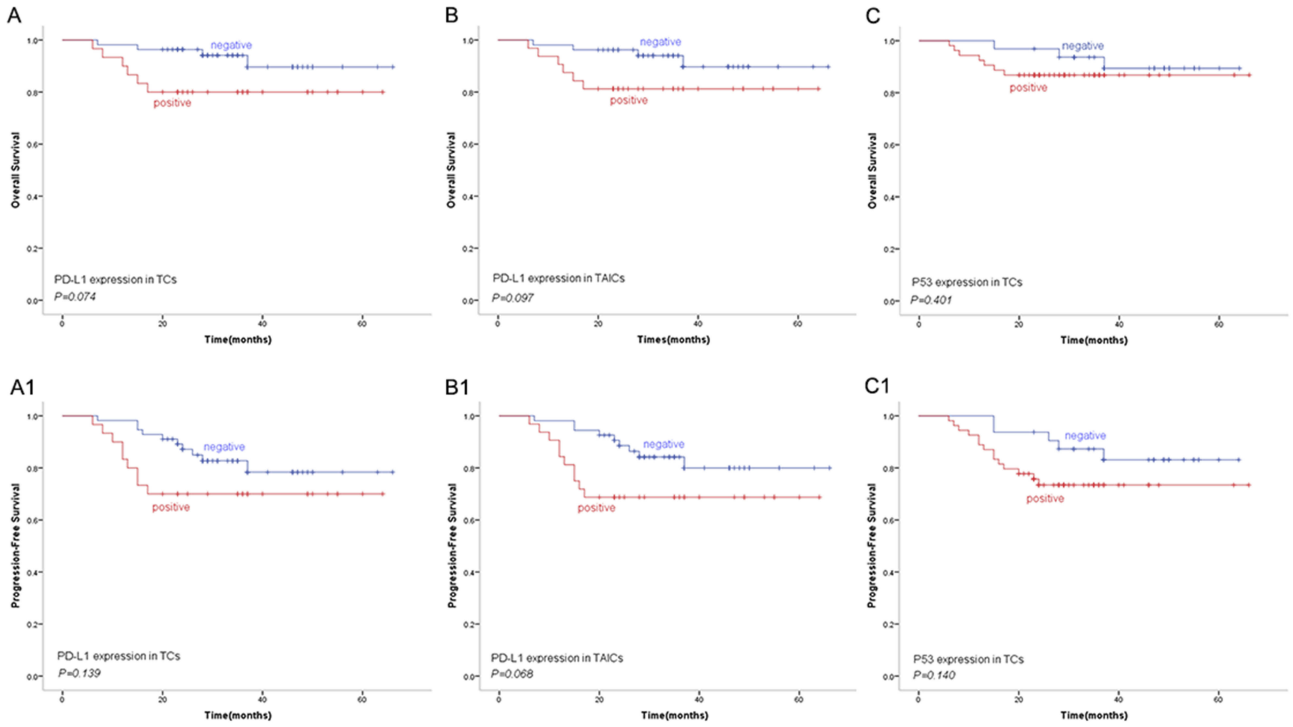

Figure 4 Kaplan–Meier analysis of OS and PFS in TNBC.
Notes: Kaplan–Meier analysis of OS and PFS according to: (**A and A1**): PD-L1 expression in TCs; (**B and B1**): PD-L1 expression in TAICs; (**C and C1**): p53 expression. Plus sign (+) indicates data censored.
Abbreviations: OS, overall survival; PFS, progression-free survival; PD-L1, programmed death ligand-1; TNBC, triple-negative breast cancer; TCs, tumor cells; TAICs, tumor-associated immune cells.

A Phase Ib clinical trial with pembrolizumab in 27 patients with TNBC positive for PD-L1 in TCs and TAICs achieved one complete response, four partial responses and seven cases with stable disease,[20] which suggests that patients with positive PD-L1 in TCs and TAICs may have a better response to anti-PD-1/PD-L1 therapy, just like in cancers with brain metastases,[23] and lung cancer.[24] Elevated PD-L1 may be caused by high mutation load and increased neoantigen burden. A research showed that the predicted neoantigen load was proportional to the mutational load in statistically and also PD-L1 expression was common in intraepithelial immune cells and more frequent in POLE-mutated and Microsatellite-Instable tumors.[25] In high grade serous ovarian cancer, BRCA1/2-mutated tumors exhibiting significantly elevated expression of PD-1 and PD-L1 in TAICs compared to HR-proficient tumors were demonstrated.[26] As a most frequently mutated gene, p53 appeared to be immunogenic and represents an attractive candidate for evaluating targeted immune cancer therapies.[27] p53 also plays an important role in DNA damage pathways, which is one of the mechanisms inducing the up-regulation of PD-L1 expression. It may be due to the activation of STING-dependent innate immune signaling.[28] In our study, both TCs and TAICs PD-L1 were positively correlated with the expression of p53, suggesting that there is a synergistic effect between PD-L1 and p53 in the occurrence and development of tumors, which has also been demonstrated in NSCLC.[14,29] p53 can bind to the PD-L1 3′-untranslated region via miR-34 to regulate PD-L1 in NSCLC models.[14] However, the relationship between these two factors in TNBC is not clear at present, and the specific regulatory mode needs to be further analyzed. Our study has laid a preliminary foundation for further study on the relationship between PD-L1 and p53 in TNBC.

Moreover, we recognize many limitations in our study. First, bias can be caused by difference between antibodies. Studies have shown that PD-L1 detection in cancer cells and immune cells varied by antibody clone. PD-L1 (E1L3N) and PD-L1 (28–8) were proved to have better dyeing effect in TNBC and GC (gastric cancer) respectively. The concordance rate between these two monoclonal PD-L1 antibodies was higher.[15,30] Therefore, PD-L1 (E1L3N) antibody was chosen to strive for the most accurate results in our study. Second, Whether the expression of p53 protein detected

by IHC can reflect the content and mutation of p53 gene. Wild-type p53 protein has a short half-life, which is difficult to be detected by IHC. Hence, the positive expression detected by IHC is mutated p53 proteins with a long half-life. Besides, studies showed there was a 59.5% concordance between p53 gene mutations and p53 immunopositivity.[31] The level of mutated p53 at the genetic level was barely replaced by the level of protein detected by IHC.

Conclusion

Elevated expression of PD-L1 and p53 is demonstrated in TNBC compared with non-TNBC and correlates with key prognosis factors. A positive correlation is found between PD-L1 and p53 in TNBC and co-inhibition of PD-L1 and mutated p53 is expected to be a new strategy for anticancer therapy in TNBC.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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