

## Programmed Cell Death Ligand 1 Expression in Osteosarcoma

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### Abstract

Programmed cell death ligand 1 (PDL1, also known as B7H1) is a cell-surface protein that suppresses the cytotoxic CD8<sup>+</sup> T-cell-mediated immune response. PDL1 expression and its clinical relevance in sarcomas are not well understood. Therefore, we sought to measure RNA expression levels for PDL1 in 38 clinically annotated osteosarcoma tumor samples and aimed to determine if PDL1 expression correlates with clinical features and tumor-infiltrating lymphocytes (TIL). Quantitative real-time RT-PCR for PDL1 was optimized in 18 cell lines, of which 5 were osteosarcoma derived. qRT-PCR results were validated via flow cytometry and immunohistochemistry (IHC) in select cell lines. Total RNA was isolated from 38 human osteosarcoma samples for qRT-PCR analysis. Clinical data were sorted, and significance was determined by the Student *t* test. TILs were examined in patient samples by tissue microarray hematoxylin-eosin staining. We confirmed the constitutive PDL1 mRNA expression in cell lines by qRT-PCR, flow cytometry, and IHC. Across human osteosarcoma samples, PDL1 mRNA gene expression ranged over 4 log (>5,000-fold difference). Relative expression levels were evaluated against clinical factors such as age/gender, metastasis, recurrence, chemotherapy, percentage of necrosis, and survival; no significant associations were identified. The presence of TILs was associated with high PDL1 expression ( $R^2 = 0.37$ ;  $P = 0.01$ ). In summary, we developed an RNA-based assay to determine PDL1 expression levels, and we show, for the first time, that high levels of PDL1 are expressed in a subset of osteosarcoma, and PDL1 expression is positively correlated with TILs. Multiple agents targeting PD1/PDL1 are in clinical development, and this may be a novel immunotherapeutic strategy for osteosarcoma clinical trials. *Cancer Immunol Res*; 2(7); 690–8. ©2014 AACR.

### Introduction

Osteosarcoma is an aggressive malignant tumor of the bone thought to arise from mesenchymal stem cells (1). Though it is a rare tumor, osteosarcoma is the most common primary malignancy of the bone and the eighth most common form of childhood cancer (2, 3). With modern multimodality therapies, the 5-year survival rate has increased to 70% (4). However, progress has slowed over the past 30 years, and efforts to improve outcomes with intensifying regimens or adding novel agents have brought disappointing results (5–9). Moreover, for patients with metastatic osteosarcoma at diagnosis and for

those with relapsed disease, outcomes are remarkably poor with 4- to 5-year overall survival rate at less than 20% (10, 11). Therefore, development of novel therapeutic strategies is critical for this patient population.

There is growing interest in the oncology community in the immunoregulatory receptor PD1 and the corresponding B7 family of ligands as a potential mechanism of tumor immune tolerance and escape. In the normal physiologic state, a T-cell response is a complex sequence of events involving clonal T-cell selection, activation, proliferation, and trafficking to antigen sites to ultimately deliver immune effector functions (12). This is initiated through the T-cell receptor followed by a series of highly regulated signals to either stimulate or inhibit the T-cell response. These inhibitory signals are critical to maintaining self-tolerance and protecting neighboring tissues during the immune response (12). PD1 is a cell-surface receptor expressed on subsets of T and B lymphocytes, as well as other immune cells. In the inflammatory microenvironment, stimuli such as IFN $\gamma$  (13–15) may upregulate programmed cell death ligand 1 (PDL1) expression in peripheral tissues and immune cells to suppress the immune response.

Interestingly, many different malignancies can co-opt this checkpoint system by upregulating PDL1 constitutively or in response to inflammation. Examples include melanoma, lung cancer, renal cell carcinoma, ovarian cancer, and colorectal

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cancers (14); however, the expression of PDL1 in osteosarcoma remains unknown.

Multiple agents targeting the PD1/PDL1 system are currently at different stages of clinical development (16–22). Recent early-phase studies of inhibitory antibodies (aimed at PD1 or PDL1) have shown exciting clinical activity and durability across a range of malignancy subtypes, including non-small cell lung cancer (NSCLC), a histology not typically associated with immune responses. In these previously heavily treated patients, the response rates ranged from 18% to 28%, depending on the tumor histology, and importantly the responses were durable.

At the moment, no clear biomarker exists to predict anti-PD1/PDL1 tumor responsiveness. Recent data suggest that PDL1 expression, determined by immunohistochemistry (IHC), may be an important biomarker (14, 23–26) perhaps also when combined with T-cell activation signals (17, 27, 28). Despite these results, diagnostics for PDL1 are limited by the lack of reliable protein-based assays, and the literature remains controversial. Previous studies have shown a correlation between PDL1 mRNA and protein expression (29, 30). To this end, we sought to develop an RNA-based PDL1 assay to circumvent the technical issues constraining the IHC-based strategies.

In summary, we have developed an RNA-based assay to detect PDL1 expression levels, which we used to demonstrate the expression of PDL1 in 32 of 38 human osteosarcoma tumor samples. Given the clinical activity of anti-PD1/PDL1-based therapies across a range of tumor histologies, these data may lead to a promising target for novel immunotherapy strategies.

## Materials and Methods

Institutional Review Board approval was obtained to study all osteosarcoma samples from the Partners Human Research Office (2007P-002464). Written informed consent was obtained from all patients whose specimens and clinical information were used for this research study.

### Osteosarcoma patient surgical specimens

The study consisted of 38 fresh-frozen tumor tissue samples of histologically confirmed osteosarcomas from 37 patients (15 female and 22 male) with an age range of 6 to 75 (median 29) years. Nine samples were from biopsy, 13 from primary tumor, 14 from metastases, and 2 from recurrent tumors. Two samples, P516 and P661 (clinical data are presented in Table 1), were metastases from the same patient, and therefore P661 was excluded from statistical analyses. Fourteen of 37 patients were treated with preoperative chemotherapy. Twenty-six patients died, with a median survival of 36 months (range, 1–200 months).

### Cell lines, cell culture, and antibodies

The human osteoblast cell line HOB-c was purchased from PromoCell GmbH, the human osteoblast cell line NHOS2 from ATCC, and the human osteoblast cell line hFOB from Lonza Walkersville, Inc. The human stem cell line MCS was obtained from Invitrogen. Two human muscle cell RNAs were purchased

from Ambion and Stratagene. The human osteosarcoma cell line SaOS was obtained from ATCC. The multidrug-resistant human osteosarcoma cell line U-2OSTR was established in our laboratory from human osteosarcoma cell line U-2OS, also purchased from ATCC. U-2OSTR was selected from U-2OS by stepwise increases in paclitaxel concentrations from 0.0001 to 0.1  $\mu\text{mol/L}$ . The human osteosarcoma cell lines KHOS and KHOSR2 were provided by Dr. Efsthios Gonos (Institute of Biological Research and Biotechnology, Athens, Greece). The human chondrosarcoma cell line Cs-1 was established in our laboratory from a surgically resected human high-grade chondrosarcoma removed from a 62-year-old male with metastatic chondrosarcoma. The human Ewing sarcoma TC-7 and the trabectedin-resistant TC-ET cell lines were donated by Dr. Katia Scotlandi (Rizzoli Orthopaedic Institute, Bologna, Italy). The human ovarian cancer cell line SKOV3 was purchased from ATCC; the human ovarian cancer cell lines A2780 and OVCAR8 were obtained from Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA) and from Dr. Patricia Donahue (Massachusetts General Hospital, Boston, MA), respectively. Cell lines were grown in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin (Invitrogen). All cell lines were tested and validated to be *mycoplasma*-free. No other authentications were performed. U-2OS cells were treated with IFN $\gamma$ , as previously described (31). Briefly,  $3 \times 10^5$  cells were incubated at 37°C for 48 hours in 8 mL medium supplemented with 100 U/mL.

To detect PDL1 by flow cytometry and IHC, six antibodies were screened: four noncommercial mouse mAbs (CST1, CST2, XW, and LC), a mouse mAb from eBioscience (clone MIH1), and a rabbit polyclonal from ProSci (cat. no. 4059).

### Quantitative real-time RT-PCR

To determine the expression of PDL1 mRNA in cell lines ( $n = 18$ ) and osteosarcoma surgical specimens ( $n = 38$ ), total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. RNA samples were quantified using the ultraviolet spectrophotometer at 260 nm (Beckman DU-640; Beckman Instruments). Equal amounts of RNA were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were then amplified by real-time PCR using TaqMan gene expression assays (Applied Biosystems) for PDL1 (product ID Hs01125301\_m1; probe sequence: 5'-gtgatacacatttgaggagacgtaatccagcattggaacttctgtctcaagcaggattctcaacctgtggttaggggttcacg-3') and according to the manufacturer's protocol. PCR was performed with TaqMan gene expression master mix (Applied Biosystems) using 2  $\mu\text{L}$  of cDNA in a 20- $\mu\text{L}$  final reaction volume. The amplification cycles were performed by the Applied Biosystems StepOne-Plus System (Applied Biosystems) as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and finally 60°C for 1 minute. The housekeeping gene  $\beta$ -actin (product ID Hs01060665\_g1; probe sequence: 5'-ggcgtgatggtgggcatgggtcagaaggattctatgtggcgacaggccagagcaagaga-3') expression level was used as an internal control to evaluate the integrity of each sample. The

**Table 1.** Clinical data of RNA samples from osteosarcoma tissues

Sample	Age at diagnosis (median = 29)	Gender (female = 15; male = 23)	Preoperative chemotherapy	Percentage of necrosis	Metastasis, origin (lung = 22; other = 8)	Local relapse	Disease status	Follow-up (months; median = 36)
P176	14	Male	Yes	95	No	No	AWED	231
P180	15	Male	None	20	Yes, lung	No	DOD	29
P188	75	Female	None	n/a	Yes, other	Yes	DOD	110
P208	17	Female	Yes	60	Yes, lung	No	DOD	16
P209	15	Male	Yes	40	Yes, lung	No	DOD	14
P214	28	Male	Yes	20	Yes, lung	No	DOD	10
P222	32	Male	Yes	50	Yes, lung	No	DOD	28
P237	31	Female	Yes	40	Yes, other	Yes	DOD	89
P249	22	Male	None	n/a	Yes, lung	Yes	AWED	36
P263	45	Male	None	n/a	Yes, lung	No	AWED	234
P286	15	Male	Yes	50	Yes, lung	No	DOD	20
P291 <sup>a</sup>	30	Female	None	n/a	No	No	NED	233
P322	42	Female	Yes	90	Yes, lung	No	AWED	226
P336 <sup>a</sup>	13	Male	Yes	25	Yes, other	No	DOD	200
P351	43	Male	Yes	95	Yes, lung	No	NED	221
P360	33	Male	None	n/a	Yes, lung	No	DOD	27
P404	6	Female	Yes	50	Yes, lung	No	AWED	27
P466 <sup>a</sup>	15	Male	Yes	75	No	No	NED	156
P483	18	Female	Yes	98	Yes, lung	No	DOD	21
P514 <sup>a</sup>	24	Female	Yes	90	Yes, lung	No	DOD	39
P516 <sup>a</sup>	20	Male	None	n/a	Yes, other	No	DOD	28
P530	33	Male	Yes	50	No	No	NED	196
P531 <sup>a</sup>	61	Female	None	n/a	Yes, lung	No	DOD	14
P593	29	Male	Yes	30	Yes, other	No	DOD	33
P644	14	Female	Yes	20	Yes, other	No	NED	193
P661 <sup>a</sup>	20	Male	None	n/a	Yes, other	No	DOD	28
P784	17	Male	Yes	50	Yes, lung	No	DOD	22
P793	63	Male	Yes	20	Yes, lung	Yes	DOD	138
P794 <sup>a</sup>	37	Female	None	n/a	Yes, lung	Yes	DOD	13
P854	25	Female	Yes	25	No	No	NED	162
P860	36	Male	None	n/a	No	No	AWED	50
P885	16	Male	Yes	20	Yes, lung	No	DOD	37
P945 <sup>a</sup>	34	Female	None	n/a	Yes, lung	No	DOD	5
P969 <sup>a</sup>	73	Male	None	n/a	No	No	DOD	1
P974	38	Male	Yes	20	Yes, lung	Yes	DOD	36
P983	26	Female	None	n/a	Yes, lung	Yes	DOD	10
P1028	70	Female	None	n/a	No	Yes	DOD	47
P1037	37	Male	Yes	n/a	Yes, lung	Yes	DOD	16

Abbreviations: AWED, alive with evidence of disease; DOD, dead of disease; n/a, not available; NED, no evidence of disease.

<sup>a</sup>No TIL data. Note that P516 and P661 are metastatic tumors from the same patient.

relative expression level of mRNA of PDL1 was calculated as follows:  $2^{(\text{threshold cycle of } \beta\text{-actin} - \text{threshold cycle of PDL1})}$ . Relative expression was scored on a log<sub>10</sub> scale (0–4).

#### Flow cytometry

Selected cell lines were examined by flow cytometry to validate a correlation of PDL1 mRNA and protein expression. MCF7 and SKOV3 cells were collected in 1 mmol/L EDTA/PBS and then washed with PBS. Cells ( $2 \times 10^5$ ) of each were blocked

in 2% BSA/PBS for 30 minutes at 4°C. The cells were then probed with 1 µg of the primary antibody (eBioscience, XW, or ProSci) for 30 minutes at 4°C, washed, and then incubated at 1:100 with the secondary antibody (R-Phycoerythrin-AffiniPure F(ab')<sub>2</sub> Frag Goat Anti-Mouse IgG, Fcγ Frag Spec (min X Hu, Bov, Hrs Sr Prot), Jackson ImmunoResearch; or Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) Antibody, Invitrogen). After washing and fixation in 2% PFA/PBS, cells were sorted using the BD FACSCalibur flow cytometer.

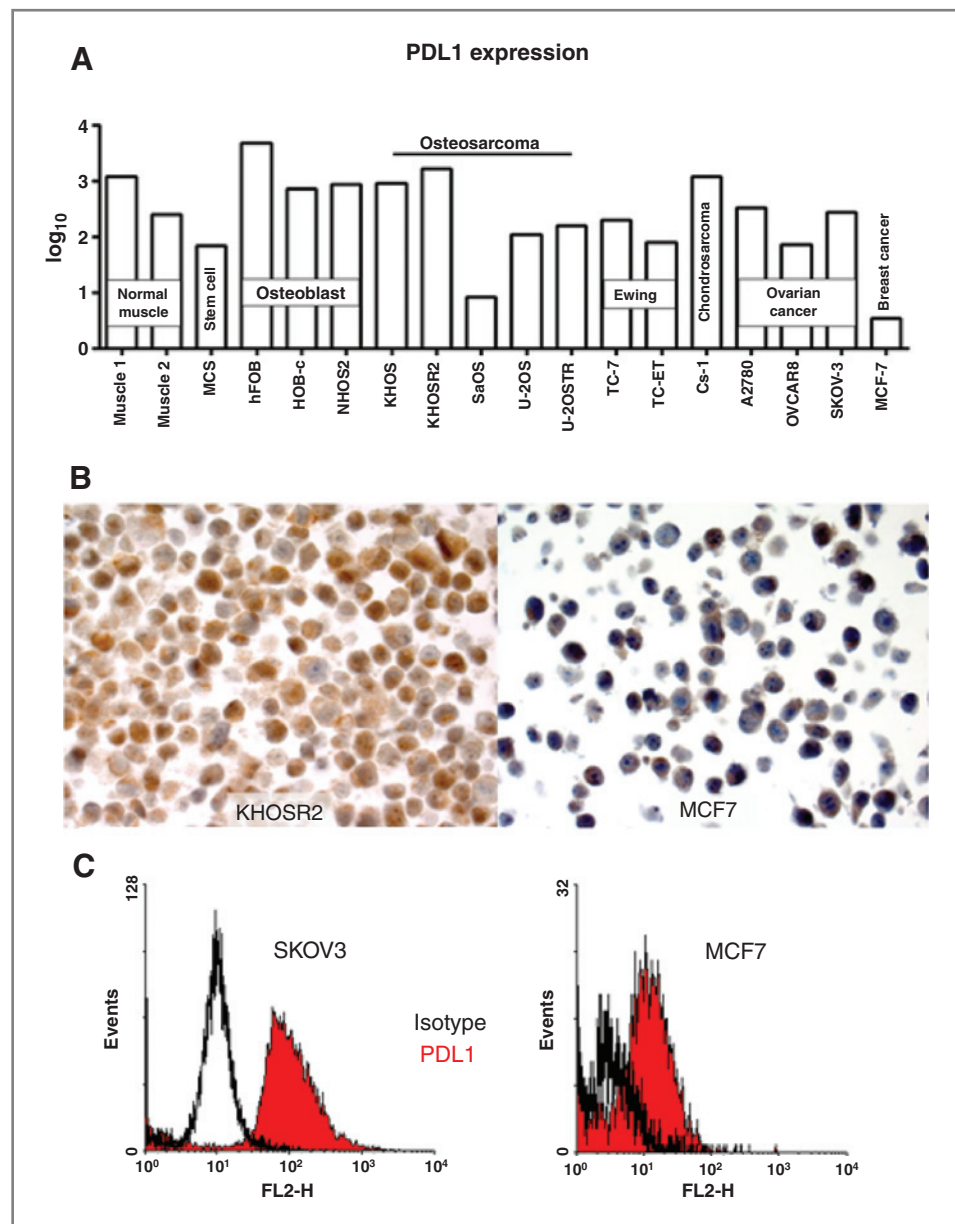
**Tissue microarray construction**

Representative areas of 100 paraffin-embedded osteosarcoma tumor specimens for each case were selected and prepared as previously described (32). Briefly, three areas of tumor parts per case were selected for assembling the recipient master block to ensure accurate representation of the selected cores. Each target area on the selected block was punched to form a 0.5-mm diameter tissue core and was placed consecutively on the recipient master block. The osteosarcoma tissue microarray (TMA) was constructed by the Tissue Microarray and Imaging Core at the Dana-Farber/Harvard Cancer Center (<http://genepath.med.harvard.edu:8080/pathcore/>).

**PDL1 detection by IHC**

TMA slides were stained using the Cell and Tissue Staining Kit (R&D Systems) with slight modifications. Briefly, slides were dewaxed and rehydrated in xylene and graded ethanol solutions for antigen retrieval. Slides were then blocked with 3% H<sub>2</sub>O<sub>2</sub>, goat serum, avidin solution, and biotin solution. Primary antibody (eBioscience; CST1, CST2, and LC) was added and then probed with biotinylated goat anti-mouse secondary antibody (Vector Laboratories) and high-sensitivity streptavidin-HRP conjugate. To visualize staining, slides were incubated in 3,3'-diaminobenzidine in 0.1% H<sub>2</sub>O<sub>2</sub> in Tris-HCl buffer, and subsequently counterstained with Hematoxylin QS (Vector Laboratories). PDL1-positive samples were defined as

Figure 1. PDL1 expression in cancer and noncancer cell lines. A, relative expression of PDL1 from total RNA isolated from cell lines. B, representative PDL1 protein expression levels in corresponding high- and low-expressing cell lines by IHC. C, representative PDL1 protein expression levels in corresponding high- and low-expressing cell lines by flow cytometry.



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those showing membrane and cytoplasmic staining pattern of tumor tissue. PDL1 staining intensity was graded into four groups: no staining (0), weak staining (1+), moderate staining (2+), and intense staining (3+). The immunostained slide was evaluated under the microscope. The staining intensity of cells showing positive membrane and cytoplasmic staining for PDL1 was calculated by reviewing the entire spot.

#### Detection of immune infiltrates

The tumor-infiltrating lymphocytes (TIL) were examined on a hematoxylin and eosin-stained osteosarcoma TMA slide. TMA was evaluated for the presence of TILs at  $\times 400$  magnification and scored semiquantitatively as follows: score 0, no TILs; 1, rare/few TILs; 2, brisk/prominent TILs.

#### Statistical analysis

Statistical analysis was done using GraphPad Prism 4 software. The 75th percentile was used as cutoff for high or low PDL1 mRNA expression, as previous studies have suggested that high-expression patients represented approximately 25% in other cancers (33, 34). For comparison between PDL1 expression and clinical and pathologic variables, a two-sided Student *t* test was used. The overall cancer-specific follow-up time was calculated in months from the date of surgery to the date of death due to osteosarcoma or the last follow-up date. Survival analysis was performed using the Kaplan–Meier method, and significance was determined by the log-rank test. For comparison between PDL1 expression and immune infiltrates, a one-way ANOVA analysis was used. A *P* value of  $<0.05$  was considered as statistically significant.

## Results

### PDL1 expression in cell lines

PDL1 gene expression was quantitatively confirmed in all 18 cell lines. Absolute PDL1 expression was normalized to that of the housekeeping gene  $\beta$ -actin. Breast cancer cell line MCF7 has been shown previously to have relatively low PDL1 expression (35). Drug-resistant osteosarcoma cell line KHOSR2 and virally derived osteoblast cell line hFOB had high (3 log) PDL1 gene expression, and osteosarcoma cell line SaOS and breast

cancer cell line MCF7 had low ( $<1$  log) expression. Other cell lines, including stem cell (MCS), osteoblast (Obs; NHOS2), ovarian cancer (SKOV3, OVCAR8, and A2780), Ewing sarcoma (TC-7 and TC-ET), chondrosarcoma (Cs-1), and osteosarcoma (U-2OS, U-2OSTR, and KHOS), ranged from intermediate (1 log) to high (2 log) expression of PDL1 (Fig. 1A). Each tumor cell type generally demonstrated varying PDL1 expression. In nonmalignant cell lines such as muscle, stem cells, and osteoblasts, PDL1 gene expression ranged from moderate to high. Osteosarcoma cell lines ranged from low to high PDL1 expression, with slightly higher expression from drug-resistant variants (KHOSR2 and U-2OSTR) than their parental cell lines (KHOS and U-2OS). Ewing sarcoma cell lines expressed moderate levels of PDL1, and ovarian cancer cell lines demonstrated moderate PDL1 expression as well. The one chondrosarcoma cell line showed high PDL1 levels, and one breast cancer cell line exhibited low PDL1 expression; however, conclusions on the expression of PDL1 on these two tumor types cannot be drawn from one cell line. PDL1 gene expression was induced 5-fold by IFN $\gamma$  treatment in osteosarcoma cell line U-2OS (data not shown).

To validate the RT-PCR data, IHC and flow cytometry assays were performed on selected cell lines. Consistent with the RT-PCR data, there was low-level staining in the negative control MCF7 cells and intense staining in the positive control KHOSR2 cells by IHC using mouse mAb LC (Fig. 1B). This staining was both membranous and cytoplasmic. Flow cytometry of negative control MCF7 and positive control SKOV3 cells using the eBioscience clone MIH1 also showed consistent patterns of expression between RNA and protein (Fig. 1C). Unfortunately, the ProSci rabbit polyclonal failed to detect PDL1 on the positive control cell line SKOV3, and the XW mouse mAb exhibited unspecific binding on the negative control cell line MCF7 (Supplementary Fig. S1). Although the eBioscience mouse mAb demonstrated the expected results by flow cytometry, it produced weak staining by IHC TMA. The CST1, CST2, and LC antibodies also failed to show sufficient specificity for PDL1 by IHC TMA.

### PDL1 expression in osteosarcoma tumor tissue

The PDL1 RT-PCR assay was performed on total RNA isolated from 38 osteosarcoma human tumor samples (Fig. 2). Absolute expression was normalized to that of housekeeping

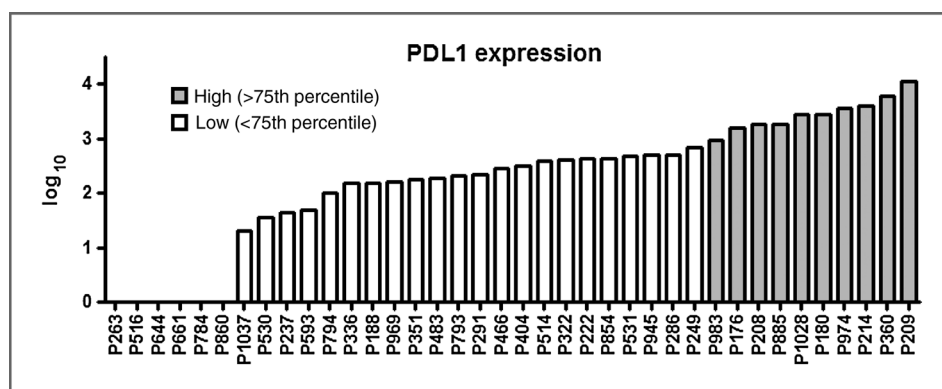
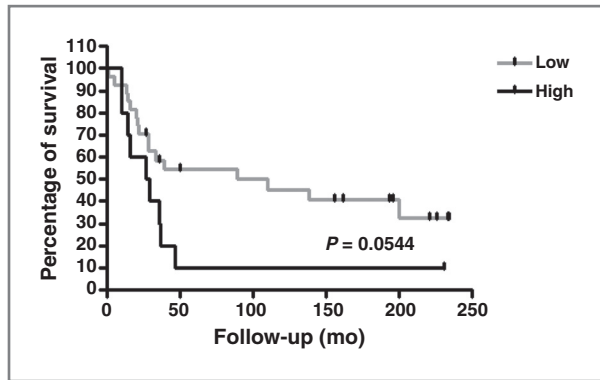


Figure 2. Relative expression of PDL1 in 38 osteosarcoma specimens. PDL1 expression was evaluated from total RNA by quantitative real-time RT-PCR and showed that the expression levels range over 4 log (5,000-fold).



**Figure 3.** Overall survival of 37 patients with osteosarcoma in relation to PDL1 gene expression. The median overall survival for PDL1-low patients was 89 months compared with 28 months for PDL1-high patients, which showed a trend but was not statistically significant ( $P = 0.0544$ ).

gene  $\beta$ -actin and categorized by log-transcript detection: low, 1 log; intermediate, 2 log; and high, 3 and 4 log. We found 9 samples with high-level expression (24%). There were 19 samples (50.0%) with intermediate expression, 4 (10.5%) with low-level expression, and 6 (15.8%) were negative.

**Prognostic value of PDL1 expression**

We evaluated the clinicopathologic features of the human tumor samples and found no significant relationship between PDL1 gene expression and age at surgery, gender, neoadjuvant chemotherapy treatment, percentage of necrosis, metastatic status, relapse status, or survival. The median overall survival for PDL1-low patients was 89 months compared with 28 months for PDL1-high patients, exhibiting a borderline trend, but not achieving statistical significance ( $P = 0.054$ ; Fig. 3).

**Correlation between PDL1 and tumor-infiltrating T lymphocytes**

IHC identified TILs in 28 of 38 tumor samples on the TMA. The TIL patterns were diffused within the 0.5-mm TMA punches of the osteosarcoma tissues. Semiquantitative scoring of the degree of TILs was determined by a pathologist, who was blinded to the PDL1 results. There was a positive correlation between PDL1 mRNA expression and TILs ( $R^2 = 0.37$ ;  $P = 0.01$ ; Fig. 4).

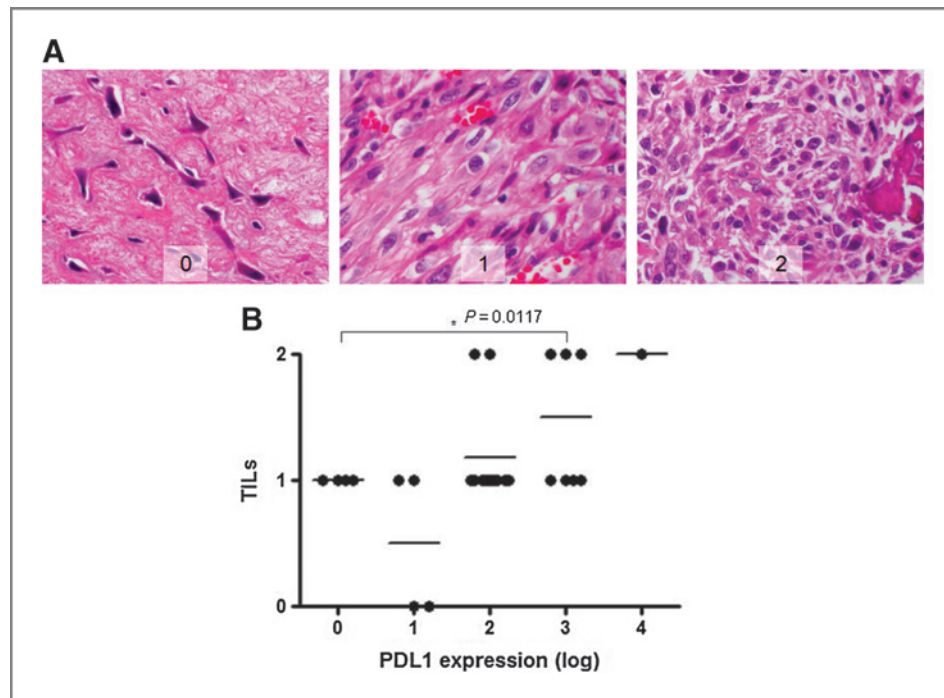
**Characterization of origin of metastases**

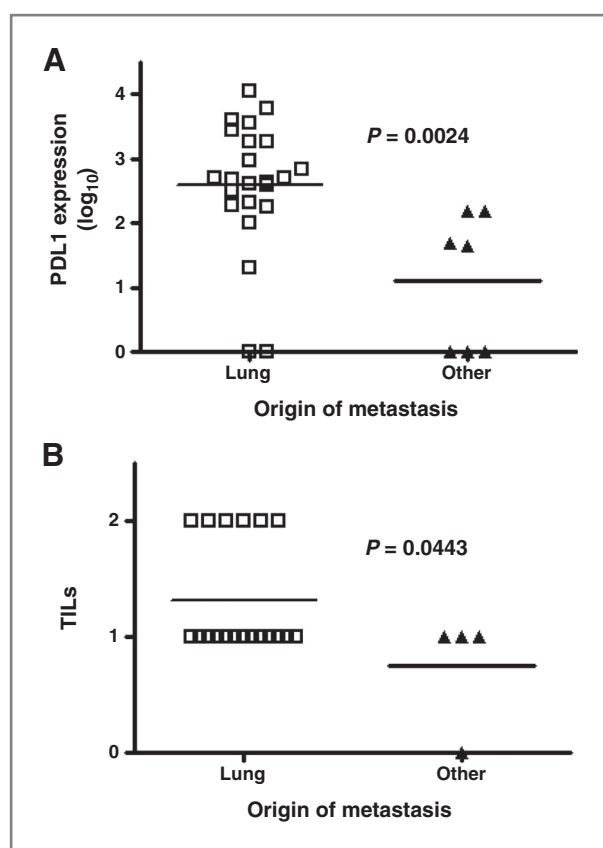
Thirty osteosarcoma tissue samples were derived from metastatic lesions (23 pulmonary and 7 nonpulmonary). Nonpulmonary metastases included the pelvis, humerus, ilium, flank, and anterior mediastinum. The mean PDL1 expression for pulmonary metastases ( $2.6 \pm 0.21$  log) was significantly higher than the mean PDL1 expression for nonpulmonary metastases ( $1.1 \pm 0.40$  log;  $P = 0.0024$ ; Fig. 5A). Furthermore, the origin of the metastasis (pulmonary vs. nonpulmonary) correlated with TILs ( $P = 0.044$ ; Fig. 5B).

**Discussion**

The field of immunotherapeutics is rapidly evolving, with the recent successful early clinical studies targeting the PDL1–PDL1 axis. There is much interest to see if these agents could be applied to other tumor subtypes. It has been suggested that tumor PDL1 expression may be an adequate biomarker to predict responsiveness to these therapies. One limiting factor, however, has been a lack of reliable, and widely available, anti-PDL1 antibodies for this important diagnostic step. In the first part of this study, our objective was to develop an RNA-based assay to circumvent this issue.

**Figure 4.** Correlation between PDL1 gene expression and TILs by IHC. A, representative TILs in osteosarcoma tissues ( $\times 400$ ); score 0, no TILs; 1, rare/few TILs; 2, brisk/prominent TILs. B, significant positive correlation was shown between PDL1 gene expression and TILs in patients with osteosarcoma ( $P = 0.0117$ ).





**Figure 5.** Characterization of the origins of metastases. A, PDL1 expression is significantly higher in metastatic osteosarcoma tumors that originate from lung than from other locations ( $P = 0.0024$ ). B, TILs also exhibit a positive correlation with pulmonary osteosarcoma metastasis compared with nonpulmonary metastasis ( $P = 0.0443$ ).

With our quantitative RNA assay, we were able to show a wide range of expression in cell lines, including low levels in MCF7 cells, which is consistent with the literature. Moreover, we could demonstrate induction of PDL1 transcript expression with IFN $\gamma$ , which is an important control for immune activation and regulation. The protein-based assays presented in Fig. 2 were used to validate our RNA findings, as previous studies have demonstrated a correlation between PDL1 mRNA and protein expression (29, 30). As other investigators have experienced, these were challenging experiments, and six antibodies were used for these studies. However, despite the difficulties, we were able to reliably and consistently show a correlation between PDL1 RNA and protein expression in these cell lines.

In the second part of this study, we applied our quantitative PDL1 RNA assay to human osteosarcoma samples. These tumor samples were intentionally selected heterogeneously to represent a wide range of ages and clinical scenarios (e.g., localized/metastatic, pretreatment/on-treatment, and new diagnoses/relapsed disease). We show for the first time that PDL1 is expressed in 32 of 38 (84.2%) osteosarcoma samples. One challenge to analyzing these data is determining how to normalize the expression levels. We used absolute values normalized to  $\beta$ -actin, as there is no specimen or comparable

"normal" tissue to establish a baseline level of PDL1 expression. With this approach, we found 24% of patients had very high levels of PDL1 transcript (3 log or more). This finding perhaps suggests that there is a subset of patients for whom PDL1-directed therapies could be relevant.

In our analyses, there was no correlation of PDL1 expression with clinicopathologic features, such as age at surgery, gender, neoadjuvant chemotherapy, percentage of necrosis, metastatic status, relapse status, or disease status at the time of biopsy. There was a slight trend for poorer overall survival for patients with osteosarcoma with high PDL1 expression. These data are likely confounded by the small, heterogeneous sample size, and larger studies with similar osteosarcoma patient samples are required. Several previous studies have demonstrated conflicting results varying from positive correlation of tumor aggressiveness in renal cell carcinoma (33, 36), to observing no correlation with clinicopathologic variables (24), to an inverse relationship in which high PDL1 expression was associated with increased survival in colorectal cancer, melanoma, and NSCLC (15, 30, 37). In addition, the role that TILs play in the mechanisms of PDL1 expression and survival for patients with osteosarcoma is not yet known.

In anti-PDL1 clinical trial samples, emerging data suggest that PDL1 expression plus a T-cell activation gene signature, including CD8 and IFN $\gamma$ , may be associated with treatment response (17). We found a correlation between TILs and PDL1 expression in our tumor samples. In future studies, we will examine the subtypes of lymphocyte present in biopsies, including B and T cells (CD20 and CD3), CD4/CD8 T-cell response, and macrophages (CD163 or CD68), as well as evidence for T-cell activation.

We found higher levels of expression of PDL1 and TILs in metastases that originated in the lung versus other sites, including the pelvis, humerus, ilium, flank, and anterior mediastinum. The significance of this finding remains unclear, but it could be related to the lung microenvironment or perhaps the prior treatments (e.g., lung metastases are typically an early event in osteosarcoma). Future studies with larger patient samples will be needed to explore this further.

In summary, we have developed a sensitive quantitative RNA-based assay to detect PDL1 expression. We showed PDL1 expression in over 80% of osteosarcoma patient samples, with high levels in 24%, and that PDL1 expression correlated with the presence of TILs. This study sets up an important framework to design anti-PD1 or anti-PDL1 immune-checkpoint therapies in patients with osteosarcoma.

#### Disclosure of Potential Conflicts of Interest

E. Choy is a consultant/advisory board member for Amgen and Pfizer. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

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