

PD-L1 gene amplification and focality: relationship with protein expression

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ABSTRACT

PD-L1 (CD274) amplification occurs in a small subset of malignancies and may predict anti-PD-1/PD-L1 immunotherapy responsiveness. We hypothesized that both copy number (CN) and focality of cancer-related PD-L1 amplifications impact protein expression, and, thus, analyzed solid tumors that underwent comprehensive genomic profiling between March 2016 and February 2022 at Foundation Medicine. PD-L1 CN alterations were detected using a comparative genomic hybridization-like method. PD-L1 CN changes were correlated with PD-L1 protein expression (DAKO 22C3 antibody) by immunohistochemistry (IHC). Overall, 60,793 samples were analyzed (most frequent histologies: lung adenocarcinoma (20%), colon adenocarcinoma (12%), lung squamous carcinoma (8%)). Using a definition of CD274 CN \geq specimen ploidy +4 (6 copies), 1.21% of tumors (738/60,793) were PD-L1 amplified. Focality category distribution was as follows: <0.1 mB (n=18 (2.4%)), \geq 0.1 to <4 mB (n=230 (31.1%)), \geq 4 to <20 mB (n=310 (42%)), \geq 20mB (n=180 (24.4%)). Lower levels of PD-L1 amplification (below specimen ploidy +4) were more frequently non-focal amplifications compared to higher levels. In addition, more focal amplification (<0.1 mB) correlated with higher PD-L1 IHC expression. Median tumor proportion score (TPS) for samples with PD-L1 amplification (ploidy \geq +4) according to focality were 87.5% (<0.1 mB), 80% (\geq 0.1 to <4 mB), 40% (\geq 4 to <20 mB), 1% (\geq 20mB). In specimens with PD-L1 ploidy less than +4, but highly focal (<0.1 mB), the 75th percentile of PD-L1 expression by TPS was 80%. Conversely, non-focal (\geq 20 mB) PD-L1 amplification (ploidy \geq +4) can present high PD-L1 expression (TPS \geq 50%), albeit infrequently (0.09% of our cohort). In conclusion, PD-L1 expression measured by IHC is influenced by PD-L1 amplification level and focality. Further correlation between amplification, focality, protein expression and therapeutic outcome for PD-L1 and other targetable genes warrants exploration.

BACKGROUND

Programmed cell death protein pathway (PD-1/PD-L1) inhibitors are frequently employed for cancer treatment. A plethora of biomarkers of response and resistance to these agents are subject of intense research and new strategies are needed.^{1–3} PD-L1 protein overexpression can predict responses to PD-1/PD-L1 inhibitors, although several biological and technical challenges are

still unsolved.⁴ PD-L1 (CD274) amplification can be present in approximately 1% of solid tumors, and may also predict high response rates to immune checkpoint inhibitors (ICIs), independent of other immune biomarkers.^{5–7} In fact, Hodgkin's disease is exquisitely responsive to ICIs, and a hallmark of the disease is amplification of *PD-L1*.⁸

The potential effects of a cancer gene amplification on its RNA and protein expression are not completely understood. Several technical considerations as well as biological phenomena can influence the detection of gene amplification using next generation sequencing (NGS),⁹ as well as the subsequent transcription and translation of genes to RNA and protein. One possible mechanism is epigenetic modulation of genes, though other mechanisms may also be operative. For instance, 13% of putative driver somatic single nucleotide variants were unexpectedly not expressed as RNA in cancer specimens.¹⁰ Moreover, a recent paper demonstrated that 6% of cancer-related gene amplifications (\geq 6 copies) were silenced (ie, not expressed at RNA level compared with adjacent normal tissue).¹¹ Counterintuitively, focal amplifications (defined as <0.1 megabases (mB) in that report) were more frequently silenced than non-focal amplifications (9% vs 5%). The relationship between copy number (CN) changes, focality and protein expression may vary on a gene-by-gene basis and depending on the definition of 'focality'. Because protein expression may have therapeutic implications, in the current study, we analyzed PD-L1 protein (by immunohistochemistry (IHC)) vs CD274 (*PD-L1*) gene amplification CN and focality (thresholds of 0.1, 4 and 20 mB) using an NGS platform with 60,793 solid tumor specimens.

METHODS

Comprehensive genomic profiling (CGP) of 60,793 solid tumor clinical cases was performed using the FoundationOne (F1)

and FoundationOne CDx (FICDx) assays (Foundation Medicine, Cambridge, Massachusetts, USA) as described previously, in a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP) accredited laboratory, between March 2016 and February 2022.¹² All de-identified research consented samples submitted for sequencing featured >20% tumor cells and yielded >50ng of extracted DNA. CGP was performed on hybridization-captured, adapter ligation-based libraries (median exon coverage depth >800X), to identify genomic alterations (base substitutions, small insertions/deletions, CN alterations, and rearrangements) in >300 cancer-associated genes.

CD274 (PD-L1) CN alterations were detected using a comparative genomic hybridization-like method applied to NGS data.^{12,13} Each specimen was analyzed alongside a process-matched normal control (an internally validated mixture of 10 heterozygous diploid samples from the HapMap project), with custom algorithms to normalize the sequence coverage distribution across captured DNA regions. Log-ratios of normalized coverage data for exonic, intronic, and single nucleotide polymorphisms (SNPs) targets accounting for stromal admixture, as well as genome-wide SNP frequencies, were used to generate the CN profiles. Circular binary segmentation and proprietary algorithms further clustered groups of targets and SNP frequencies to define upper and lower bounds of genomic segments. Empirical Bayesian algorithms used a distribution of parameters including purity and base ploidy and probability matrices were derived using different statistical sampling methodologies to fit these data. Specimen-level ploidy was estimated as described by Sun *et al.*¹³ Computational models were reviewed by expert curators for each specimen.

CD274 (PD-L1) amplification was defined as ploidy +4 (CN 6), though other cut-off's were explored such as ploidy +2 (CN 4).^{6–11} Amplifications were classified as 'non-focal' when the length of the amplified CN segment was ≥ 20 Mb,¹⁴ but other thresholds (0.1 and 4Mb) were analyzed consistent with prior publications.^{9,11} PD-L1 IHC was run and interpreted by experienced board-certified pathologists according to the manufacturer's instructions in a CLIA-certified and CAP-accredited laboratory (Foundation Medicine, Morrisville, North Carolina, USA) (DAKO PD-L1 IHC 22C3 pharmDx) (tumor proportion score (TPS) method).¹⁵ TPS scores cut-offs were defined as 1% and 50% as per usage in non-small cell lung cancer (NSCLC).^{16,17} Kruskal-Wallis χ^2 test was used to test the association between the continuous PDL1 score and CD274 focality bins.

As self-reported race was not available, genomic ancestry was determined for each patient sample. For each profiling platform (F1 and FICDx), >40,000 SNP sites sequenced by CGP were identified. To remove biases due to linkage disequilibrium (LD), LD pruning was performed using PLINK (using the `-indep` flag with a window size of 50, a step size of 5, and a variance inflation factor threshold of 2). A random forest classifier was

trained on 1000 genomes to identify ancestral populations (European, African, Admixed American (Hispanic), East Asian, South Asian) using genetic variation at the SNP sites. Genetic variation was defined by 10 features that captured allele-count variation as determined by principal component analysis. This classifier was applied to CGP patient samples to assign them to one of the ancestral populations.¹⁸

RESULTS

Data from 60,793 clinical samples were analyzed in a CLIA laboratory (online supplemental table 1). Samples were derived from primary (44%) and metastatic tissue (39%) (rest unknown). Median age was 66 years; 54% were women. Most samples (78%) were from patients of European genetic ancestry. The most frequent histologies included lung adenocarcinoma (20%), colon adenocarcinoma (12%), lung squamous carcinoma, (8%) and ovarian serous carcinoma (5%).

Prevalence of *PD-L1* amplification varied according to ploidy category adopted. Using a definition of *CD274* $\text{CN} \geq \text{specimen ploidy} + 4$ (equivalent to >6 CN), 738 of 60,793 (1.21%) were considered *PD-L1* amplified (figure 1 and online supplemental table 2). These 738 samples were distributed according to focality category, from more to less focal: <0.1 mB ($n=18$ (2.4%)), ≥ 0.1 to <4 mB ($n=230$ (31.1%)), ≥ 4 to <20 mB ($n=310$ (42%)), ≥ 20 mB ($n=180$ (24.4%)). Using a less stringent threshold for determining CN positivity of *CD274* $\text{CN} \geq \text{specimen ploidy} + 2$ (CN >4) not only increased the prevalence of *PD-L1* amplification ($n=4,167$ (6.9% of all samples)), but also altered the distribution in focality categories: <0.1 mB ($n=30$ (0.7%)), ≥ 0.1 to <4 mB ($n=430$ (10.4%)), ≥ 4 to <20 mB ($n=1346$ (32.3%)), ≥ 20 mB ($n=2,380$ (56.6%)) (online supplemental table 3). From this analysis, lower levels of *PD-L1* amplification (below specimen ploidy +4) are more frequently non-focal amplifications compared with higher levels.

Using PD-L1 expression (DAKO 22C3 IHC assay; TPS), we demonstrated that more focal amplification (<0.1 mB) was associated with higher PD-L1 expression levels, which was observed using different ploidy categories (all p values $< 2.2 \times 10^{-16}$, figure 1). For samples with *CD274* $\text{CN} < 6$ ($<$ specimen ploidy +4), but highly focal (<0.1 mB), the 75th percentile of PD-L1 expression by TPS was 80% (figure 1A and B), meaning a reasonable proportion of samples expressing PD-L1; however, median expression was 1%. Tumors with *CD274* amplified (≥ 6 CN or \geq specimen ploidy +4) in a non-focal fashion (≥ 20 mB) may be currently considered as a variant of unknown significance. Even in this category, the 75th percentile of PD-L1 expression was 62.5%, suggesting a proportion of high PD-L1 expressors within this group; however, median expression level was 1%. Numerically, non-focal (≥ 20 Mb) *CD274* amplified presenting high PD-L1 expression (TPS $\geq 50\%$) encompassed 52 samples (0.09% of our cohort; online supplemental table 2). In contrast, median PD-L1

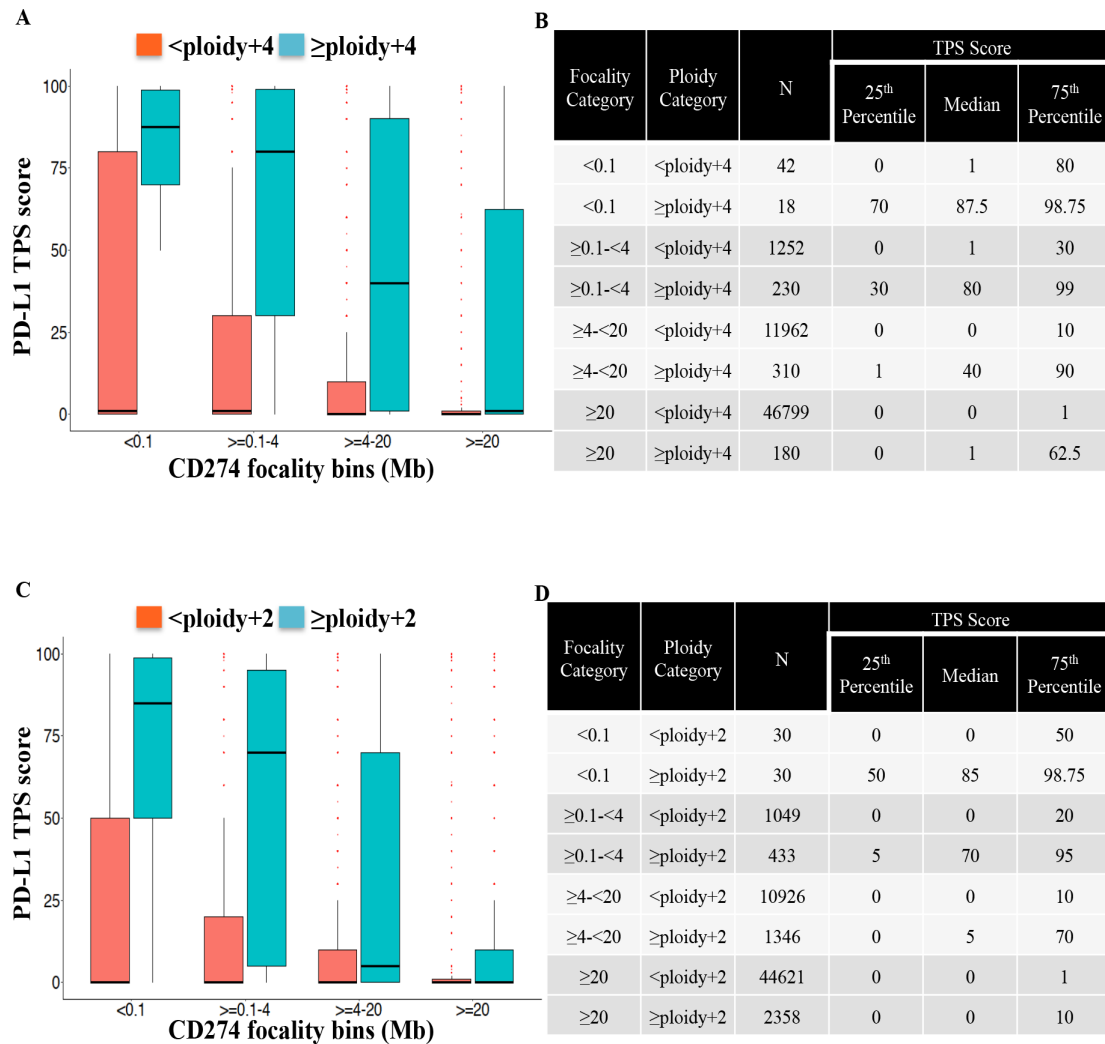


Figure 1 Levels of PD-L1 expression as measure by immunohistochemistry using TPS score and *CD274* (*PD-L1*) amplification focality. Distribution of PD-L1 TPS score (numbers represent per cent) over *CD274* focality bins at a *CD274* ploidy gain threshold of +4 (CN 6) (A) and +2 (CN 4) (C). Numbers of patient samples according to focality and ploidy category and interquartile distribution of TPS score for ploidy gain threshold of +4 (CN 6) (B) and +2 (CN 4) (D). Association between the continuous PD-L1 score and *CD274* focality bins ($\lt;0.1$, >math>\geq 0.1-4</math>, >math>\geq 4-20</math>, >math>\geq 20</math>) at ploidy+2 and ploidy+4 was significant at p value $\lt;2.2e-16$ (Kruskal-Wallis χ^2). CN, copy number; mB, megabases; TPS, tumor proportion score.

expression level was 87.5%, 80%, and 40% for >specimen ploidy +4 with focality of $\lt;0.1$, >math>\geq 0.1-4</math>, and >math>\geq 4-20</math> mB.

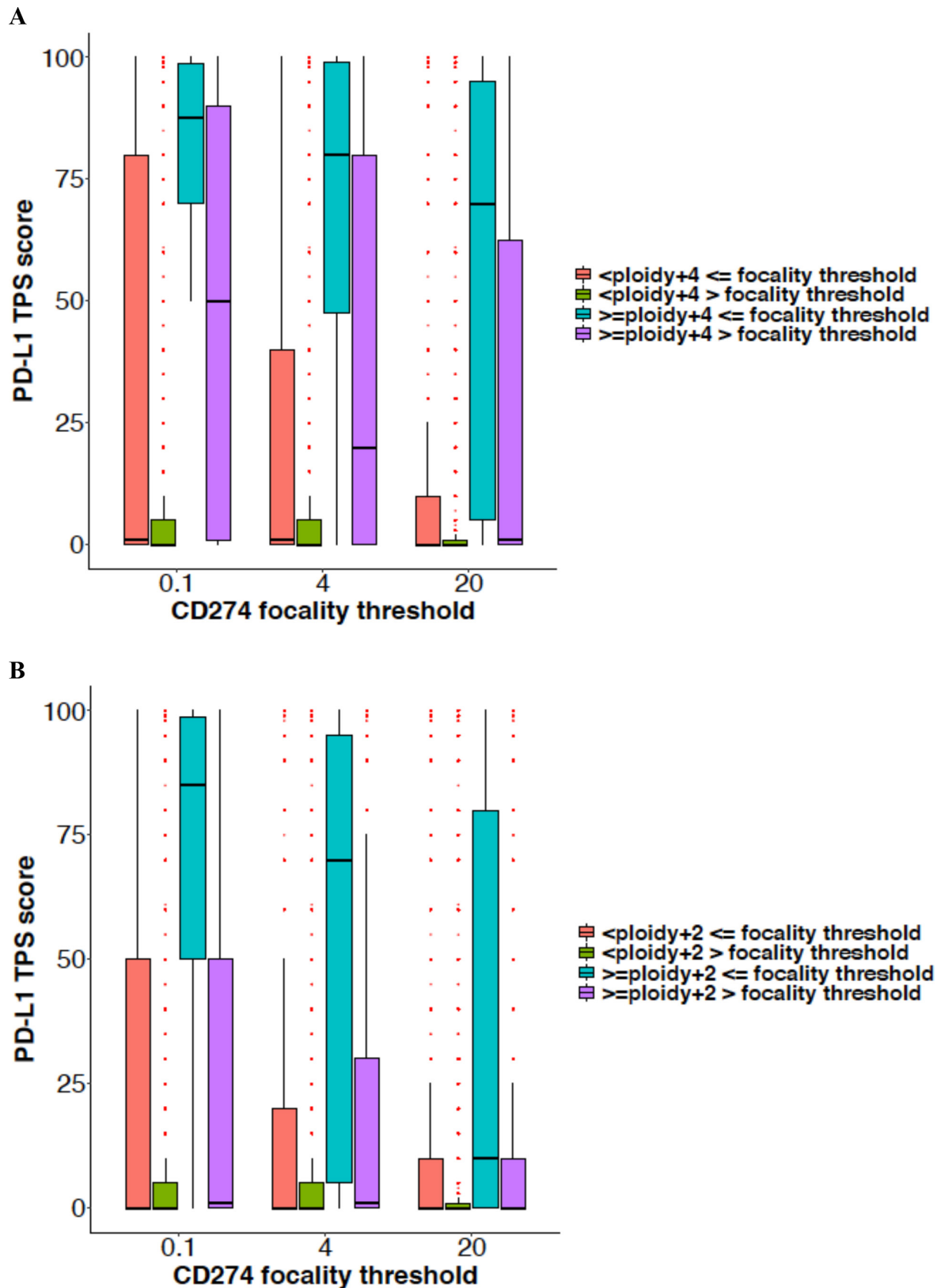
Within different *CD274* focality categories, the amplification level is also driving PD-L1 expression (figure 2). The presence of *PD-L1* amplification above the CN threshold is correlated to higher TPS score, and this effect is even clearer with lower levels of *CD274* gene ploidy, such as +2 (figure 2B and online supplemental table 3). We analyzed the possible influence of histology on the correlation of *PD-L1* gene amplification and protein expression. Separate analysis of the two most frequent histologic groups (NSCLC and colorectal cancer) and other histologies produced similar results (online supplemental table 4).

DISCUSSION

PD-L1 amplification is correlated with PD-L1 expression and also responsiveness to immunotherapy. To our

knowledge, this is the first data set of patients also indicating that focality of *PD-L1* amplification also influences protein expression. We demonstrated that *PD-L1* amplification occurred in 1.21% of patients, which is similar to the 1.1% previously reported in a large contemporary cohort.⁶ The focality level of a gene amplification can impact the likelihood of messenger RNA (mRNA) overexpression on a gene-by-gene basis.¹¹ It is plausible that focality may also reflect PD-L1 protein levels. Using PD-L1 expression (DAKO 22C3 IHC assay; TPS), we demonstrated that more focal amplification ($\lt;0.1$ mB) was associated with higher PD-L1 expression levels.

Tumors with *PD-L1* amplified (≥ 6 CN or \geq specimen ploidy +4) in a non-focal fashion (≥ 20 mB) may be currently considered as a variant of unknown significance. Even in this category, the 75th percentile of PD-L1 expression was 62.5%, suggesting a proportion of high



PD-L1 expressors within this group; however, median expression level was 1%. This finding might help for interpretation of genomic reports and can indicate that

in some cases a correlation with PD-L1 protein expression is warranted. Several quantitative parameters of CN gains were suggested for NGS reports, but focalinity levels

are currently not recommended.⁹ Our data suggested that focality could add additional granularity for clinical interpretation, at least for *PD-L1*. If focality demonstrates clinical impact on therapeutic decisions, it is feasible to incorporate this potential biomarker from NGS tests commercially available. This factor should be evaluated in prospective studies.

The directed relationship of *CD274* CN gains and PD-L1 expression was described as highly correlated in multiple tumor types.⁶ In addition, *CD274* CN $\geq +4$ (specimen ploidy +2) predict responses to checkpoint inhibitors in NSCLC.¹⁹ However, the role of *PD-L1* gene amplification focality on clinical responses to PD-1/PD-L1 inhibitors is unknown, and merits evaluation. Assessing cancer gene amplification using NGS can offer information that is useful for therapy. For *ERBB2*, information about CN and gene co-amplification can be predictive of trastuzumab clinical efficacy.^{20–21} Mesenchymal Epithelial Transition (*MET*) gene amplifications can be found in multiple tumors types and, at least in lung cancer, focality of amplification is correlated with responses to MET inhibitors.^{22–23} Herein, we described this interplay between an additional genomic layer (focality) and protein expression of PD-L1, which could also be explored for other cancer genes. Prior data suggested that, in general, genomic biomarkers are more predictive of responses to anticancer therapies as compared with protein biomarkers,²⁴ perhaps due to technical limitations for protein assessment, but variations are expected on a gene-by-gene basis. One limitation of our data is lack of RNA expression, and correlation between *PD-L1* gene amplification and focality with *PD-L1* mRNA expression and impact on proteins levels. This analysis merits further studies.

Conclusions

Our data indicate that *PD-L1* amplification level correlates with PD-L1 expression measured by IHC. Importantly, focality of *PD-L1* amplification is also important for PD-L1 expression, as more focal amplification increases PD-L1 expression in different ploidy thresholds. Nonetheless, even above higher thresholds of focality (≥ 20 mB), PD-L1 overexpression can be detected, especially in the presence of increased CN, although less frequently. It is therefore important to correlate genomic findings from *PD-L1* amplification and focality with PD-L1 proteins levels and to further examine correlation between amplification, focality, and immunotherapy outcome. The impact of focality and amplification level on protein expression and therapeutic outcomes for other targetable genes also merits exploration.

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Contributors Conceptualization: DLJ, KM, RSPH and RK. Methodology: KM, JAE and RSPH. Investigation: DLJ, KM, JAE, RSPH and RK. Visualization: DLJ, KM, RSPH and RK. Funding acquisition: RK. Supervision: RSPH and RK. Writing—original draft: DLJ. Writing—review and editing: DLJ, KM, JAE, RSPH and RK.

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Patient consent for publication Not applicable.

Ethics approval Approval for this study was obtained from the Western Institutional Review Board (IRB protocol No. 20152817). Approval for this analysis, including a waiver of informed consent and a Health Insurance Portability and Accountability Act (HIPAA) waiver, was obtained from the Western Institutional Review Board (IRB protocol No. 20152817).

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Data availability statement Data are available upon reasonable request. Not applicable.

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