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Original Research Article

PD-L1 pfeRNAs as blood-based predictors of treatment response of unresectable malignant pleural mesothelioma patients administered Durvalumab with cisplatin and pemetrexed as first-line therapy

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ABSTRACT

Background: A new therapeutic avenue combining Durvalumab with cisplatin-pemetrexed (Durva-CP) has delivered a promising outcome for previously untreated patients with unresectable malignant pleural mesothelioma (MPM) in clinical trials. However, the limited patient response to Durva-CP needs predictors to select optimal candidates and monitor the developed resistance. Protein functional effector sncRNA (pfeRNA) reveals a fundamental mechanism underlying the regulation of protein activity. The common mechanisms underlying durvalumab, cisplatin, and pemetrexed indicate that PD-L1 pfeRNAs (PDLpfeRNAs) are key molecules that control the treatment response.

Methods: We specified PDLpfeRNAs by sncRNA deep sequencing, confirmed their binding to PD-L1 by immunoprecipitation and reverse pull-down assays, and demonstrated their roles in controlling the interaction behaviors of PD1/L1 through quality-controlled drug development assays. Following the standards required for the CLIA-compliant LDT, we measured their expression levels in 60 plasma biospecimens from 30 unresectable MPM patients enrolled in the PrE0505 Phase II multicenter study. Using the Cox proportional hazards model and Kaplan-Meier analyses, we described their significance in predicting the treatment response of unresectable MPM patients administered Durva-CP as first-line therapy.

Results: Two PDLpfeRNAs, PDLpfeRNAa and PDLpfeRNAb, were characterized, confirmed to bind to PD-L1, and identified to control the interaction behaviors of PD-1/L1. Their plasma relative expression levels (REL) demonstrated significant prognostic value for both overall survival ($p = 0.0019$) and progression-free survival ($p = 0.019$), and the association remained significant after adjusting for histological subtype (HR 2.59, 95 % CI: 1.00–6.70, $p = 0.050$) and age (HR 1.03, 95 % CI: 0.98–1.07, $p = 0.269$).

Conclusions: Plasma PDLpfeRNAs are predictors of treatment response of unresectable MPM patients treated with Durva-CP as first-line therapy to select optimal candidates and monitor the developed resistance.

Abbreviations: MPM, malignant pleural mesothelioma; Durva-CP, Durvalumab with Cisplatin-Pemetrexed; pfeRNAs, protein functional effector sncRNAs; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PDLpfeRNAs, PD-L1 pfeRNAs; PD1/L1, PD-1 and PD-L1; CxD1, the first day of treatment cycle X; PR, partial response; SD, stable disease; PD, progressive disease; OS, overall survival; PFS, progression-free survival; CLIA, clinical laboratory improvement amendments; LDT, laboratory developed test; REL, relative expression levels; HR, hazard ratio; IP, immunoprecipitation.

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1. Introduction

Malignant Pleural Mesothelioma (MPM) is a relatively rare malignancy characterized by an aggressive clinical course. It is particularly lethal and challenging to treat, as the treatment of MPM has historically advanced at a slow pace [1–3]. However, recent technological and therapeutic advances with U.S. Food and Drug Administration (FDA) approval have reshaped the standard-of-care landscape [3,4]. A new therapeutic avenue combining Durvalumab (Imfinzi, AstraZeneca, a PD-L1 inhibitor) with Cisplatin-Pemetrexed (Durva-CP) as first-line therapy has delivered a promising outcome for previously untreated patients with unresectable MPM [5–9]. However, as in many other tumor types, there is always a refractory population of patients who do not respond to therapy, here the therapy is Durva-CP, which puts forward a need for predictors to select optimal candidates and monitor the developed resistance in MPM patients.

To identify predictors for MPM patients treated with Durva-CP, we need to understand the common mechanisms underlying Durvalumab, Cisplatin, and Pemetrexed. Durvalumab, an anti-PD-L1 monoclonal antibody, blocks the functional interaction between programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) to release the inhibition of immune responses. Cisplatin alters antitumor immunity and synergizes with PD1/L1 inhibition to enhance antitumor immunity by mechanisms other than direct tumor cell killing [10–14]. Activation of the mTOR/P70S6K and STAT pathways by pemetrexed induces the transcriptional activation of PD-L1, promotes an intra-tumor immune response, and increases the secretion of cytokines that stimulate a further increase in PD-L1 levels [15–18]. Thus, the common mechanism of these three regimens in the new Durva-CP therapy is directly related to the functional behavior of PD1/L1.

Protein functional effector sncRNA (pfeRNA) reveals a fundamental principle governing how protein activity is regulated [19–22]. To identify predictors for MPM patients treated with Durva-CP, we need to specify pfeRNAs that control the functional interaction behaviors of PD/L1. We first extracted sncRNAs binding to PD-L1, specified their sequences by sncRNA deep sequencing, and compared PD-L1 pfeRNAs (PDLpfeRNAs) in patient-matched cancerous tissue and histologically normal adjacent tissues. We then confirmed that PDLpfeRNAs bound to PD-L1, screened the roles of PDLpfeRNAs in controlling the interaction behaviors of PD1/L1 using quality-controlled drug development assays, and measured their levels in the plasma of unresectable MPM patients treated with Durva-CP as first-line therapy. Finally, we applied their relative expression levels (REL) to these patients and found that PDLpfeRNAs were predictive biomarkers for predicting treatment response. Importantly, we performed the study following the requirements and standards of the Clinical Laboratory Improvement Amendments (CLIA)-compliant laboratory developed test (LDT) [23–25], providing evidence for potential translational studies on applying PDLpfeRNAs to select optimal candidates and monitor the developed resistance in the clinic.

2. Materials and methods

2.1. Ethics

The study was approved by the Institutional Review Board of Johns Hopkins University (IRB approvals: NA_00005998, IRB00125554, CR00024815). Informed consent was obtained from all participants prior to sample collection, and all procedures were conducted in accordance with the Declaration of Helsinki and its amendments.

2.2. Patients and treatment

We analyzed plasma samples from 30 patients with unresectable MPM enrolled in the PrE0505 phase II multi-center study of Durva-CP conducted at 15 U S. sites between June 2017 and June 2018 [5–9]. Plasma samples were collected on the first day of treatment cycles (CxD1). Each patient contributed two timepoints: either C1D1/C2D1 or C1D1/C5D1, depending on their specific treatment course.

2.3. Immunoprecipitation (IP) and PDLpfeRNAs extraction from tissues

Washed tissues with cold phosphate-buffered saline (PBS, Thermo Fisher Scientific, pH 7.4), added ice cold Pierce IP Lysis Buffer (Thermo Fisher Scientific), Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and RNasin Plus RNase Inhibitor (Promega Corporation) to the tube, put the tube on ice, and disrupted tissues using TissueRuptor II (Qiagen) with a TissueRuptor disposable probe (Qiagen). Incubated disrupted tissues on ice for 30 min with periodic mixing and removed cell debris by centrifugation at ~13,000×*g* for 10 min at 4 °C. Transferred supernatant to a new tube for pre-clearing with Pierce protein A/G magnetic beads (Thermo Scientific), kept rotating for 2 h at 4 °C, then placed the tube into a magnetic stand to collect the supernatant, and discarded the beads. Equally divided each supernatant sample for PD-L1 monoclonal antibody (Thermo Fisher Scientific) and Rabbit IgG1 isotype control (Thermo Fisher Scientific), respectively, kept rotating overnight at 4 °C. Placed the tube into a magnetic stand to collect the beads against the side of the tube, discarded the supernatant, and washed the beads using binding/wash buffer (Thermo Fisher Scientific). Finally, added 200 μL of water to the beads and extracted the interacted pfeRNAs using our well-developed method [24].

2.4. pfeRNA library for sncRNA deep-Seq

The 5′- and 3′-end adaptor ligation, reverse transcription (RT), and PCR were performed according to the instructions of the kit (True Small RNA kit, Illumina). After pfeRNA purification, the pooled library was sequenced using the NextSeq 500 sequencer at Johns Hopkins University as we described previously [19,21,23].

2.5. Characteristics of PDLpfeRNAs from sncRNA deep-Seq

We identified unique sequences from reads using miRDeep2 software, aligned them to the human reference GRCh38, determined the genomic locus and number of transcripts using Genomics Workbench 10.1.1 software package, and analyzed known and novel sequences by the Partek Genomics Suite and TIBCO Spotfire DecisionSite platforms. Differentially expressed pfeRNAs were obtained as previously described [19,21,23].

2.6. Cell culture

Human H1975 cells were obtained from the American Type Culture Collection (ATCC) (Manassas) and cultured in RPMI 1640 with 10 % fetal bovine serum (FBS) (Sigma-Aldrich). The cell line was genotyped for the authentication. Mycoplasma contaminations were tested regularly, and cells were routinely treated to prevent mycoplasma growth.

2.7. IP and pull-down assays in cells

For each condition, used 1×10^7 H1975 cells, 10 μg PD-L1 antibody or isotype control with 10 μL Protein A/G magnetic beads, or 10 μg biotin-ligated oligonucleotides with 10 μL streptavidin beads for IP or pull-down assays. PDLpfeRNAa-Biotin, PDLpfeRNAb-Biotin, and scrambled control-Biotin were synthesized at IDT (Supplementary Table 1). All other steps were similar to IP from tissues described above.

2.8. PD1/L1 cell-based screening assay

The roles of PDLpfeRNAs in regulating the interactive activities of PD1/L1 were tested by PD-1: PD-L1 Cell-Based Inhibitor Screening Assay (BPS Bioscience). The assay consists of two main components of

effector cells (expressing firefly luciferase and human PD-1) and expression vectors (encoding TCR activator and human PD-L1). Following its instructions, we used a serial gradient concentration of PDLpfeRNAs to screen for their roles in regulating the interaction efficacy of PD1/L1. Fold induction= (luminescence of stimulated cells − average background)/(luminescence of unstimulated cells − average background).

2.9. RT and QuantStudio Dx PCR

The specific adaptor modifications, GSP sequences, common reverse primers, and pfeRNA forward primers were listed in Supplementary Table 1. Ligation, RT, qPCR, and QuantStudio Dx PCR were performed as previously described [21,23,24]. We used single-strand truncated T4 RNA ligase 2 (New England Biolabs) for ligation, SuperScript II first-strand synthesis system (ThermoFisher Scientific) for RT, and QuantStudio PCR for measuring pfeRNA levels. Levels were measured as follows: denaturation for 10 min of the first cycle at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 12 s.

2.10. Relative levels of plasma PDLpfeRNAs

We have established a method that can effectively and objectively measure plasma pfeRNA levels using QuantStudio Dx PCR [21,23,24]. While results generated by our method did not depend on spike-in controls, we used spike-in sncRNAs [26,27] as positive controls to evaluate efficiency of RT and Dx PCR, and we also included no template negative controls. At each time point, the relative expression levels (REL) of PDpfeRNAa to PDpfeRNAb were calculated, and the changes in REL at the two time points of the same patient were compared.

2.11. Statistical analysis on the significance of PDLpfeRNAs in treatment response

Based on the data of patients' responses to Durva-CP [5–9], we grouped patient responses to Durva-CP into three anti-tumor activities

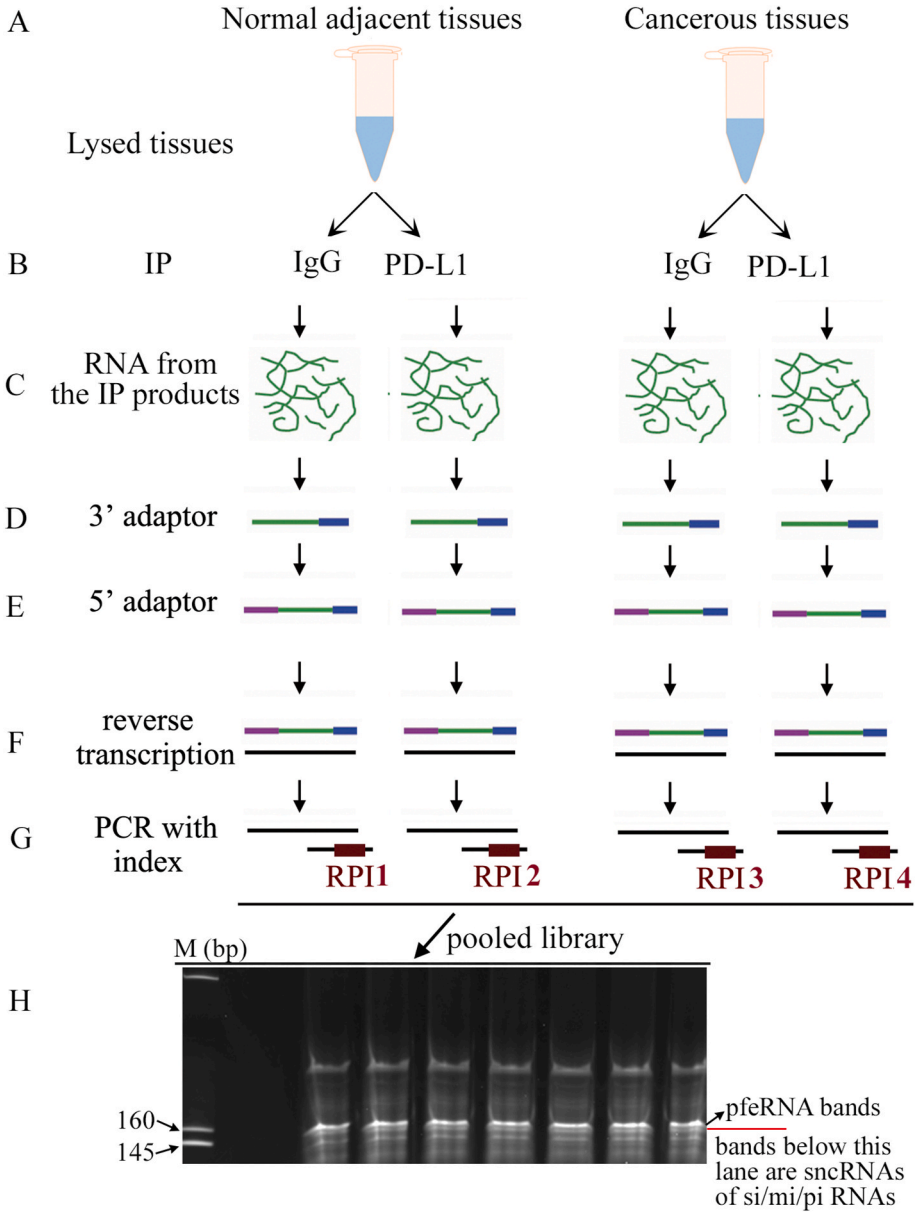

Fig. 1. Specify specific sequences of PD-L1 pfeRNAs. (A–C) pfeRNAs were precipitated with PD-L1 antibody and its corresponding isotype controls, respectively. (D–H) Specific steps of sncRNA library preparation. (I) Purification of pfeRNAs for sncRNA deep sequencing using Illumina MidSeq.

including (a), complete and partial response (PR), (b), Stable disease (SD), and (c) Progressive disease (PD). The association between levels of PDLpfeRNAs and patients' responses, progression-free survival (PFS), and OS was assessed by Univariate and Multivariate analysis using the Cox proportional hazards model, and the Cox proportional hazards regression model was used to study whether each time point's measure was associated with OS and PFS. We calculated the change in levels of PDLpfeRNAs by taking the difference between the second and first timepoint measurements. The median of this difference was used as the cutpoint to classify patients into high versus low PDLpfeRNA groups. We performed survival analyses using R software (version 4.0.5). Cox proportional hazards regression models were constructed using the 'survival' package, with the Efron method for tie handling. We calculated Hazard ratios (HR) with 95 % confidence intervals (CI). The model included PDLpfeRNA level (binary: above vs below median), histological subtype (sarcomatoid/biphasic vs epithelioid), and age as covariates. Kaplan-Meier survival curves were generated and compared using log-rank tests. All statistical tests were two-sided with significance set at $p < 0.05$.

3. Results

3.1. Specific sequences and characteristics of PDLpfeRNAs

To specify the PDL1 pfeRNAs that control the functional interaction of PD-1 and PD-L1, we precipitated the interacted sncRNAs of PD-L1 in patient-matched cancerous tissues and histologically normal adjacent tissues (Fig. 1A, Supplementary Table 2), used a PD-L1 antibody and its isotype control to precipitate their potential interacted sncRNAs (Fig. 1B), and extracted sncRNAs from the IP products (Fig. 1C). We then ligated the RNA with two end adaptors (Fig. 1D and E), performed reverse transcription (Fig. 1F), and used a unique index PCR Primer Indexes (RPI) as a barcode for each treatment to different sncRNAs from either control, patients, IgG, or antibody (Fig. 1G). We separated the PCR product library in a PAGE gel, purified the pfeRNA bands, and processed them for sncRNA deep sequencing (Fig. 1H). After reads were post-processed, normalized, identified, blasted, and filtered by bioinformatics and biostatistical analyses, we used copy numbers of mapped sequences to assemble and quantify each pfeRNA. We identified two PD-L1 pfeRNAs, referred to as PDLpfeRNAa and PDLpfeRNAb (Table 1). Their copy numbers were 407 and 419 in the IP products using PD-L1 antibody precipitated from normal adjacent tissues, but showed 1,315,332 and 12,417 in the IP products using PD-L1 antibody precipitated from cancerous tissues, indicating that PDLpfeRNAs were endogenously induced during tumorigenesis and cancer development. Also, they showed 0 copies in the IP products using an isotype control, indicating that they specifically interact with PD-L1. Together, these results suggested that PDLpfeRNAs interact with PD-L1 and are induced during tumorigenesis.

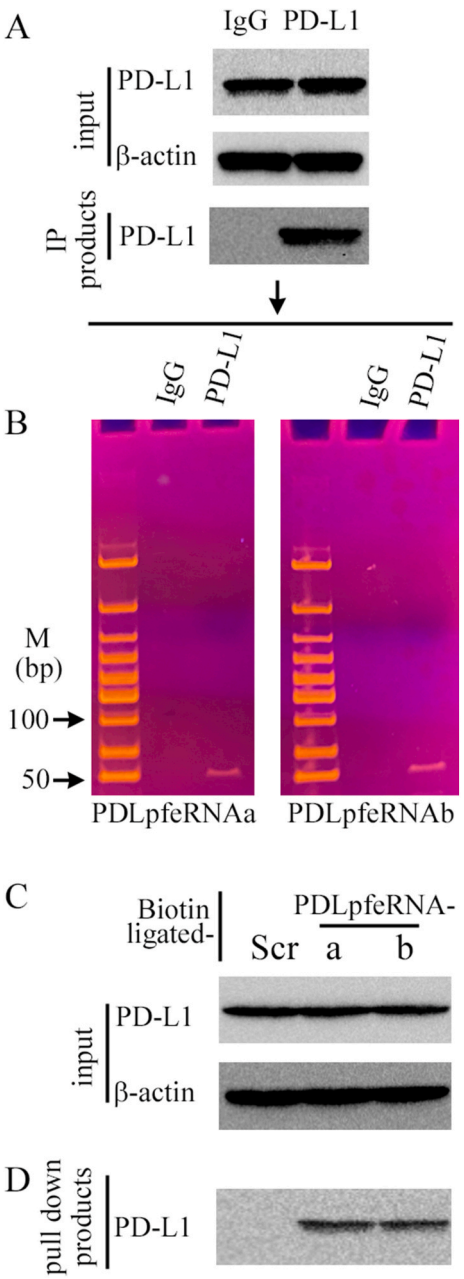

Fig. 2. PDLpfeRNAs bind to PD-L1. (A, B) PDLpfeRNAs detected in RNA purified from IP products of PD-L1 antibody. (C, D) PD-L1 detected in pull-down products of biotin ligated PDLpfeRNAs.

3.2. PDLpfeRNAs bind to PD-L1

To confirm the binding between PDLpfeRNAs and PD-L1, we used H1975 cell line, which is known to express high levels of PD-L1 [28,29], to perform an IP assay using PD-L1 antibody and its isotype immunoglobulin G (IgG) followed by RT-PCR using primers specific for PDLpfeRNAs (Fig. 2A, Supplementary Fig. 1A, Supplementary Table 1). While PDLpfeRNAs were not detectable in IP products using a control IgG antibody, PDLpfeRNAs were readily detected in IP products using an antibody specific for PD-L1 (Fig. 2B), suggesting that PDLpfeRNAs bind to PD-L1. We then performed the reverse pull-down assay followed by Western blot analysis. Biotin-ligated PDLpfeRNAs, scrambled RNA oligonucleotides (Supplementary Table 1), and streptavidin beads were used to pull down proteins to determine whether the pull-down products contained PD-L1(Fig. 2C). PD-L1 was only detected in the pull-down

Table 1
PDLpfeRNAs identified by sncRNA deep sequencing.

Name	sequences (5′-3′)	Normal tissue		Malignant tissue	
		IgG	PD-L1 Ab	IgG	PD-L1 Ab
PDLpfeRNAa	AUAUCAUUGGUCGUGGUUGUAGUCCGUGCGAGAAUACCA	0	407	0	1,315,332
PDLpfeRNAb	AGAAGACCCUGUUGAGCUUGACUCUAGUCUGGCACGGUGAA	0	419	0	12,417

products from PDLpfeRNAs but not from the scrambled RNA oligonucleotides (Fig. 2D), thereby supporting the binding between PDLpfeRNAs and PD-L1.

3.3. PDLpfeRNAs control the functional interaction activities of PD-1 and PD-L1

To measure the potency and stability of PDLpfeRNAs in regulating the interactive activities of PD-1 and PD-L1, we used a bioluminescent cell-based assay that overcomes the limitations of other existing assays. This assay system consists of PD-1 effector cells (Reporter Jurkat cells) and PD-L1 T cell receptor activator cells (overexpressing PD-L1) to generate luminescence for measuring the efficacy of PD-1/L1 functional interaction behaviors. Luminescence light was induced by the PD-1/L1 inhibitor and reduced by the PD-1/L1 enhancer. Serial gradient concentration of 0.0066, 0.066, 0.66, 6.6, and 66 ng/μL were used to screen their role in controlling the functional interaction behaviors of PD-1 and PD-L. While the scrambled control (Scr Con) showed no changes in the relative luminescence, PDLpfeRNAa treatment significantly reduced the luminescent signal (Fig. 3A), suggesting that PDLpfeRNAa is an enhancer of the PD1/L1 interaction. Also, the luminescence signal was induced by PDLpfeRNAb treatment (Fig. 3B), indicating that PDLpfeRNAb is an inhibitor of the PD1/L1 interaction. In addition, these results directly confirmed the functional specificity of PDLpfeRNAs in controlling the interaction behaviors and activities of PD-1 and PD-L1; otherwise, they could not change the luminescence signal.

3.4. PDLpfeRNAs as predictors for treatment response

From the PrE0505 phase II multi-center study (Supplementary Fig. 1), we analyzed 60 plasma biospecimens from 30 patients with unresectable MPM who received Durva-CP as first-line therapy, and we included almost all available participants who had two cycles of treatment, as not all participants had two cycles of treatments [5–9]. The average age of the 30 patients (4 female and 26 male) was 68 ± 11.8 years (range 35–82 years), and the race included 26 White (86.7 %), two Asian (6.7 %), one Black (3.3 %), and one Mixed (3.3 %, more than one race). There were 21 (70 %) patients with epithelioid, 6 (20 %) patients with sarcomatoid, and three (10 %) patients with biphasic. The two time points of plasma collection were 10 (33.3 %) patients with C1D1 plus C2D1, and 20 (66.7 %) patients with C1D1 plus C5D1. The patterns of response to first-line Durva-CP therapy included 15 (50 %) partial response (PR), 12 (40 %) stable disease (SD), and three (10 %) progressive disease (PD) (Supplementary Table 3).

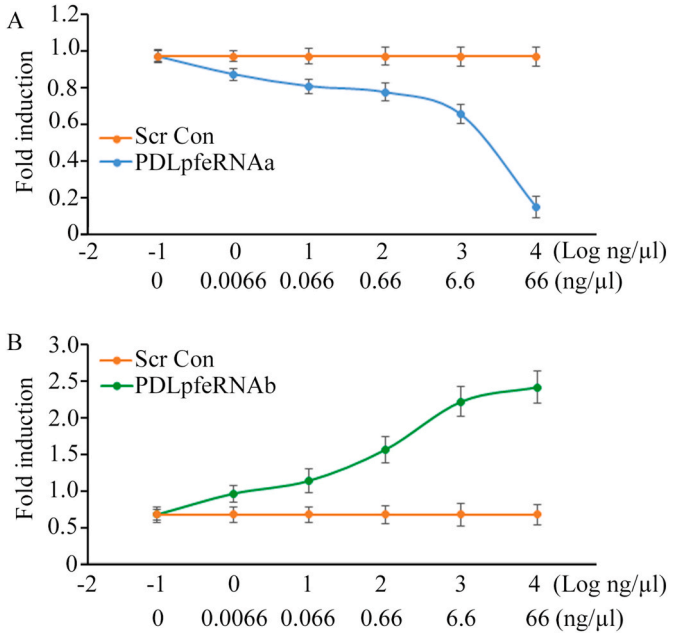

Fig. 3. PDLpfeRNAs control the interaction activities of PD-1 and PD-L1. Relative luminescence of PDLpfeRNAa (A) and PDLpfeRNAb (B) at different concentrations. All data are mean ± standard error of the mean (s.e.m.).

Following the requirements of a CLIA-compliant LDT, we measured levels of PDLpfeRNAs by qPCR. Both PDLpfeRNAa and PDLpfeRNAb were specifically and efficiently measured (Supplementary Fig. 2), and the REL of PDLpfeRNAa to PDLpfeRNAb of each biospecimen was calculated. The REL of PDLpfeRNAs demonstrated significant prognostic value. Patients with high REL showed significantly worse overall survival compared to those with low levels (HR 4.31, 95 % CI: 1.49–12.42, $p = 0.007$). Kaplan-Meier analysis demonstrated a significant separation of survival curves between high and low REL groups for both overall survival (OS, $p = 0.0019$) (Fig. 4A, Table 2) and progression-free survival (PFS, $p = 0.019$) (Fig. 4B–Table 2). This association remained significant after adjusting for histological subtype (sarcomatoid/biphasic vs epithelioid, HR 2.59, 95 % CI: 1.00–6.70, $p = 0.050$) and age (HR 1.03, 95 % CI: 0.98–1.07, $p = 0.269$) (Fig. 4C). Overall, the low REL group showed markedly survival probability compared to the high REL group.

4. Discussion

While immune checkpoint inhibitors targeting PD-1/PD-L1 have already substantially improved the outcomes of patients with many types of cancer, only 20–40 % of patients benefit from these therapies [30–32]. Durvalumab in combination with chemotherapy has been identified as an effective and valuable treatment option for previously untreated patients, including those with lung cancer, gastric/gastroesophageal junction cancer, head and neck cancer, *etc* [33–36]. In the treatment field of this rare MPM, platinum-based chemotherapy in combination with pemetrexed had been the mainstay of first-line treatment for many years before immunotherapy available. Durva-CP is among the first therapeutic advances that have reshaped the standard-of-care landscape for this notorious cancer because of its aggressive nature and poor prognosis [1–4]. However, not all patients derive durable benefits, and resistance to combined therapy is common. Thus, predictors of treatment response can be used to select optimal candidates and monitor the developed resistance in the clinic.

To develop effective predictors for these rare MPM patients treated with Durva-CP as first-line therapy, we first analyzed the common mechanism underlying Durvalumab, Cisplatin, and Pemetrexed, and we found that the molecules that control the functional interactive activities of PD-1/L1 are potential predictors [10–18]. We then precipitated the pfeRNAs binding to PD-L1 in patient-matched cancerous tissues and histologically normal adjacent tissues, specified sequences, and characterized them by sncRNA deep sequencing (Fig. 1, Table 1). To confirm that PDLpfeRNAs bind to PD-L1, we performed IP and reverse pull-down assays (Fig. 2). To determine that the identified PDLpfeRNAs regulate the interaction activities of PD-1/L1, we screened their roles using quality-controlled drug development assays, and we identified one PDLpfeRNA as an enhancer and the other as an inhibitor of PD-1/L1 (Fig. 3). These results suggest that PDLpfeRNAs bind to PD-L1 to regulate its interaction with PD-1, resulting in functional activities of PD1/L1 interaction.

In normal tissues, the total expression values of these two PDLpfeRNAs were similar (Table 1), suggesting a balanced status between the enhancer PDLpfeRNAa and the inhibitor PDLpfeRNAb. During tumorigenesis and cancer development, both are endogenously induced. However, the enhancer PDLpfeRNAa was increased significantly higher than that of the inhibitor PDLpfeRNAb (Table 1), the balance was broken, and the induced PDLpfeRNAs led to the functional interaction of PD1/L1, resulting in the immune escape of cancerous cells (Supplementary Fig. 3). Thus, these two PDLpfeRNAs are potential

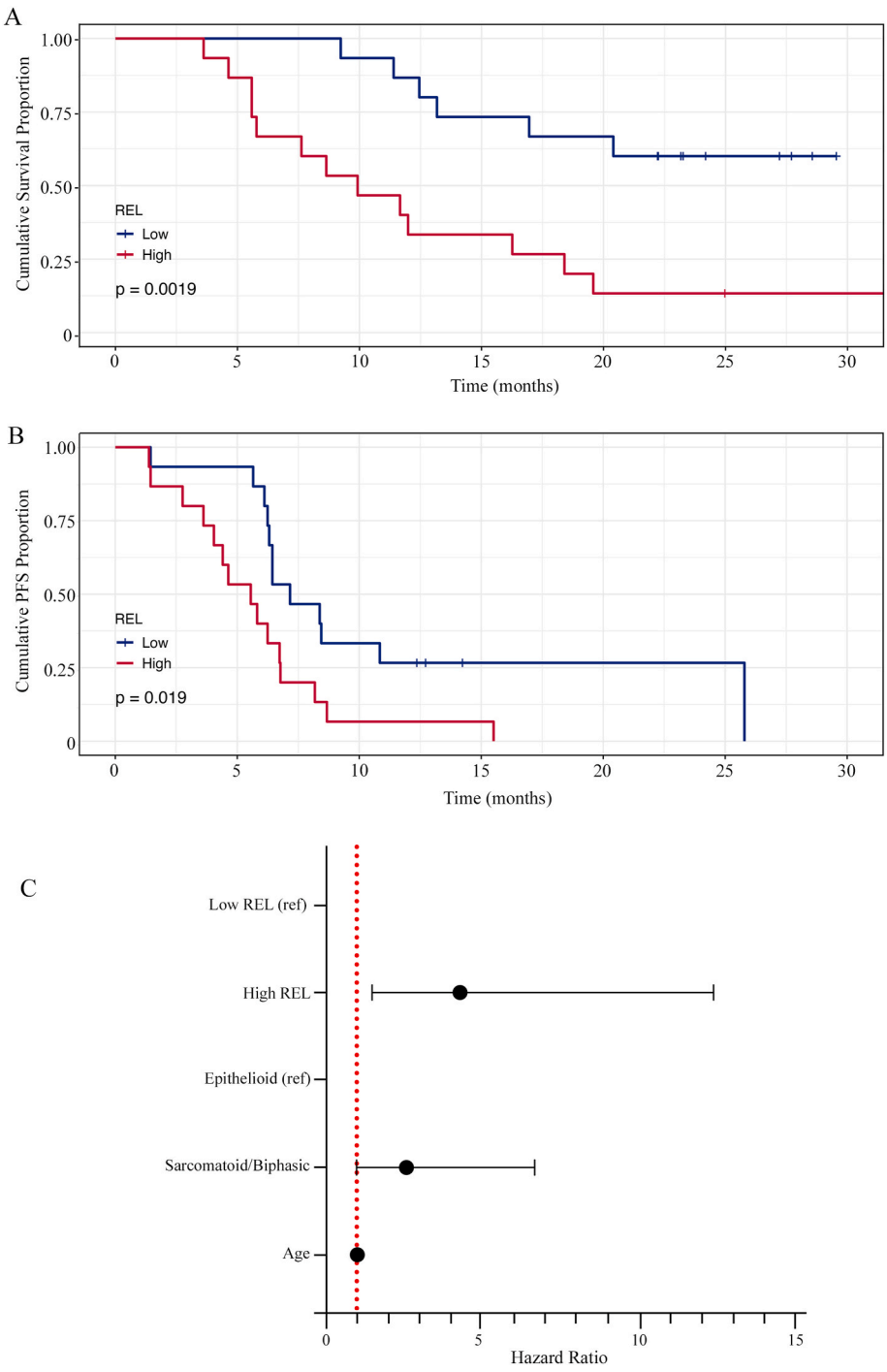

Fig. 4. REL of PDLpfeRNAs demonstrated significant prognostic value. (A) Kaplan-Meier Curves for overall survival by REL. Overall survival probability stratified by high versus low REL. Patients with high REL showed significantly worse overall survival ($p = 0.0019$). (B) Kaplan-Meier Curves for progression-free survival (PFS) by REL. Progression-free survival probability stratified by high versus low REL. Patients with high REL demonstrated significantly shorter progression-free survival ($p = 0.019$). (C) Forest Plot of Multivariate Cox Proportional Hazards Analysis for overall survival. Shows hazard ratios and 95 % confidence intervals from multivariate analysis. High REL (HR 4.31, 95 % CI: 1.49–12.42, $p = 0.007$) was significantly associated with worse overall survival after adjusting for age and histology. Reference groups are indicated by (ref).

predictors in patients treated with Durva-CP. There are two possibilities that PDLpfeRNAa and PDLpfeRNAb regulate the functional interactions of PD-1 and PD-L1 to contribute to the treatment response of patients with unresectable MPM. (1) Through the secondary and tertiary structures, the structure of PDLpfeRNAa was able to bind to structures of PD-L1, improving the functional interaction between PD-1 and PD-L1 to result in the immune escape of cancer cells. The effects of PDLpfeRNAb were the opposite. (2) Through the complex structures induced by protein-protein (PD-1 to PD-L1) interactions, PD-L1 is generally a monomer but forms complex structures by interacting with PD-1. The formed complex structures may have different binding affinities to PDLpfeRNAa and PDLpfeRNAb.

Given that MPM is a rare malignancy and the number of participants in clinical trials of Durva-CP was limited, we have collected almost all available participants who had two cycles of treatments, as not all participants had two cycles of treatments [5–9]. We finally analyzed the

Table 2
Number of patients at risk stratified by PD-L1 pfeRNA REL over time for overall and progression-free survival analyses, with log-rank test p-values.

	Time (months)						
	0	5	10	15	20	25	30
Overall Survival (p = 0.0019)							
Low REL	15	15	14	11	10	4	0
High REL	15	13	7	5	2	1	1
Progression-free Survival (p = 0.019)							
Low REL	15	14	5	1	1	1	0
High REL	15	8	1	1	0	0	0

clinical significance of the REL of these two PDLpfeRNAs, and we found that significant separation of survival curves between high and low REL groups for both OS and PFS (Fig. 4A and B, Table 2). In addition, this association remained significant after adjusting for histological subtype and age (Fig. 4C).

To bridge gaps and meet clinical needs in predictors of Durva-CP response for selecting optimal candidates and monitoring the developed resistance, we measured PDLpfeRNAs following the requirements of a CLIA-compliant LDT. The estimated cost of our qPCR-based assay was less than $15 per sample and only need 200 μL of plasma [23–25]. This assay has cost-effective and non-invasive advantage for future clinical application. The limitation of our study is its retrospective nature, and further validation of PDLpfeRNAs in a multicenter prospective trial is needed. Also, the roles of PDpfeRNAs capable of regulating the interaction between PD-1 and PD-L1 could have important applications to patients with different types of cancers as treatment response predictors and therapeutic targets because PDpfeRNAs might: (1) improve the effectiveness of existing PD-1/L1 inhibitors by modulating the interaction in ways current therapies cannot; (2) overcome resistance to benefit patients who have stopped responding to PD-1/L1 inhibitors; (3) reduce immune-related adverse events by allowing for more precise regulation of the PD-1/PD-L1 interaction.

5. Conclusion

In summary, our data on the functional roles and mechanisms of PDLpfeRNAs reveal the translational potential of PDLpfeRNAs. Following the standards of the CLIA-compliant LDT, we identified that plasma PDLpfeRNAs are predictors of treatment response of unresectable MPM patients treated with Durva-CP as first-line therapy to select optimal candidates and monitor developed resistance.

CRediT authorship contribution statement

Andrei Gurau: Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Suguru Yamauchi:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Kaitlyn Ecoff:** Investigation, Resources, Writing – review & editing. **Kristen P. Rodgers:** Writing – review & editing, Writing – original draft, Validation, Data curation. **James R. Eshleman:** Writing – review & editing, Writing – original draft, Validation, Data curation. **Charles Conover Talbot Jr:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Peng Huang:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Joshua Choi:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Patrick M. Forde:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Valsamo Anagnostou:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Malcolm Brock:** Writing – review & editing, Writing – original draft, Validation, Resources, Investigation, Data curation. **Yuping Mei:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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