INTRODUCTION

Studies have historically revealed an immune component of the tumor microenvironment. Recently, several studies showed the effectiveness of blocking the programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) pathway of immune checkpoint inhibitors in adult patients with multiple PD-L1-expressing cancers.[1–4] PD-1, expressed on activated T cells, suppresses T-cell responses upon binding to PD-L1 or PD-L2. Therefore, in PD-L1-expressing tumors, anti-PD-1 antibodies could counteract the inhibitory pathway that blocks effective antitumor T-cell responses, thus inducing an antitumor effect.[5] Furthermore, tumor-infiltrating lymphocytes (effector cells) are important factors in prognosis, PD-L1 expression, and the prediction of responses to PD-1/PD-L1 blockade.[5–9]

Although PD-1/PD-L1 blockade is also desirable for patients with pediatric cancers, particularly those with a poor prognosis and long-term toxic effects of current therapies, little is known about the immunological parameters for PD-1/PD-L1 blockade in pediatric cancers.[10] Herein, we assessed the intensity of PD-L1 expression and tumor-infiltrating CD8+ T cells in pediatric cancers to determine the potential usefulness of PD-1/PD-L1 blockade.

METHODS

Patients

This retrospective study was conducted in accordance with the Declaration of Helsinki, with approval from the institutional review board at Saitama Children’s Medical Center. We evaluated formalin-fixed, paraffin-embedded (FFPE) tumor specimens from patients with pediatric solid tumors who underwent biopsy or resection and were treated at Saitama Children’s Medical Center from 2009 to 2011. We excluded patients whose pretreatment paraffin blocks could not be obtained and included two patients with a rhabdomyosarcoma or an atypical teratoid/rhabdoid tumor (AT/RT) because of the small number. We evaluated specimens from 53 patients, including 18 neuroblastomas, eight extracranial malignant germ cell tumors, seven germinomas, seven hepatoblastomas, four renal tumors, four medulloblastomas, three rhabdomyosarcomas, and two AT/RTs.

Low Frequency of Programmed Death Ligand 1 Expression in Pediatric Cancers

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Key words: PD-1/PD-L1 blockade; pediatric cancer; tumor-infiltrating lymphocyte

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Patients were diagnosed at a median age of 2 years (range: 0 months–16 years), and 17 presented with distant metastases. During a median follow-up period of 52 months (range: 1–75 months), event-free survival and overall survival rates were 74% (39/53) and 89% (47/53), respectively. The characteristics of the neuroblastoma cases are shown in Supplementary Table S1.

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Abbreviations: AT/RT, atypical teratoid/rhabdoid tumor; FFPE, formalin-fixed, paraffin-embedded; HPF, high-power field; PD-1, programmed death 1; PD-L, programmed death ligand

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Fig. 1. Images of commercially available control tissue sample and pediatric cancers stained to detect PD-L1. (A) Images of SignalSlide® PD-L1 IHC Controls (Cell Signaling Technology): Left, positive (E1L3N); right, negative (E1L3N). (B) Classical Hodgkin lymphoma: Left, positive (E1L3N); right, negative (isotype). (C) Rhabdomyosarcoma: Left, positive (E1L3N); middle, negative (isotype); right, negative (E1L3N). (D) Germinoma: Left, negative (E1L3N); middle, negative (isotype); right, CD68 staining. (E) Hepatoblastoma: left, granularly positive (E1L3N); right, negative (isotype). (F) Normal hepatocytes: Left, negative (E1L3N); right, negative (isotype). (G) Upper left, neuroblastoma: negative (E1L3N); upper middle, Wilms tumor: negative (E1L3N); upper right, clear cell sarcoma of the kidney: negative (E1L3N); lower left, yolk sac tumor: negative (E1L3N); lower middle, medulloblastoma: negative (E1L3N); lower right, atypical teratoid/rhabdoid tumor: negative (E1L3N). Original magnification, 400×.

Immunohistochemistry

Immunohistochemical staining was performed using an automated staining system (XT iVIEW DAB v3 SP; Ventana, Oro Valley, AZ) with anti-PD-L1 (E1L3N; Cell Signaling Technology, Danvers, MA), anti-CD8 antibody (already diluted, Nichirei Biosciences, Tokyo, Japan), and anti-CD68 antibody (dilution: 1:100, Dako, Glostrup, Denmark). Three micrometers thick FFPE sections were deparaffinized, subjected to heat-induced antigen retrieval (100°C for 90 min), and stained with anti-PD-L1 antibody (1:200 dilution) for 32 min.

To confirm sensitivity, anti-PD-L1 antibody staining was validated using PD-L1 IHC Controls (Cell Signaling Technology; Fig. 1A) and classical Hodgkin lymphomas (Fig. 1B)[3,11] which were embedded using standard procedures at Saitama Children’s Medical Center.

Sample Evaluation

Pathologic diagnoses were confirmed by a pathologist (HK) who reviewed FFPE tissue sections stained with hematoxylin.
TABLE I. PD-L1 Expression and Tumor-Infiltrating CD8+ T-Cell Intensity

| Cancer type                        | Patient number (N = 53) | Distant metastasis (N = 17) | PD-L1+ immune cell containing tumor (N = 1) | Tumor-infiltrating CD8+ T-cell intensitya | CD8+ T-cell count/HPF | Mean | Range |
|------------------------------------|-------------------------|-----------------------------|---------------------------------------------|------------------------------------------|----------------------|------|-------|
| Neuroblastoma                      | 18                      | 12                          | 0                                           | 0                                        | 2.1                  | 10  | 5     |
| Extracranial germ cell tumor       | 8                       | 2                           | 0                                           | 0                                        | 2                    | 2   | 0     |
| Yolk sac tumor                     | 4                       | 1                           | 0                                           | 0                                        | 1                    | 1   | 0     |
| Teratoma and Yolk sac tumor        | 3                       | 1                           | 0                                           | 0                                        | 1                    | 1   | 0     |
| Teratoma and mixed germ cell tumor | 1                       | 0                           | 0                                           | 0                                        | 1                    | 0   | 0     |
| Germinoma                          | 7                       | 0                           | 0                                           | 7                                        | 1                    | 0   | 5     |
| Hepatoblastoma                     | 7                       | 1                           | 0                                           | 0                                        | 3                    | 4   | 0     |
| Medulloblastoma                    | 4                       | 0                           | 0                                           | 3                                        | 1                    | 0   | 0     |
| Renal tumor                        | 4                       | 1                           | 0                                           | 2                                        | 1                    | 1   | 0     |
| Wilms tumor                        | 2                       | 0                           | 0                                           | 1                                        | 1                    | 0   | 0     |
| Clear cell sarcoma of the kidney   | 2                       | 1                           | 0                                           | 1                                        | 1                    | 0   | 0     |
| Rhabdomyosarcoma                   | 3                       | 1                           | 0                                           | 0                                        | 1                    | 2   | 0     |
| Atypical teratoid/rhabdoid tumor   | 2                       | 0                           | 0                                           | 1                                        | 1                    | 0   | 0     |

aUnder the column titled “Tumor-infiltrating CD8+ T-cell intensity,” the numbers 0, 1+, 2+, and 3+ represent <2, 2–5, 5–20, and ≥20 CD8+ T-cell count/HPF, respectively.

Statistical Analysis

Tumor-infiltrating CD8+ T-cell counts were analyzed using the t-test. All probability (P) values were two-sided and the significance level was set at α = 0.05.

RESULTS

PD-L1 Expression

Table I presents the study results. One rhabdomyosarcoma specimen exhibited PD-L1 expression (Fig. 1C). Although the patient’s histological subtype could not be specified, he presented with distant metastasis and died within 6 months despite chemotherapy, the shortest survival duration in this cohort. The remaining 52 pediatric solid tumors did not express membranous PD-L1. Although germinomas contained PD-L1+ cells, the tumor cells did not directly express PD-L1; rather, PD-L1+ cells were identified as CD68-positive macrophages [13] (Fig. 1D). Although tumor cells in two hepatoblastomas exhibited strong PD-L1 staining (Fig. 1E), compared with normal hepatocytes (Fig. 1F), PD-L1 staining was granular and restricted to the cytoplasm. The other hepatoblastomas exhibited weak cytoplasmic PD-L1 expression, similar to normal hepatocytes. The remaining cancer types did not express PD-L1 (Fig. 1G).

Tumor-Infiltrating CD8+ T-Cell Intensity

Table I also presents the mean numbers of tumor-infiltrating CD8+ T cells per HPF in each cancer type. Germinomas had the highest numbers of tumor-infiltrating CD8+ T cells, followed by neuroblastomas. Excluding patients with primary brain tumors, patients with distant metastases had higher numbers of tumor-infiltrating CD8+ T cells (P = 0.019, Supplementary Table SII).

DISCUSSION

We showed a low frequency of PD-L1 expression in pediatric cancers, which is consistent with two previous reports. Routh et al. described PD-L1 expression in 11 (14%) of 81 Wilms tumors; moreover, only tumors from seven (10%) of 71 patients with favorable histology expressed PD-L1.[14] Uehara et al. also reported a low frequency of PD-L1 expression in neuroblastomas [five (12%) of 41 pretreatment specimens].[15] In contrast, Chowdhury et al. reported membranous PD-L1 expression in 66 (57%) of 115 pediatric solid tumors, including high-risk neuroblastomas, rhabdomyosarcomas, Ewing sarcomas, and osteosarcomas.[12] Differences in staining antibodies, staining procedures, and antigen retrieval techniques could explain this discrepancy; however, 10 high-risk neuroblastomas in our study were also negative when stained with another anti-PD-L1 antibody (ab58810; abcam, Cambridge, UK) at the dilution reported by Chowdhury et al. As our PD-L1-positive rhabdomyosarcoma exhibited the most aggressive clinical course, and Wilms tumors and rhabdomyosarcomas with unfavorable histologies exhibited stronger PD-L1 expression,[12,14] PD-L1 expression may correlate with a poor prognostic subtype.

Immune cells including macrophages and lymphocytes that infiltrated germinomas expressed PD-L1, a phenomenon previously described as a predictive factor of response to anti-PD-L1 antibody therapy.[16] This abundance of immune cells indicates
that immunomodulatory drugs may play an important role in future germinoma treatment strategies. Although we observed stronger PD-L1 staining in hepatoblastoma cells, the staining pattern differed from previous reports of other cancers, including hepatocellular carcinoma.[17] therefore, we could not confirm this staining to be meaningful.

Considering neuroblastomas have low to absent MHC class I expression,[18,19] a low frequency of cytotoxic T lymphocyte is suspected, although CD8\(^+\) T cells may be present when stimulated by IL-2 secreted by other immune cells.[20] However, if we can create an immunogenic environment, we could use immune checkpoint inhibitors effectively and unleash the T-cell response.[9] For example, treatment of neuroblastoma cell lines with toll-like receptor 3 ligands or interferon-gamma induces immune checkpoint inhibitor effectiveness and unleashes the T-cell response.[9] For example, treatment of neuroblastoma cell lines stimulated by IL-2 secreted by other immune cells.[20] However, if we can create an immunogenic environment, we could use immune checkpoint inhibitors effectively and unleash the T-cell response.

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