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Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor infiltration between paired primary lesions and brain metastases in lung cancer

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Background: The dynamics of PD-L1 expression may limit its use as a tissue-based predictive biomarker. We sought to expand our understanding of the dynamics of PD-L1 expression and tumor-infiltrating lymphocytes (TILs) in patients with lung cancer-related brain metastases.

Experimental design: Paired primary lung cancers and brain metastases were identified and assessed for PD-L1 and CD3 expression by immunohistochemistry. Lesions with 5% or greater PD-L1 expression were considered positive. Agreement statistics and the χ^2 or Fisher's exact test were used for analysis.

Results: We analyzed 146 paired lesions from 73 cases. There was disagreement of tumor cell PD-L1 expression in 10 cases (14%, $\kappa = 0.71$), and disagreement of TIL PD-L1 expression in 19 cases (26%, $\kappa = 0.38$). Most paired lesions with discordant tumor cell expression of PD-L1 were obtained 6 or more months apart. When specimens were categorized using a proposed tumor microenvironment categorization scheme based on PD-L1 expression and TILs, there were significant changes in the classifications because many of the brain metastases lacked either PD-L1 expression, tumor lymphocyte infiltration or both even when they were present in the primary lung cancer specimens ($P = 0.009$).

Conclusions: We identified that there are significant differences between the tumor microenvironment of paired primary lung cancers and brain metastases. When physicians decide to treat patients with lung cancer with a PD-1 or PD-L1 inhibitor, they must do so in the context of the spatial and temporal heterogeneity of the tumor microenvironment.

Key words: lung cancer, tumor immunology, PD-L1, heterogeneity, metastasis

introduction

Immunotherapy has been rapidly adopted for the treatment of non-small-cell lung cancer (NSCLC). Monoclonal antibodies that disrupt signaling between programmed cell death protein 1 (PD-1,

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otherwise known as CD279) and its ligand PD-L1 (otherwise known as B7-H1 and CD274) have been proven superior to docetaxel in the second-line setting [1–3] and are being explored in the first-line setting in clinical trials. There have been significant efforts to develop PD-L1 as a predictive biomarker to select patients for treatment with PD-1 or PD-L1 inhibitors, but there is substantial confusion over the applicability of a PD-L1 antibody clone to a non-companion therapeutic agent, how interval therapy or sequencing of therapy affects rates of detection of PD-L1, and how heterogeneity of PD-L1 expression may hinder appropriate selection of patients for treatment [4, 5].

The dynamics of PD-L1 expression may also limit its use as a tissue-based predictive biomarker in NSCLC, yet its expression is being incorporated into companion and complementary testing strategies to select which patients should receive PD-1 or PD-L1 inhibitors [6]. PD-L1 is an immunologic marker that is critical for immunologic tolerance [7], and its expression by tumors results in apoptosis of tumor-specific T-lymphocytes [8]. PD-L1 can be expressed adaptively in response to stimuli such as IFN-γ [8, 9], or constitutively (intrinsically) due to oncogenic signaling such as loss of phosphatase and tensin homolog (PTEN) [10], activating mutations in epidermal growth factor receptor (EGFR) [11, 12] or other mechanisms [13]. Immunologic categorizations of tumors and their microenvironments have been proposed based on the combination of PD-L1 expression and the presence of tumor-infiltrating lymphocytes (TILs) [14, 15]. It is not certain if both patients with intrinsic or adaptive expression of PD-L1 benefit from PD-1 or PD-L1 inhibition, or if the immunologic milieu and classification of tumors is dynamic. With the incorporation of PD-L1 expression as a test for treatment selection with PD-1 inhibitors in NSCLC, it remains uncertain whether archival or new tumor samples should be used for testing.

PD-L1 is primarily detected at the tumor-stromal interface, and it is not commonly expressed by all tumor cells [16]. This intratumoral heterogeneity of PD-L1 limits its detection in NSCLC by biopsy [17]. Furthermore, we have shown that there was poor concordance of PD-L1 expression between independent primary lung cancers, but good concordance between genomically similar lesions in patients with multifocal lung cancer [18]. Since patients with multifocal lung cancer are not necessarily the ones most commonly being considered for and treated with PD-1 inhibitors, we sought to expand our understanding of intertumoral heterogeneity of PD-L1 expression in patients with metastatic NSCLC. Furthermore, we sought to consider the effects of time on the concordance of PD-L1 expression between lesions. Accordingly, we identified a large cohort of patients with paired primary lung cancers and brain metastases, the majority of which were fully resected at different clinical time points. We also assessed the agreement of PD-L1 expression and immunologic categorization between the paired tumors over time.

materials and methods

patients

The Tissue Registry at Mayo Clinic was searched between January 1994 and December 2015 to identify brain metastases for which paired primary lung cancer specimens were available. We favored inclusion of whole-tissue sections, but allowed paired biopsy specimens in a few cases. A pathologist

(MCA) reviewed tissue sections for adequacy. Patients with a history of multiple malignancies were excluded to reduce the possibility of including cancers other than NSCLC.

immunohistochemistry

Blocks were sectioned at 5 μm. Deparaffinization and immunohistochemistry (IHC) staining were carried out online. IHC staining for PD-L1 was carried out using the Leica Bond RX stainer (Leica, Buffalo, IL). Slides for PD-L1 stain were retrieved for 20 min using Epitope Retrieval 2 (EDTA; Leica). PD-L1, Rabbit Monoclonal (Clone E1L3N; Cell Signaling #13684) was diluted 1/600 and incubated for 15 min at RT. The detection system used was Polymer Refine Detection System (Leica). IHC staining for CD3 was carried out on the Ventana Benchmark XT (Ventana Medical Systems, Tucson, AZ). Slides for CD3 were retrieved with CC1 for 32 min. CD3, Mouse Monoclonal (Clone LN10, Leica, #NCL-L-CD3-565) was diluted 1/250 and incubated for 15 min at 37C. For CD3, the detection system used was OptiView DAB (Ventana Medical Systems). Normal tonsil was used as a positive control and normal tonsil without primary antibody was used as a negative control.

IHC expression scoring

PD-L1 was considered as expressed in tumor cells only if membranous staining was present with an intensity staining of 2 to 3+. The scoring of PD-L1 in tumor cells was expressed as a percentage of stained cells in the overall section of tumor and estimated in increments of 5%. Immune cells, both intratumoral and peritumoral at the interface between tumor and lung, positive for PD-L1 were also scored. A low-power magnification area with greatest intensity of staining was identified. The percent of positive immune cells was estimated in increments of 5%. Patients with at least moderate 5% or greater PD-L1 staining of tumor cells or immune cells were considered positive, consistent with many other studies [14, 19–21]. The number of CD3+ TILs was counted and averaged over three high-powered fields.

classification of tumors

Tumors were classified based on a proposed immunologic classification that defines tumors based on the presence of PD-L1 expression and TILs (Table 1) [9, 15]. Since there is no clear-cut value for how many TILs are required to consider a tumor infiltrated, we used the median number of TILs in primary lung cancers ($n = 11$) as a cut off for being infiltrated. We did not consider the presence of a few individual TILs to warrant classification of tumors as being infiltrated.

statistics

Descriptive statistics were used to summarize patient demographics and results. Agreement statistics [Cohen's κ coefficient, limits of agreement (Bland–Altman analysis)] were used to assess heterogeneity of expression between paired lesions. Confidence intervals (CIs) were calculated with the modified Wald method. Fisher's exact test was used to compare proportions within groups when classifications contained fewer than five subjects, and

Table 1. Immunologic classification of tumors

	PD-L1$^+$	PD-L1$^-$
TIL$^+$	Adaptive immune resistance	Tolerance (other suppressors)
TIL$^-$	Intrinsic induction	Immunologic ignorance

The immunologic classification is summarized above based on the presence of tumor cell expression of PD-L1 and tumor-infiltrating lymphocytes as proposed by others [9, 15].

the χ^2 test was used to compare the differences in proportions of immunologically categorized tumors. The Wilcoxon matched-pairs signed-rank test was used to compare TILs between groups. All tests were two-tailed. Prism 6 for Mac OS X (GraphPad Software, Inc., La Jolla, CA) was used for analysis. Circos diagrams were created using an online interface [22] and modified for clarity with Adobe Photoshop CC 2014. This project was approved by Mayo Clinic's Institutional Review Board (#13-007990).

results

patient demographics

We analyzed 146 paired primary lung cancers and brain metastases from 73 cases (supplementary Table S1, available at *Annals of Oncology* online). There were 54 adenocarcinomas, 17 squamous cell carcinomas, 1 adenosquamous and 1 combined small cell included in this study. The majority of our specimens were whole-tissue sections ($n = 133$, 91%); however, a few biopsies were included ($n = 13$, 9%; lung = 10, brain = 3). Eight of the biopsies were obtained from the lung from cases with synchronous brain metastases, and five of the biopsies (3 brain and 2 lung) were obtained 3 or more months after the original diagnosis. The median interval between acquisitions of paired lesions was 11 months (inter-quartile range 2–26 months, complete range 0–84 months).

spatial heterogeneity of PD-L1 expression

The tumor cells of 56 lesions (39% of all lesions, 95% CI 32% to 47%) were positive for PD-L1, with 32 positive primary lung cancer lesions (44% of lung cancers) and 24 positive brain metastases (33% of brain metastases; Figure 1, supplementary Figures S1 and S2, available at *Annals of Oncology* online). Among all 73 cases, there was agreement of PD-L1 expression by the tumor cells in the paired lesions of 63 cases (86%, 95% CI 76% to 93%), and disagreement in 10 cases (14%, 95% CI 7% to 24%) ($\kappa = 0.71$, 95% CI 0.55–0.87) (Figure 2A, supplementary Table S2, available at *Annals of Oncology* online). In 23 cases, both tumor specimens were positive for PD-L1 expression, and

in 40 cases, both tumor specimens were negative. The expression of PD-L1 was concordant with the whole-tissue sections in 11 of the 13 biopsies (6 were both positive, 5 were both negative). A bias of 3.4% (95% CI −24.5% to 31.2%) was identified when the differences of percent PD-L1 expression were plotted over the average percent expression between pairs (supplementary Figure S3A, available at *Annals of Oncology* online).

The immune cells within or surrounding the primary lung cancers or brain metastases were positive for PD-L1 in 41 lesions (28% of all 146 lesions, 95% CI 21% to 36%) (Figure 2B). Among the 73 cases, there was agreement of PD-L1 expression by immune cells in paired lesions of 54 patients (74%, 95% CI 63% to 83%) and disagreement in 19 cases (26%, 95% CI 17% to 37%) ($\kappa = 0.38$, 95% CI 0.17–0.59) (supplementary Table S3, available at *Annals of Oncology* online). In 11 cases, both lesions were positive, and in 43 cases, they were both negative or had no detectable immune cells.

temporal discordance of PD-L1 expression

In regard to tumor cell expression of PD-L1, there were fewer discordant pairs that were obtained <6 months apart ($n = 2$, 3%, 95% CI 0% to 10%) than 6 or more months apart ($n = 8$, 11%, 95% CI 5% to 20%), but this difference in proportions was not statistically significant (Fisher's exact test $P = 0.48$; supplementary Figure S4 and Table S4, available at *Annals of Oncology* online). In regard to immune cell expression of PD-L1, there were fewer discordant pairs that were obtained <6 months apart ($n = 8$, 11%, 95% CI 5% to 20%) than 6 or more months apart ($n = 11$, 15%, 95% CI 8% to 25%), but this difference in proportions was not statistically significant (Fisher's exact test $P = 0.40$; supplementary Table S5, available at *Annals of Oncology* online).

immunologic categorizations

Primary lung cancers overall contained more TILs (median 11, IQR 3–30) than brain metastases (median 5, IQR 1–15;
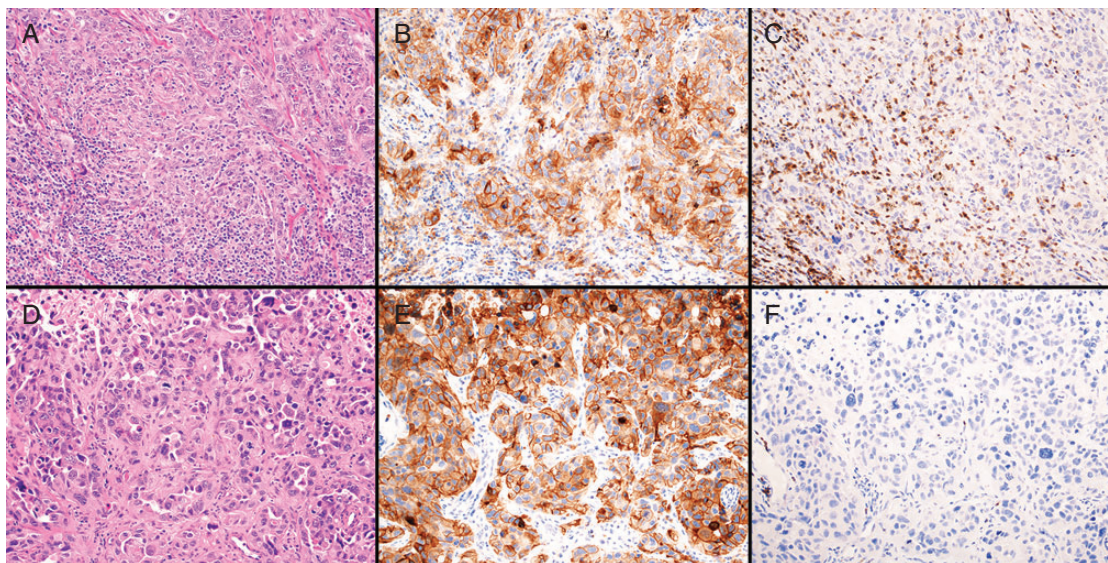

Figure 1. Representative figure of concordant PD-L1 expression between lesions.

$P < 0.0001$). Using a proposed immunologic categorization scheme based on PD-L1 expression and TILs (Table 1) [9, 15], we grouped each specimen as adaptive, intrinsic, tolerant or ignorant. There were significantly more primary lesions ($n = 22$, 15%, 95% CI 10% to 22%) than brain metastases ($n = 13$, 9%, 95% CI 5% to 15%) categorized as adaptive, and fewer primary lesions ($n = 23$, 16%, 95% CI 11% to 23%) than brain metastases ($n = 35$, 24%, 95% CI 18% to 32%; $P = 0.009$) categorized as ignorant (Figure 3).

discussion

The tumor microenvironment, as categorized by TILs and PD-L1 expression, is significantly different between primary lung cancers and their paired brain metastases primarily because many of the brain metastases lost PD-L1 expression, tumor lymphocyte infiltration or both despite their presence in the primary lung cancer specimens. We identified agreement in PD-L1 expression by tumor cells between paired primary and metastatic lesions in 86% of the cases that we included in our study, which was much greater agreement than expected by chance alone. Similarly, there was agreement in PD-L1 expression by immune cells between paired lesions in 74% of the cases. Overall, there was greater expression of PD-L1 by tumor cells or immune cells in primary lung cancers compared with their paired brain metastases. In only a few cases, we observed PD-L1 expression in metastatic lesions, but not the primary lung cancer. Although there is agreement of PD-L1 expression between paired lesions in the majority of patients, this degree of discrepant expression challenges the current practices to select which patients should receive a PD-1 or PD-L1 inhibitor.

The cases in our series came from patients with lung cancer that metastasized to the brain, which is a very common scenario for this disease. The brain metastases were either diagnosed concurrently with or months to years after an intended curative resection of the primary disease. Even though we included biopsies from a few of our patients (9% of specimens), the majority of these biopsies were in agreement with their paired fully resected lesion. The majority of subjects treated on clinical trials with PD-1 inhibitors were not required to undergo fresh biopsies for PD-L1 detection [1–3]. It is possible that the spatial and temporal heterogeneity of PD-L1 expression explains why some subjects without detectable PD-L1 respond to treatment. Overall, the diminished expression of PD-L1 and tumor lymphocyte infiltration in brain metastases suggests that there may be differential responses between intracranial and extracranial disease when these patients receive PD-1 or PD-L1 inhibitors. Given that PD-L1 may be adaptively expressed in response to IFN-γ, it is not certain whether 'ignorant' brain metastases would have inducible expression of PD-L1 if lymphocytes could traffic appropriately to the sites of metastases. Since the patients in this series were not treated with PD-1 or PD-L1 inhibitors, we cannot comment on the predictive significance of PD-L1 expression in the discrepant tumor microenvironments. Also, since we used the E1L3N PD-L1 clone, we are not certain at this time how it compares with the companion and complementary PD-L1 diagnostic tests associated with the approved PD-1 inhibitors.

There are many antibodies available for the detection of PD-L1. We found that 39% of the lesions in our series were positive for PD-L1, which is similar to the rates detected by members of our team and others with the same [18] or different antibodies [23, 24] by IHC. The heterogeneity of PD-L1 expression has been explored in other malignancies as well. In a separate series of lung
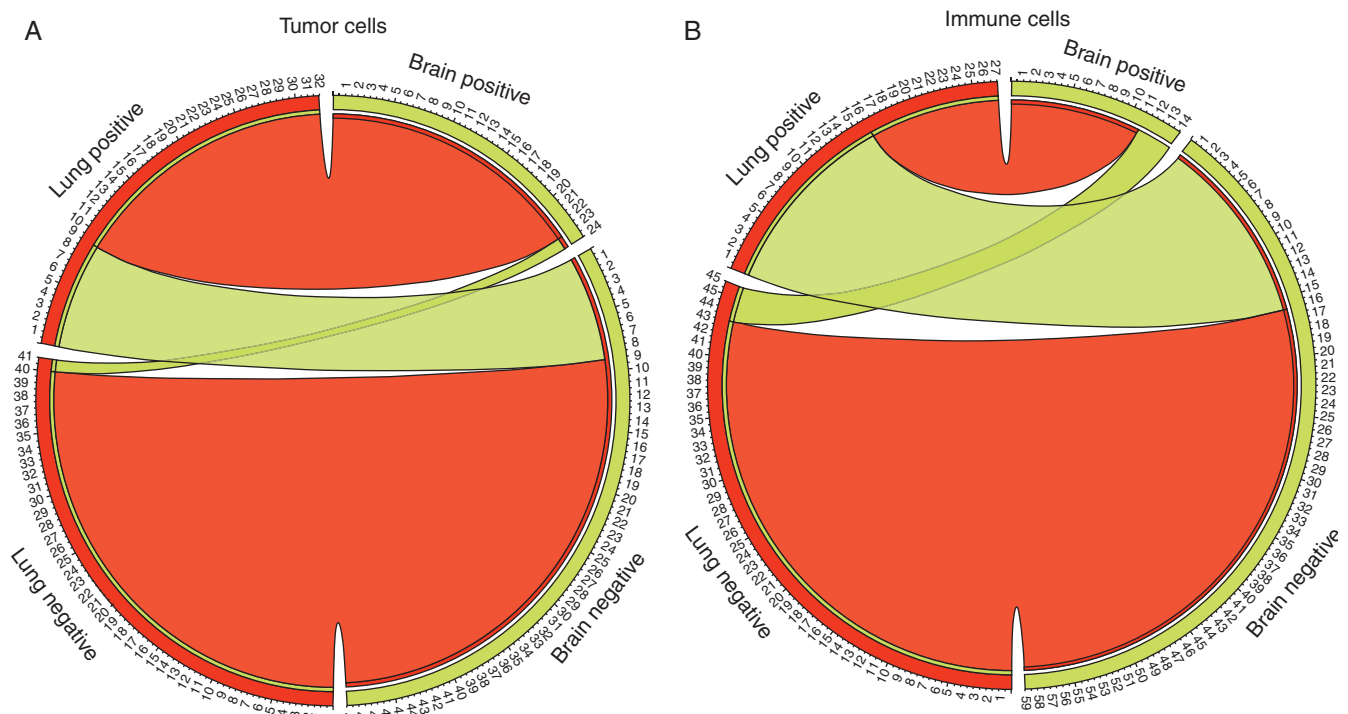

Figure 2. Circos diagrams of tumor cell and immune cell PD-L1 expression between paired lesions.
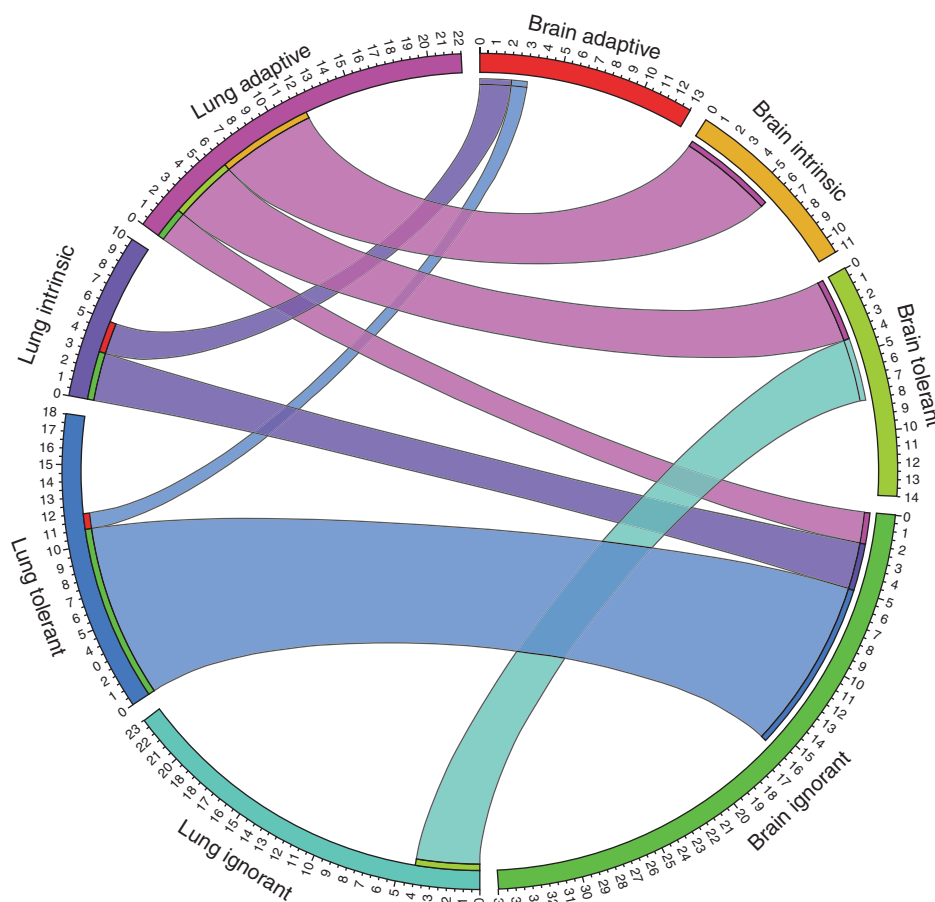
Figure 3. Circos diagram of tumor microenvironment categorizations between paired lesions.

cancers that looked at paired primary lung cancers and lymph node metastases, almost 20% of cases lost tumor cell expression of PD-L1 with metastasis to the lymph nodes [25], which is within range of the 14% rate of discordance, we found between primary lung cancers and paired brain metastases. In renal cell carcinoma, there was discordance in tumor cell PD-L1 expression between 20.8% of the paired primary and metastatic lesions [26]. Additionally, brain metastases in melanoma had few TILs [27], which is concordant with our findings demonstrating that the majority of the brain metastases were immunologically ignorant because of the lack of TILs and PD-L1 expression. Overall, across malignancies, it seems that there is a trend for metastases to less commonly express PD-L1 or be infiltrated with lymphocytes. For tumor types that require a companion diagnostic test for a PD-1 or PD-L1 inhibitor, these results call into question which lesions should be assessed for PD-L1 expression or tumor lymphocyte infiltration. Furthermore, our results suggest that there may be discordant responses to immunotherapies between primary lesions and metastases based on the discrepancies in their tumor microenvironments.

When physicians decide to treat patients with a PD-1 or PD-L1 inhibitor based on PD-L1 expression, they must do so in the context of the spatial and temporal heterogeneity. With the data that we have presented here and previously [18] on intertumoral and temporal heterogeneity of PD-L1 expression, and the data that others have presented on intratumoral heterogeneity [16,

17], novel tissue acquisition may be useful to guide treatment selection for patients with small or negative archival specimens. Hopefully, ongoing studies or blood-based biomarkers will improve treatment selection strategies for patients with lung cancer [28].

In this figure, a case of lung adenocarcinoma (A—hematoxylin and eosin, 200×) was resected 1 month following removal of a brain metastasis (D—hematoxylin and eosin, 200×). Both the lung adenocarcinoma and brain metastasis displayed concordant PD-L1 immunostaining with diffuse and strong membranous staining (B—lung PD-L1, 200×; E—brain PD-L1, 200×); however, TILs as demonstrated with CD3 were present in the lung primary (C—CD3, 200×) but not the brain metastasis (F—CD3, 200×).

These circos diagrams display the agreement of PD-L1 expression by tumor cells (A) and immune cells (B) between paired primary lung cancers and brain metastases. The left sides of the circos diagrams represent the primary lung cancers, and the right sides represent the paired brain metastases. The positive and negative specimens are demonstrated by the labeled segments. Ribbons within the circos diagram connect paired specimens. Red ribbons connect pairs of lesions with concordant PD-L1 expression and chartreuse ribbons connect pairs of lesions with discordant expression.

The tumor microenvironments of the paired lesions in our series were classified according to Table 1 based on tumor cell expression of PD-L1 and TILs. The circular segments are labeled

with these classifications, and the numbers of lesions within each classification are shown with tick marks around the segments. Cases with discordant classifications between the paired primary lung cancers and brain metastases are connected by colored ribbons to demonstrate the dynamics of the discrepant tumor microenvironment classifications between pairs. Concordant cases are not connected by ribbons. Overall, many of the brain metastases lost PD-L1 expression or tumor lymphocyte infiltration that was present in the primary lung cancer specimens.

funding

disclosure

The authors have declared no conflicts of interest.

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