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Clinical Significance of *PD1* and *PDL1* in Human Breast Cancer

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Abstract. *Background/Aim: Programmed death 1 (PD1) and its ligand programmed death ligand 1 (PDL1) form a pathway which when activated is thought to result in suppression of antitumor adaptive responses, influencing antitumor immunity. With potential targeted therapies emerging against PDL1, we investigated the clinical significance of mRNA expression levels of PD1 and PDL1 in our breast cancer cohort to explore its association with disease progression and prognosis. Previous studies evaluating the expression of PD1 and PDL1 (mRNA or protein) and its association with prognosis in breast cancer showed both positive and negative correlations and hence remain controversial. Materials and Methods: Quantitative polymerase chain reaction was used to determine transcript expression levels of PD1 and PDL1 in a cohort consisting of primary breast cancer tissues (n=127) and matching non-neoplastic background tissues (n=33) with available clinical and pathological information. Two-sample two-tailed t-test, Kaplan–Meier survival analysis and Wilcoxon tests were performed. Results: Significant PDL1 transcript level reductions were seen in patients who developed metastases, as well as those who had local recurrence, compared to patients who remained disease-free. Higher PDL1 transcript levels were also associated with better overall and disease-free survival. Significantly higher transcript expression levels of PD1 were found in tumor tissue, whilst a general increase in PDL1 expression was found in tumor tissues, although this did not reach statistical significance. Conclusion: Our study demonstrates higher levels of expression of PDL1 are associated with favorable clinical outcome.*

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Key Words: PD1, PDL1, breast cancer, metastasis.

Programmed death 1 (PD1) is a 55-kDa transmembrane protein and a member of the B7/CD28 co-regulatory factor family. It is widely expressed on T-cells, B-cells and natural killer cells (1) and acts as an immune checkpoint receptor. Its associated receptor, programmed death ligand 1 (PDL1), appears to be up-regulated in multiple solid malignancies (2) and is typically expressed on the surface of tumor cells (3). It suppresses autoimmunity, and is expressed by T- and B-cells, dendritic cells, macrophages, mesenchymal stem cells, and mast cells (4).

Evidence indicates that activation of the PD1/PDL1 pathway results in suppression of antitumor adaptive responses through mechanisms involving induction of cytotoxic T-cell anergy, exhaustion, apoptosis and decreased cytokine production (5-7). Thus, the interaction of PD1 with PDL1 leads to increased tumor cell resistance to pro-apoptotic signals (8) and immune escape of tumor cells, ultimately leading to poor prognosis (9). Tumor-infiltrating lymphocytes (TILs) in many types of epithelial cancer express PD1, indicating that the PD1/PDL1 pathway may influence antitumor immunity. Blockade of immune checkpoints using monoclonal antibodies targeting the PD1/PDL1 pathway have demonstrated very promising results. Several PD1 or PDL1 targeted antibodies are currently being examined in clinical trials for a variety of malignancies (10-17), alongside immunotherapies, re-activation of the tumor immune response remains a highly relevant research topic.

In the current study, we aimed to identify the clinical significance of the transcript expression levels of *PD1* and *PDL1* in a breast cancer cohort.

Table I. *Programmed cell death 1 (PD1) and programmed death ligand 1 (PDL1) transcript expression (mean relative transcript copy numbers) in cancerous tissue and association with clinical Nottingham Prognostic Index (NPI) stage, grade and TNM stage.*

Variable	*PD1*				*PDL1*			
	n	Mean	SEM	*p*-Value*	n	Mean	SEM	*p*-Value*
Tissue								
Background	26	10.01	3.90		25	66.8	46.2	
Tumor	103	39.9	10.1	0.007	90	144.3	47.9	0.250
NPI								
1	53	30.17	7.82		46	176.7	76.0	
2	30	74.6	31.0	0.170	27	149.6	93.6	0.820
3	15	15.58	4.87	0.120	13	46	19.7	0.100
Grade								
1	17	66.8	47.9		15	55.5	17.5	
2	31	31.8	11.6	0.490	27	235	127	0.170
3	53	37.0	10.4	0.550	46	122.2	56.5	0.260
TNM1								
1	56	59.6	17.9		51	157.6	73.1	
2	30	17.83	5.19	0.029	23	195.0	93.9	0.750
3	7	12.82	5.19	0.015	6	26.4	11.4	0.082
4	4	19.6	15.4	0.110	4	55.7	40.7	0.230

* *Versus* background, NPI1, grade 1, and TNM1, correspondingly.

Materials and Methods

Collection and processing of breast tissues. Primary breast cancer tissues (n=127) and matching non-neoplastic background tissues (n=33), taken from the same mastectomy samples were collected immediately following surgery and stored at −80˚C until processing and use in this study. Ethical approval was obtained from the Bro Taf Health Authority local ethics committee (reference 01/4303) and all patients gave their written informed consent to use of their data and tissues. Patients were routinely followed-up and the median follow-up period was 120 months. Clinical pathological information was also collected.

Tissue samples were homogenized using a hand-held homogenizer (Cole Parmer UK, London, UK) in ice-cold tri reagent (Sigma-Aldrich, Poole, Dorset, UK) and RNA extracted in line with the manufacturer's guidelines. RNA concentrations were determined using a spectrophotometer and samples were standardized before undergoing reverse transcription, using a high-capacity cDNA reverse transcription kit (Life Technologies, Paisley, UK) to obtain cDNA.

Quantitative polymerase chain reaction (qPCR). PD1 and *PDL1* expression within the clinical cohort was determined using qPCR based on Ampliflor technology. This method was modified based on previously described reports from our group (18, 19). In brief, primers were designed to detect *PD1* and *PDL1* transcripts and a Z sequence (5'-ACTGAACCTGACCGTACA) was added to the reverse primer of each pair to facilitate incorporation of the Uniprimer probe. Primers used were as follows; *PD1* forward: 5'-ATGGTTCTTAGACTCCCCAG, reverse: 5'-*ACTGAACCTGACCGTACA*CTCCGATGTGTTGGAGAAGC; *PDL1* forward: 5'-AAAGTCAATGCCCCATACAA, reverse: 5'-*ACTGAACCTGACCGTACA*ACATGTCAGTTCATGTTCAGAG. Primers were combined with Precision FAST qPCR Mastermix (Primer Design, Eastleigh, UK), a fluorescently-tagged Uniprimer probe (Intergen Inc, Oxford, UK) and cDNA samples in the reaction mix. Subsequently, the reaction was placed in a StepOne plus qPCR system (Life technologies, Paisley, UK) and amplified under the following conditions: initial denaturing for 10 min at 94˚C, followed by 100 cycles of 94˚C for 15 seconds, 55˚C for 40 sec and 72˚C for 15 sec. Unknown samples were run simultaneously alongside a standard of known concentration allowing for determination of mean relative transcript copy numbers per sample.

Statistical analysis. Statistical analysis was performed using the Minitab statistical software package (Minitab Ltd, Coventry, UK). Statistical comparisons were drawn between groups using a two-sample two-tailed *t*-test. Kaplan–Meier survival analysis and Wilcoxon tests were performed using the SPSS statistical software package (SPSS, Chicago, IL, USA). Statistical significance was considered at $p<0.05$.

Results

Association between PD1 and PDL1 with clinical stage, grade and estrogen receptor (ER) status. Transcript expression levels of *PD1* and *PDL1* within the breast cancer cohort were analyzed and compared according to patient clinical pathological information (Table I). Transcript expression levels of *PD1* were found to be significantly higher in tumor tissue compared to normal background tissue ($p=0.007$). No significant differences were seen in *PD1* transcript expression when compared by Nottingham Prognostic Index (NPI) status, grade (Table I), nor ER status

(data not shown, ERα: p=0.170 and ERβ: p=0.190), although significantly reduced *PD1* expression was seen in TNM2 (p=0.029) and TNM3 (p=0.015) but not TNM4 (p=0.110) compared to TNM1 stage tumor tissues (Table I).

Analysis of *PDL1* expression in conjunction with patient clinical pathological information indicated a general increase in *PDL1* expression in tumor tissues compared to normal background tissue, although this was not statistically significant (p=0.250). No significant differences were seen in *PDL1* expression according to NPI, grade or TNM stage (Table I), nor ER status (data not shown, ERα: p=0.890 and ERβ: p=0.270).

Association of PD1 and PDL1 expression with patient prognosis and survival. Further analysis was undertaken to explore the potential association of *PD1* and *PDL1* with patient prognosis and survival. No associations were found between mean *PD1* transcript levels and patient prognostic factors and no significant differences in *PD1* levels were seen in patients who developed metastases, had local recurrence or who died of breast cancer when compared to disease-free patients (p=0.08, p=0.15 and p=0.93, respectively), although levels were generally lower in those who developed metastasis or local recurrence (Figure 1A).

Generally, in our clinical cohort, significant reduction of expression of *PDL1* was seen in poorer prognostic groups, with significant reductions seen in patients who developed metastasis and those who had local recurrences (p=0.048 and p=0.014, respectively, *vs.* patients who remained disease-free); reduced levels were similarly seen in those patients who died of breast cancer compared to disease-free patients, although this was not found to be statistically significant (p=0.12, Figure 1B).

Kaplan–Meier survival analysis, following dichotomization of samples into high and low expression groups, indicated that a higher level of *PDL1* was associated with statistically significant better overall survival (OS) (p=0.017), although this was not the case for *PD1*, which had no significant association (p=0.144, Figure 2). Similarly, higher *PDL1* expression was also found to be associated with better patient disease-free survival (DFS) (p=0.007), whereas again no significant association of DFS was seen with *PD1* expression (p=0.220, Figure 3).

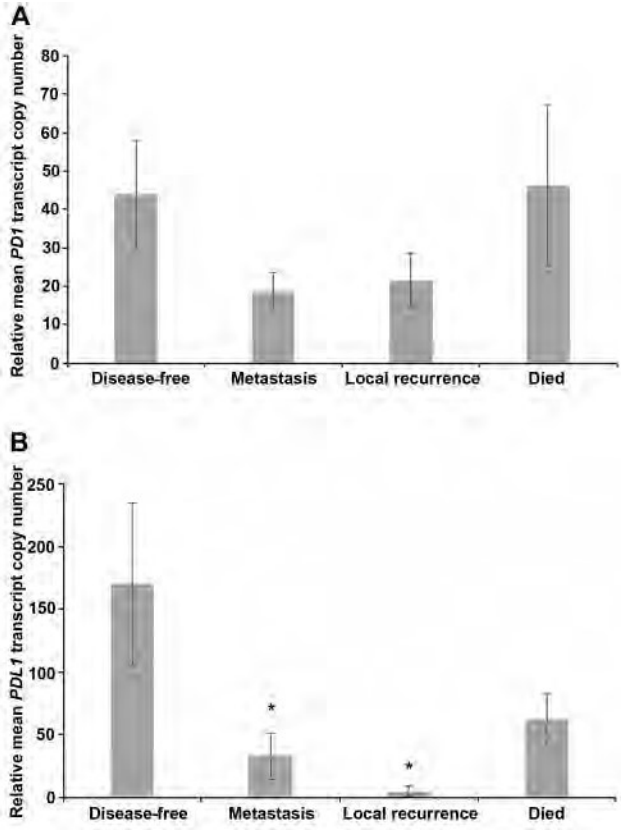

Figure 1. *Impact of programmed cell death 1 (PD1) and programmed death ligand 1 (PDL1) on the prognosis of patients with breast cancer. A: PD1 did not significantly impact on prognosis of patients with breast cancer, although reduced levels were observed in patients who suffered metastasis or local recurrence. B: PDL1 expression was significantly reduced in patients with metastasis or local recurrence of breast cancer compared to disease-free patients. Mean transcript copy numbers relative to internal standard are shown, error bars represent SEM. Significantly different at *p<0.05.*

Discussion

We demonstrated that the mRNA expressions of *PD1* and *PDL1* are up-regulated in breast cancer tissues and higher expression of *PDL1* is associated with favorable prognosis, in terms of metastasis and local recurrence, and improved clinical outcome as indicated by the Kaplan–Meier survival analysis. Our findings are consistent with previous reports examining the mRNA of *PDL1* in breast cancer (2, 20, 21). Using antibody-independent tissue compartment-specific assay, Schalper *et al.* reported that *in situ* tumor *PDL1* mRNA expression was associated with increased TILs and a better clinical outcome in patients with breast cancer (2). Bae *et al.*, in their IHC-based study, observed that PDL1 protein expression in breast cancer was associated with better DFS and OS but was not an independent prognostic indicator (20). Furthermore, recent meta-analyses considering the mRNA tumor expression or the TIL expression of *PDL1* reported improved clinical outcome with increased expression (22, 23). Given the immunosuppressive effect of PDL1 on antitumor activity of the immune system, our observations can be explained by the fact that our methodology determined the mRNA expression in the tumor cells and the tumor microenvironment, which includes immune system response cells such as cytotoxic $CD8^+$, natural killer and antigen-presenting cells. These cells are

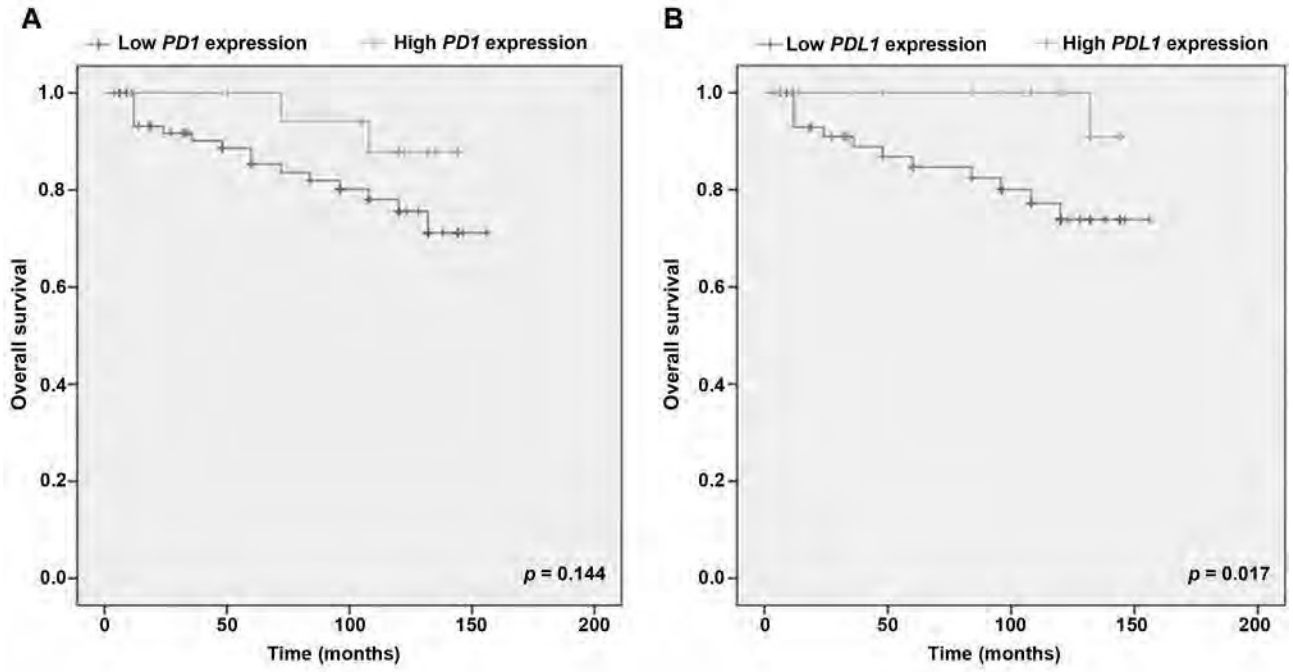

Figure 2. *Kaplan–Meier analysis of overall survival according to programmed cell death 1 (PD1) (A) and programmed death ligand 1 (PDL1) (B) expression in patients with breast cancer. No significant associations were seen between PD1 expression and overall survival. High PDL1 expression was significantly associated with better overall patient survival when compared to those with low PDL1 expression (p=0.017).*

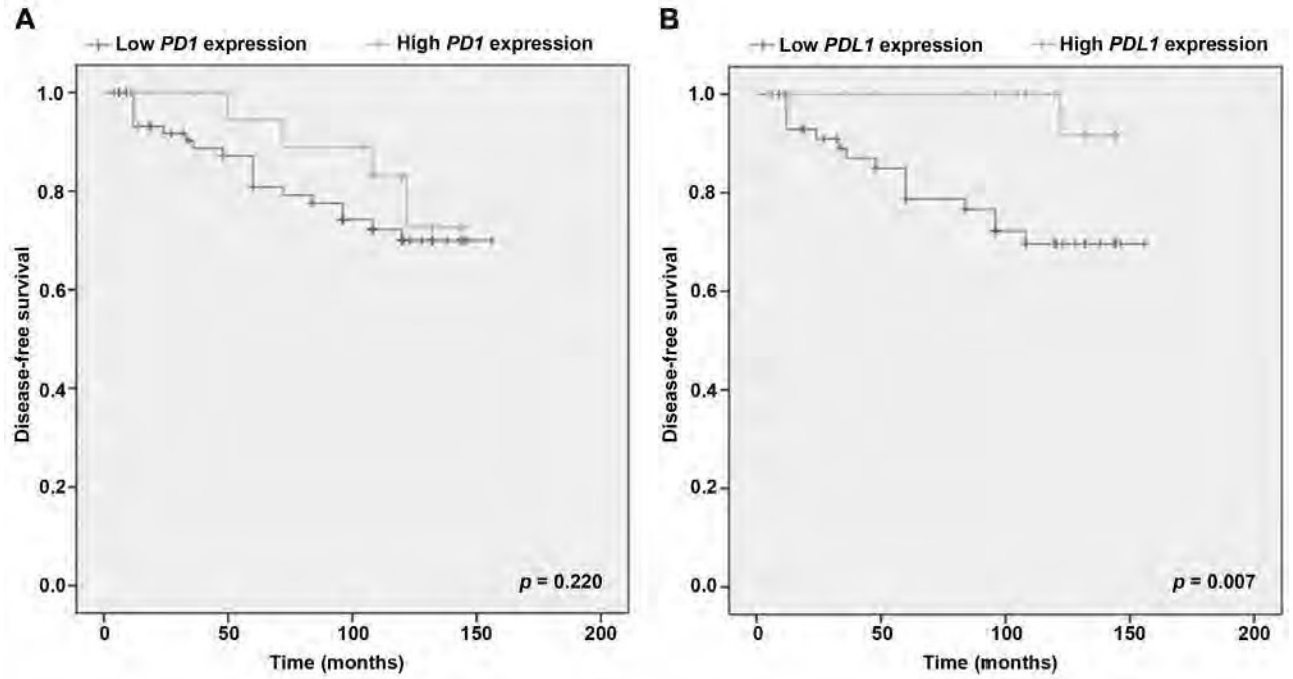

Figure 3. *Kaplan–Meier survival analysis of disease-free survival according to programmed cell death 1 (PD1) (A) and programmed death ligand 1 (PDL1) (B) expression. No significant association was seen between PD1 expression and patient disease-free survival. High PDL1 expression was significantly associated with better patient disease-free survival in comparison to those who had low PDL1 expression (p=0.007).*

known to express PDL1. The magnitude of the immune response is therefore expected to correlate with PDL1 expression and better prognosis. It has been demonstrated that TILs up-regulate the PD1/PDL1 pathway in the tumor microenvironment through the release of certain cytokines such interferon gamma and interleukin 17A (4). This could also explain the results of other studies which demonstrated that higher expression of the PDL1 protein in tumor cells was associated with a worse clinical outcome (24). An IHC study conducted by Muenst *et al.* found that PDL1 expression was associated with a significantly worse OS and remained an independent negative prognostic factor for OS (24). A similar result was reached in a recent meta-analysis which showed that high PDL1 protein expression was associated with a shorter survival in patients with breast cancer (25).

Consistent with our hypothesis, Zhao *et al.* observed that PDL1 expression in tumor-infiltrating immune cells was related to better survival in a breast cancer subgroup (23). The strength of our study lies in its originality, the use of a robust methodology (qPCR to quantify the transcript expression of *PD1* and *PDL1*) and relatively long clinical follow-up. The limitations include its retrospective nature, the small sample size and the fact that we did not determine the expression of *PD1* and *PDL1* separately in tumor cells and in TILs. Furthermore, our study did not examine the protein expression, however, this has been reported to correlate well with the mRNA level (2).

Breast tumors are heterogeneous and their microenvironment contains other cells such as immune cells which express PDL1. Therefore the favorable prognostic role of *PD1* and *PDL1* expression observed in our study seems to be a reflection of increased TILs and an enhanced immune response against the tumor.

Our findings suggest that the mRNA expression of *PD1* and, in particular *PDL1*, may be useful prognostic parameters for predicting patient survival and disease progression and that loss of *PDL1* is associated with a more aggressive cancer phenotype and worse clinical outcome. The expression of PD1/PDL1 pathway members as a prognostic indicator in human breast cancer should be included in future validation studies. Finally the observed up-regulation of this pathway in human breast cancer lends further support to ongoing clinical trials evaluating the potential therapeutic role of antibodies to PDL1 in patients with breast cancer (4).

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