

RESEARCH PAPER



PD1 protein expression in tumor infiltrated lymphocytes rather than PDL1 in tumor cells predicts survival in triple-negative breast cancer

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ABSTRACT

To determine PD1/PDL1 expression status in triple-negative breast cancer (TNBC) at both protein and mRNA levels, and to analyze the relationship between their expression and clinical parameters of the TNBC patients.

Immunohistochemistry and RNAscope were used to semi quantitatively evaluate PD1/PDL1 protein and mRNA expression in 195 TNBC cases on tissue microarrays. Tumor infiltrating lymphocyte (TILs) abundance was assessed using hematoxylin-eosin staining. Both tumor cells and TILs expressed PDL1. PDL1 protein and mRNA positivity was 6.7% and 74.4% respectively in tumor cells, and 31.3% and 50.9% respectively in TILs. PDL1 protein and mRNA expressions had no significant association with patient prognosis. PD1 protein was only detected in TILs (70.3% positivity). PD1 protein expression was significantly related to PDL1 expression, higher TIL abundance, Ki-67 index, basal-like subtypes, and distant metastasis. Furthermore, it was significantly associated with longer disease free survival ($P < 0.001$) and overall survival ($P = 0.004$). There was no significant association between PD1 mRNA expression and clinicopathological characteristics. PD1/PDL1 protein and mRNA expressions were inconsistent ($\kappa = 0.705$ and 0.061 , respectively). PD1 protein expression in TILs, but not PDL1 in tumor cells, was a favorable prognostic factor in TNBC. PD1/PDL1 mRNA and protein expressions were inconsistent.

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Introduction

Breast cancer lacking the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) is termed triple-negative breast cancer (TNBC). It is a clinically and molecularly heterogeneous disease that encompasses more than one entity.^{1,2} In total, 70–80% of the TNBCs belong to the basal-like subtype.^{3,4} Basal-like breast cancers express at least one basal marker, such as cytokeratin (CK) 5/6, CK14, or epidermal growth factor receptor (EGFR).^{5,6} TNBC, especially the basal-like subtype, does not benefit from any routinely available targeted therapy. And it brings about the poorest disease-free survival (DFS) and overall survival (OS) of any breast cancer.^{7,8} Lymphocytes were often seen in the tumor stroma of TNBC, namely tumor-infiltrating lymphocytes (TILs). Fridman et al reviewed the impact of TILs on the clinical outcomes of various epithelial cancers. In breast cancer, some reports showed TILs were associated better outcome.⁹

As is well known, programmed cell death protein 1/programmed cell death ligand 1 (PD1/PDL1) are important immune checkpoint components that mainly regulate the function of tumor cells and TILs.¹⁰ PD1/PDL1 activation in tumor cells and TILs attenuates local immune responses, thus shielding the tumor from T cell-mediated killing.^{11–13} Clinical trials of agents targeting PD1/PDL1 in these tumors have displayed obvious tumor regression and extended stabilization of disease in some patients.^{14–16}

Most research focuses on the relationship between PD1/PDL1 expression and targeted therapeutic effectiveness. The prognosis value of PDL1 expression mostly were based on mRNA level and the result were controversial in breast cancer.^{17,18} The correlations between PD1/PDL1 protein expression and clinicopathological factors such as TIL abundance, basal-like markers and prognosis are seldom researched.

In light of previous researches, and with the interest in finding an association between PD1/PDL1 expression and TNBC clinicopathological factors, we analyzed PD1/PDL1 expression status in TNBC on both the protein and mRNA level in tumor cells and TILs. We then analyzed the expression data in relation to clinicopathological parameters and prognosis of TNBC. Finally, the consistency of mRNA and protein of PD1/PDL1 expression were assessed.

Results

Clinicopathological characteristics of TNBC patients

This study enrolled altogether 195 consecutive TNBC cases. The average age of the patients was 50.8 years old at diagnosis (range: 24–90). Clinicopathological characteristics of the patients are presented in Table 1. 177 patients (90.8%) were

histologically non-specific type and the majorities had a high-grade lesion. 175 (89.7%) patients underwent modified radical mastectomy. 82 (42.1%) had positive lymph node metastases in surgical specimen; 103 patients (69.2%) received adjuvant chemotherapy only, and 32 (16.4%) patients received both adjuvant chemotherapy and radiotherapy. For the 135 patients who received chemotherapy, recurrence was observed in 67 patients (34.4%). 57 of them presented distant metastases and 10 (5.1%) had local recurrence. 35 (17.9%) patients died of the disease during the follow-up.

Protein expression of PDL1 in tumor cells and PD1 in TILs and their relationships with clinicopathological features

PD1 or PDL1 protein expression assays were successful in 195 cases. PDL1 protein expression could be detected in both tumor cells and TILs. There were 13 PDL1 positive cases as evaluated by tumor cells and 61 by TILs (namely tumor cells PDL1 protein positivity and TILs PDL1 protein positivity thereafter, respectively). Tumor cells PDL1 protein positivity was significantly related to TILs PDL1 protein positivity ($P = 0.001$) (Table 2). However, no significant relationship was found between tumor cells PDL1 protein positivity and assessed clinicopathological characteristics. Kaplan–Meier and Cox analysis showed that PDL1 positivity in neither tumor cells nor TILs was correlated with recurrence or survival.

No tumor cells PD1 protein positivity was identified in the present cohort. On the other hand, TILs PD1 protein positivity was found in 70.3% of the cases. TILs PD1 protein positivity was significantly related to basal-like subtype, TILs abundance, TILs PDL1 protein positivity and distant metastasis ($P < 0.05$) (Table 3). Kaplan–Meier tests revealed that TILs PD1 protein positivity was significantly related to DFS and OS ($P = 0.000$ and 0.004 , respectively) (Fig. 1A and B). Of all factors, stage, lymph node or distant metastases, tumor diameter >5 cm, TILs abundance $\geq 30\%$, and TILs PD1 protein positivity were significantly correlated with DFS and OS in the univariate test. Multivariate analyses showed that TILs PD1 positivity was an independent prognostic factor.

PD1/PDL1 mRNA expression and its relationship with clinicopathological features

101 cases were subjected to in situ PD1 and PDL1 mRNA expression analyses with RNAscope. The successful rates for PD1 and PDL1 assays were 73.3% (74 out of 101 cases) and 82.2% (83 out of 101 cases), respectively.

Both tumor cells and TILs expressed PDL1 mRNA. There were 61 tumor cells PDL1 positive cases (74.4%), far higher than the rate of PDL1 protein expression (8.4%). Tumor cells PDL1 mRNA expression was also significantly related to TILs PDL1 mRNA positivity ($P = 0.006$), which is similar to the corresponding protein expression. Tumor cells PDL1 mRNA positive cases are more likely to appear in basal-like subtypes, tumors with higher Ki-67 index ($\geq 20\%$), and high grade cases ($P = 0.033$, 0.025 and 0.034 , respectively) (Table 2). PDL1 mRNA positivity in neither tumor cells nor TIL was significantly related to recurrence or survival by Kaplan–Meier analysis.

Table 1. Patient characteristics.

Characteristics		N (%)	
Age (years)	<50	99(50.8)	
	≥ 50	96(49.2)	
Recurrent status	Yes	71(36.4%)	
	No	117(60.0%)	
	NA	7 (3.6%)	
Living status	Yes	153(78.5%)	
	No	35(17.9%)	
	NA	7 (3.6%)	
Tumor size (cm)	>5	8(4.1%)	
	$>2, \leq 5$	106(54.4%)	
	≤ 2	79(40.5%)	
	NA	2(1.0%)	
Histopathology	Invasive carcinoma of non-specific subtype	177(90.8%)	
	others	18(9.2%)	
	Mixed ductal and lobular carcinoma	4	
	medullary carcinoma	4	
	Mucinous carcinoma	1	
	Invasive micropapillary carcinoma	2	
	Invasive lobular carcinoma	4	
	Metaplastic carcinoma(Adenosquamous carcinoma)	1	
	Adjuvant chemotherapy	Yes	135(69.2%)
		No	29(14.9%)
		NA	31(15.9%)
Adjuvant radiotherapy	Yes	33(16.9%)	
	No	142(72.8%)	
	NA	20(10.3%)	
Basal-like subtype	Yes	152(77.9%)	
	No	43(22.1%)	
Grade	Grade1	9(7.9%)	
	Grade2	52(30.7%)	
	Grade3	134(61.4%)	
	Positive	93(47.7%)	
P53	Negative	101(51.8%)	
	NA	1(0.5%)	
Ki67	<20%	35(17.9%)	
	$\geq 20\%$	160(82.1%)	
TILs amount	<30%	143(73.3%)	
	$\geq 30\%$	49(25.1%)	
	NA	3(1.5%)	
TILs PD1 protein	Positive	128(65.6%)	
	Negative	54(27.7%)	
	NA	13(6.7%)	
Tumor cells PDL1 protein	Positive	13(6.7%)	
	Negative	182(93.3)	
TILs PDL1 protein	Positive	61(31.3%)	
	Negative	134(68.7%)	
Pathological stage	I	50(25.7%)	
	II	105(53.8%)	
	III	40(20.5%)	
Lymphonodes metastasis	Yes	82(42.1%)	
	No	106(54.4%)	
	NA	7(3.5%)	
Distant metastasis	Yes	57(29.2%)	
	No	131(67.2%)	
	NA	7(3.6%)	
Death	Yes	35(17.9%)	
	No	153(78.5%)	
	NA	7(3.6%)	

Table 2. Tumor cells PDL1 protein and mRNA expression relationship with clinicopathological features.

	Clinical Parameters	Protein Positive	Negative	P value	mRNA Positive	Negative	P value
Age				0.568			0.803
	<50	8	91		27	10	
	≥50	5	91		35	11	
Tumor size				0.701			0.152
	≤2cm	5	74		22	7	
	>2cm≤5cm	8	98		38	10	
	>5cm	0	8		3	2	
P53				0.777			1.000
	positive	7	86		28	10	
	negative	6	95		34	11	
Ki-67 index				0.130			0.025
	<20%	0	35		13	10	
	≥20%	13	147		49	11	
TILs amount				1.000			0.449
	<30%	10	137		55	16	
	≥30%	3	42		7	4	
TILs PDL1 expression				0.001			0.006
	Positive	10	51		37	5	
	negative	3	131		25	16	
Basallike subtype				0.737			0.033
	Yes	11	141		46	10	
	No	2	41		16	11	
Histological type				1.000			0.488
	Invasive carcinoma of non-specific subtype	12	165		54	17	
	others	1	11		8	4	
Grade				0.352			0.034
	Grade1 or 2	2	59		18	12	
	Grade 3	11	123		44	9	
Stage				0.832			0.906
	I	3	47		13	4	
	II	8	97		37	12	
	III	2	38		12	5	
Lymphnode metastasis				0.117			0.803
	Yes	2	80		30	10	
	No	9	97		28	11	
Distant metastasis				0.538			1.000
	Yes	5	52		26	9	
	No	8	123		36	12	

PD1 mRNA positivity was mainly found in TILs. Tumor cells PD1 mRNA expression was only detected in two cases. PD1 mRNA expression did not correlate with any characteristics (Table 3), except for tumor cells and TILs PDL1 mRNA expression.

Consistencies of PD1/PDL1 mRNA and protein expression in tumor cells and lymphocytes

The tumor cells PDL1 mRNA positive rate was 74.7%, figure was much lower when evaluated at protein level (8.4%). Tumor cells PDL1 protein positive case was 100% positive for mRNA, but not vice versa (Fig 2). Some cases with high mRNA expression did not have corresponding high protein expression. The TILs PDL1 mRNA and protein positive rate was 50.6% and 21.7%, respectively. mRNA expression was not consistent with protein expression in either tumor cells or TILs. The Kappa value of PDL1 mRNA and protein consistence was 0.061 and 0.234 for tumor cells and TILs, respectively (Table 4). PDL1 positivity in tumor cells was also significantly correlated with TILs PDL1 expression at mRNA level.

The TILs PD1 mRNA positive rate was 36.5% and the protein positive rate was 51.4%. There were 11 PD1 protein positive cases that were PD1 mRNA negative. We didn't find high

consistence in PD1 mRNA and protein expression, too (with kappa value = 0.705) (Table 4, Fig 3).

Discussion

Blockade of the PD1/PDL1 pathway is a new and promising therapeutic approach in oncology. Research has focused primarily on the relationship between PDL1 expression level and response to PDL1 targeted therapy.^{14,19} However, the expression and prognostic value of PD1/PDL1 expression in TNBC has been insufficiently investigated. To our knowledge, this is the first large cohort study (n = 195) that analyzed PD1 and PDL1 expression at both the mRNA and protein levels at the same time and analyzed the relationship between expression and clinicopathological characteristics.

In this study, there was a low tumor cells PDL1 protein positivity in the 195 enrolled cases (n = 13, 6.7%). PDL1 positivity was reported to be between 19% and 64% in existing literatures.²⁰⁻²² However, the antibodies were from different companies and the positive cut off value was different. The low positivity in the present study might be attributable to the strict criteria recommended by the antibody producer, which required ≥25% tumor cell membrane staining, instead of 1% or 5% membrane or cytoplasm staining being sufficient for a case

Table 3. TILs PD1 protein and mRNA expression and their relationship with clinicopathological features.

	Clinical Parameters	Protein		P value	mRNA		P value
		positive	negative		Positive	Negative	
age							0.333
	<50	67	24	0.417	17	23	
	≥50	61	30		10	24	
Tumor size				0.247			0.557
	≤2cm	54	20		5	17	
	>2cm≤5cm	70	29		30	39	
	>5cm	3	4		0	4	
P53				0.519			1.000
	positive	66	25		13	23	
	negative	61	29		14	24	
Ki-67 index				0.012			0.487
	<20%	17	16		2	14	
	≥20%	111	38		12	37	
tumor cells PDL1				0.112			0.013
	Positive	12	1		25	33	
	Negative	116	53		1	14	
TILs amount				0.001			0.164
	<30%	89	49		11	47	
	≥30%	38	4		3	4	
TILs PDL1 positivity				0.000			0.003
	Positive	57	3		20	18	
	negative	71	51		6	29	
Basallike subtype				0.025			0.298
	Yes	108	37		21	30	
	No	20	17		6	17	
Histological type				0.084			0.716
	Invasive carcinoma of non-specific subtype	120	46		23	42	
	others	8	8		4	5	
grade				0.113			0.618
	Grade1 or 2	34	21		8	17	
	Grade 3	94	33		19	30	
Stage				0.550			0.492
	I	36	11		1	10	
	II	66	31		10	29	
	III	26	12		3	12	
Lymphnode metastasis				0.245			1.000
	Yes	49	27		12	23	
	No	73	26		13	22	
Distant metastasis				0.000			0.084
	Yes	25	27		7	23	
	No	97	26		20	24	

to be classified as PDL1 positive, as was the case in previous studies.

The results in the literature regarding the relationship between PDL1 expression and prognosis are conflicting. One

study showed that tumor cells PDL1 expression was associated with poor prognosis in breast cancer.²³ However, other groups showed that PDL1 mRNA was associated with better prognosis.^{24,25} Our mRNA expression analysis found similar results to

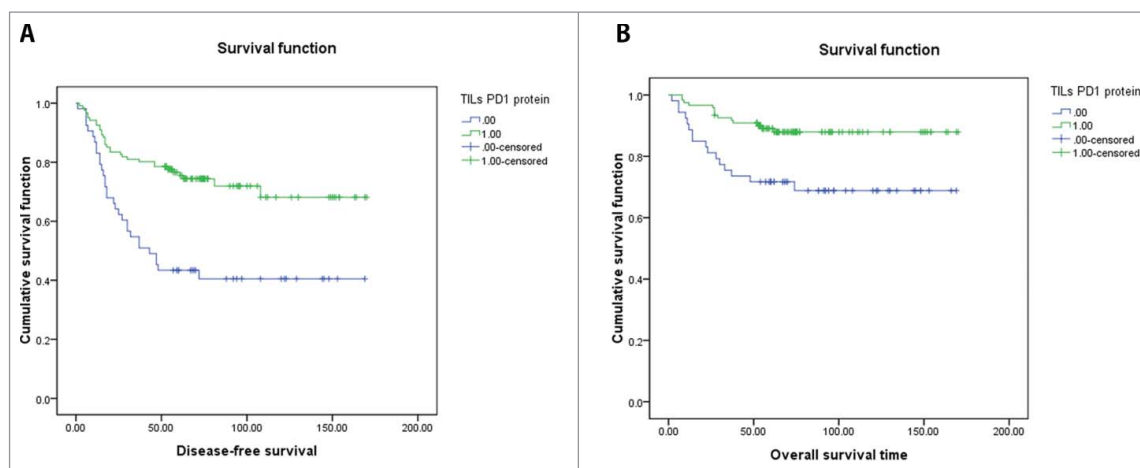


Figure 1. (A) K-M survival analysis showed that TILs PD1 protein expression was significantly influence DFS time. (B) K-M survival analysis showed that TILs PD1 protein expression was significantly prolonged OS time.

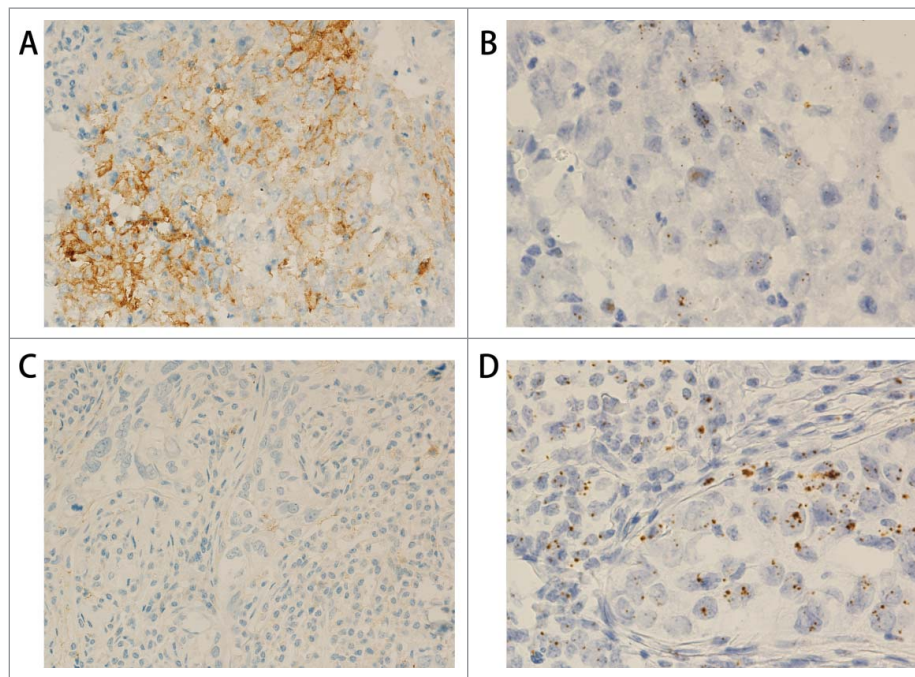


Figure 2. PDL1 protein and mRNA staining in triple negative breast cancer. (A) IHC shows PDL1 protein expression in the tumor cells (200x). (B) RNAscope shows PDL1 mRNA expression in the same case as in A (400x). (C) IHC shows negative PDL1 protein staining in the tumor cells (200x). (D) RNAscope shows positive PDL1 mRNA expression in the same case as in C (400x).

those described by Muenst et al, who found that PDL1 upregulation was associated with poor-prognostic features.²⁵ Tumor cell PDL1 mRNA expression tended to appear in cases with higher grade ($P = 0.034$), larger size ($P = 0.094$), higher Ki-67 index ($\geq 20\%$, $P = 0.025$), and basal-like subtypes ($P = 0.033$). In our study, cases that were determined to be tumor cells PDL1 mRNA positive had shorter DFS and OS on average, which was consistent with their relation to poor-prognostic features, although this prognostic relationship was not significant. But we were unable to establish an association between tumor cells PDL1 protein positivity and clinical outcome, which was different to the result of Beckers et al.²² They also noticed that PDL1 expression in stromal immune cells was associated with a lower mortality rate. Our findings in TILs also showed that PDL1 expression cases had longer DFS and OS, but this was not statistically significant.

This is the first study reporting that PD1 expression in triple negative breast cancer could be of prognostic significance. PD1 protein was only detected in lymphocytes. It was positively related to basal-like subtype, TIL abundance, and PDL1 protein expression, but negatively related to distant metastasis. More importantly, survival analysis showed PD1 protein positivity was an independent prognostic factor relating to TIL

abundance, stage, large tumor size, and lymph node metastasis. A higher abundance of TILs was considered a good prognosticator.²⁶⁻²⁸ Our data also showed that higher numbers of TILs could significantly prolong DFS and OS. Previous research has verified that TILs in breast cancer were related to the expression of CD8⁺T-cell-related genes.²⁹ PD1 itself was reported to be expressed in activated T cells.³⁰ Therefore, it is possible that PD1 may act through a TIL-mediated anti-tumor inflammatory response rather than in tumor immune evasion. PD1 expression was significantly related to TILs PDL1 expression. This might explain why the PDL1 expression in TILs tended to be associated with better prognosis. Vassiliki et al pointed out that the effectiveness of anti-PD1 therapy requires the existence of both PD1⁺CD8⁺ T cells and PDL1-expressing cells in the tumor microenvironment.³¹ Although tumor cells PDL1 expression was low; there was a subset of TILs that did show PDL1 expression (31.3%). This may indicate that anti-PD1 or PDL1 therapy could still be effective even if tumors have low expression levels overall.

As PDL1 expression was analyzed simultaneously at mRNA and protein levels, we checked the concordance between the two methodologies. PDL1 protein and mRNA expression were inconsistent. For PDL1, there were cases with positive mRNA expression but negative protein expression. PDL1 mRNA expression in both tumor cells and TILs was not significantly related to survival. PD1 mRNA and protein expression were also not having a high concordance with a kappa value of 0.705. For PD1, positivity evaluated by mRNA was lower than protein and this may be due to mRNA degradation after long-term storage of the tissue. The oldest cases in our study has been stored for 10 years; some scientists proposed that analyzing the relationship between expression and clinical outcomes by protein was more reasonable because mRNA is not always

Table 4. PD1/PDL1 mRNA and their corresponding protein comparison.

		Protein		Kappa value	
		positive	negative		
Tumor cells PDL1	mRNA	Positive	7	55	0.061
		Negative	0	21	
TILs PDL1	mRNA	Positive	14	28	0.234
		Negative	4	37	
TILs PD1	mRNA	Positive	27	0	0.705
		Negative	11	36	

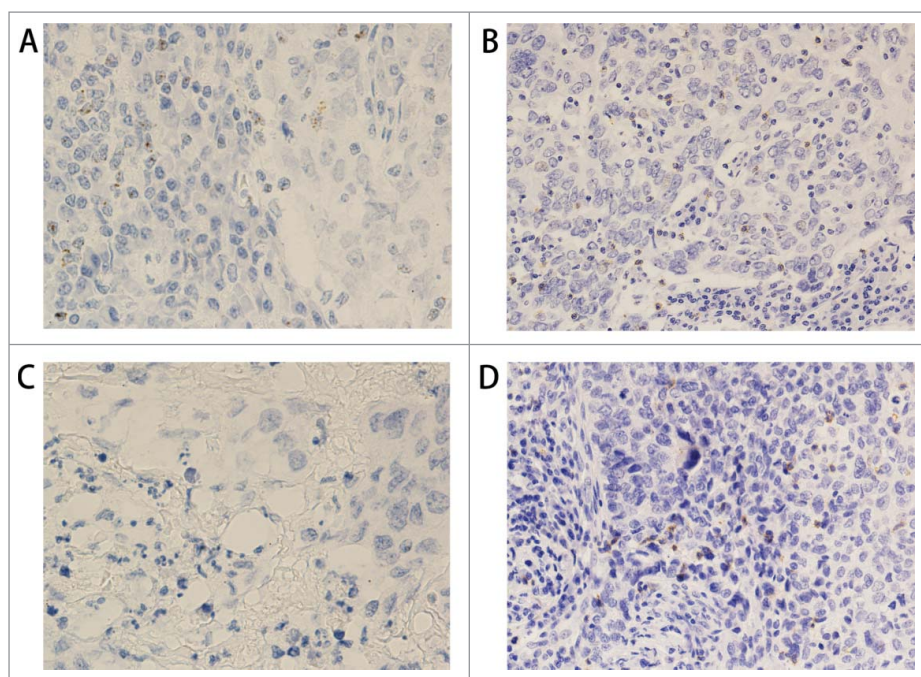


Figure 3. PD1 protein and mRNA staining in triple negative breast cancer. (A) RNAscope shows PD1 mRNA expression in TILs (400x). (B) IHC shows PD1 protein expression in the same case as in A (200x). (C) RNAscope shows negative PD1 mRNA expression in TILs (400x). (D) IHC shows PD1 protein expression in the same case as in C (200x).

translated into protein and protein is more stable than mRNA over long periods of time.³² There was no significant association between PD1 mRNA expression and clinicopathological features, indicating that protein detection is more reliable than PD1 mRNA detection.

Our research had some limitations. Firstly, the use of TMA may not detect true protein or mRNA status due to intra-tumor heterogeneity. We tried to avoid this heterogeneity by sampling 3 from representative regions in every tumor. Another limitation is that we could not obtain mRNA expression data in all cases. When we performed statistics, we included a subset cohort that contained cases with both RNA and protein detection.

In conclusion, no significant association was established between PDL1 expression and patient survival. However, higher TIL PD1 protein expression is associated with longer DFS and OS. The protein and mRNA expression levels of PD1/PDL1 were not consistent and protein detection may better reflect the true status of PD1/PDL1 gene expression.

Materials and methods

Case enrollment, Tissue Microarrays (TMAs) Preparations

Breast cancer cases that underwent curative operations between May 2002 and May 2012 and showed negative ER, PR, and HER-2 immunohistochemistry (IHC) were retrospectively enrolled from the pathology department of Peking Union Medical College Hospital (PUMCH). Cases with insufficient paraffin-embedded tumor tissue or those with preoperational neoadjuvant treatment were excluded from the study. Altogether, 195 cases with complete medical records were enrolled. Hematoxylin and Eosin (H&E) slides of all enrolled cases were reviewed by two experienced pathologists (XY Ren and HW

Wu) to confirm the diagnosis and select representative tumor areas. Pathological features, including tumor size and nodal metastasis status were obtained. The follow-up time began from the date of surgery until June 30, 2016. The primary endpoint was the progression or relapsing of disease and the secondary endpoint was death. DFS and OS were defined as the period between surgery and the date of breast cancer-related relapse and death, respectively. A tissue microarray construction machine (Quick-Ray UT-06, UNITMA) was used. Three 1mm cores per case were obtained using a needle and arrayed in a recipient block for representativeness. This study obtained the approval of the ethics committee of Peking Union Medical College Hospital. Written informed consent was obtained from each patient.

Immunohistochemistry (IHC) staining and result interpretation

4 μ m thick sections were mounted on adhesion slides from tissue microarray blocks. The immunohistochemistry staining was done on a Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, AZ) according to the manufacturer's protocols. Antibodies against ER, PR, Her-2, EGFR, CK14, CK5/6, P53, Ki-67 and their respective interpretation criteria were the same as previously described, except that we changed the cutoff value for Ki67 index to 20%.³³ Briefly, all antigens were subjected to thermal remediation by 1 mM EDTA in 10 mM Tris buffer (pH 8.5). Antibodies against PD1, PDL1 and CK14 and their respective optimizations are listed in Table 5. Positive control and negative control slide were included in each run for each antibody. IHC slides were independently evaluated by two experienced pathologists. PD1, PDL1, and CK14 positivity was defined by criteria listed in Table 5. ER, PR, Her-2, EGFR, CK5/6, P53, Ki-67 HER-2 were

Table 5. Antibodies used for the immunohistochemical analysis.

Antibodies	Clone	Dilution	Source	Heat-induced Antigen Retrieval	Incubation	Positive style	Positive control	Cut-off values (%)
PDL1	Rabbit monoclonal	Prediluted	Ventana Medical Sstems,Inc,ready-to-use ,code:263	100°C 30 min	37°C 16 min	membrane staining	placenta	≥25
PD1	Mouse monoclonal	Prediluted	OriGene, MRQ-22	100°C 30 min	37°C 16 min	plasmatic staining	tonsile	≥1
CK14	Mouse monoclonal	Prediluted	Novocatra™,Leica Biosystem, LL002	100°C 30 min	37°C 16 min	plasmatic staining	Head and neck cancer	≥1

interpreted as previously described.³³ Of note, Her-2 staining was evaluated according to the HER-2 test guide for breast cancer.³⁴ For HER-2 (2+) cases by IHC, fluorescent in situ hybridization (FISH) were conducted. HER-2–negative cases confirmed by FISH assay were enrolled in the study. Basal-like phenotype was defined by positivity for any of the following markers: CK5/6, EGFR, and CK14 in the present study.

In Situ evaluation of PD1/PDL1 RNA expression

In situ assays for PD1/PDL1 transcripts in formalin-fixed paraffin embedded (FFPE) TMA samples was performed according to the manual of the RNAscope® FFPE 2.0 HD detection kit (Brown) (Advanced Cell Diagnostics, Hayward, CA, USA, Catalog Number 310035).^{35–37} Before running the RNAscope® Assay on our samples for the first time, we ran positive and negative control probes on our samples to assess sample RNA quality and determine the optimal permeabilization conditions. Briefly, 5- μ m sections were deparaffinized, boiled with preamplification reagent for 15min, subjected to protease digestion, followed by hybridization for 2h with target probes to human PD1/PDL1 mRNA (CAT #602021 and 600861). Hybridization signals were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate. Positive staining was indicated by brown punctuate dots in the cytoplasm or nucleus. PD1/PDL1 mRNA expression levels were categorized into 5 grades according to the manufacture's scoring guidelines: score 0, no staining or <1 dot per 10 cells; score 1, 1–3 dots per cell (visible at 20–40x); score 2, 4–9 dots per cell (visible at 20–40x); score 3, 10–15 dots per cell and <10% positive cells have dot clusters (visible at 20–40x); score 4, >15 dots per cell and >10% positive cells have dot clusters (visible at 20–40x). Cases that scored higher than 0 were considered positive.

Evaluation of tumor infiltrating lymphocytes

The scoring of TILs was carried out on Hematoxylin&Eosin-stained TMA slides according to methods described previously by 2 experienced pathologists (XY Ren and HW Wu).²⁵ A score of 0 indicated virtual absence of TILs, 1+ = low TILs (<30%), 2+ = moderate (30–60%), and 3+ = marked increase in the lymphocytic infiltrate (>60%). Cases that could not be appropriately evaluated for technical reasons (e.g. bad staining, low tumor area, etc.) were designated as not evaluable. In the case of inter-observer discordance in TIL abundance, the particular slide was reviewed jointly and a agreeable score was assigned.

Statistical analysis

Statistical analysis was performed by SPSS 17.0 (SPSS, Chicago, IL, USA). Qualitative variables were compared using the chi-square test. The analysis of DFS and OS were performed firstly by a Kaplan–Meier plot and log-rank test and later with Cox regression to adjust for covariates. P-values were two sides and a $p < 0.05$ was considered statistically significant.

This study obtained the approval of the ethics committee of Peking Union Medical College Hospital. Written informed consent was obtained from each patient.

(Reagents listed in the Materials and Methods: PDL1 antibody were purchased from Ventana Medical Sstems,Inc,ready-to-use,code:263; PD1 antibody were from OriGene, MRQ-22; CK14 antibody were from Novocatra™,Leica Biosystem, LL002. RNAscope® FFPE 2.0 HD detection kit (Brown) were from (Advanced Cell Diagnostics, Hayward, CA, USA, Catalog Number 310035; human PD1/PDL1 mRNA probes were from CAT #602021 and 600861.).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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