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Dysgerminomas: germ cell tumors exhibit high expression of PD-L1 and associated with high TILs and good prognosis

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Ovarian germ cell tumors (OVGCTs) account for 28% of all diagnosed ovarian cancers, and malignant germ cell tumors specifically account for approximately 13% of diagnosed ovarian cancers in Saudi Arabia. Although most germ cell tumor patients have a high survival rate, patients who experience tumor recurrence have a poor prognosis and present with more aggressive and chemoresistant tumors. The use of immunotherapeutic agents such as PD-L1/PD-1 inhibitors for OVGCTs remains very limited because few studies have described the immunological characteristics of these tumors. This study is the first to investigate PD-L1 expression in ovarian germ cell tumors and explore the role of PD-L1 expression in tumor microenvironment cells and genetic alterations. A total of 34 ovarian germ cell tumors were collected from pathology archives. The collected tumor tissues included ten dysgerminomas, five yolk sac tumors, five immature teratomas, and one mature teratoma, and the remaining samples were mixed germ cell tumors. The tumors were analyzed using immunohistochemical analysis to determine PD-L1 expression, immune cell infiltration and cancer stem cell populations and their correlation with clinical outcome. Furthermore, the genetic alterations in different subtypes of germ cell tumors were correlated with PD-L1 expression and clinical outcome. Datasets for testicular germ cells (TGCTs) were retrieved from The Cancer Genome Atlas (TCGA) and analyzed using cBioPortal (cbioportal.org) and Gene Expression Profiling Interactive Analysis (GEPIA). Compared with yolk sac tumors, dysgerminomas highly express PD-L1 and are associated with high levels of tumor infiltrating lymphocytes (TILs) and stem cell markers. In addition, compared with PD-L1-negative yolk sac tissue, dysgerminomas/seminomas with high PD-L1 expression are associated with more genetic alterations and a better prognosis. Our findings will contribute to the knowledge about the potential benefits of ovarian cancer immunotherapy in specific subsets of germ cell tumor patients and the risk factors for resistance mediated by tumor microenvironment cells.

Ovarian cancer is one of the most common gynecological malignancies and has the third highest mortality rate after corpus uteri and cervical cancer[1]. In Saudi Arabia, ovarian cancer is the second most deadly gynecological malignancy in women[2]. Ovarian malignant neoplasms are pathologically classified as epithelial or nonepithelial in origin. Epithelial ovarian cancers comprise 92.3% of all malignant ovarian tumors. Subtypes of epithelial cells include high-grade serous, low-grade serous, mucinous, clear cell, and endometrioid ovarian carcinomas. Nonepithelial tumors comprise approximately 7% of all ovarian neoplasms, and these types are less well characterized. The two main subtypes of nonepithelial tumors are sex-cord stromal and ovarian germ cell tumors (OVGCTs). OVGCTs are rare ovarian tumors that develop from the primitive germ cells of embryonic gonads[3]. In contrast to females, in males, germ cell tumors are the most common type of cancer[4].Therefore, several studies have investigated testicular germ cell tumors (TGCTs).

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While most ovarian cancers are present in the older population, OVGCTs are commonly diagnosed at younger ages[5]. Early-diagnosed patients with OVGCT can be treated successfully. Surgery is the first-line treatment after a confirmed diagnosis of OVGCT, followed by cisplatin-based chemotherapy to decrease the possibility of metastasis and recurrence[6]. However, some OVGCT patients continue to have poor survival outcomes. Some studies have suggested a familial genetic cause for germ cell tumors, but no definite conclusions can be drawn based on the available data[7].

OVGCTs are classified into different subtypes based on their histology: benign teratoma, immature teratoma, dysgerminoma, yolk sac, embryonal, choriocarcinoma and mixed germ cell tumors[3]. In the Saudi population, dysgerminoma comprise more than 40% of OVGCTs, mirroring the worldwide distribution, along with yolk sac tumors[8,9].

Programmed death receptor 1 (PD-1) and its ligand (PD-L1) act as immune checkpoints in the tumor microenvironment and have been investigated for their immunological importance in several cancers, including TGCTs and epithelial ovarian cancer[10–12], but not in OVGCTs. PD-L1 expression is being studied as a predictive biomarker for the tumor response to anti-PD-1/PD-L1 immunotherapy (checkpoint inhibitors)[13,14]. Blocking PD-1 or PD-L1 regulates the immune response, T-cell infiltration and tumor survival[15–17]. Checkpoint molecules and their inhibitors have been studied in different types of cancer, including non-small cell lung cancer, kidney cancer, colorectal cancer, prostate cancer, bladder cancer, and melanoma[18].

In germ cell tumors, very few studies have explored the presence and prognostic value of PD-1/PD-L1. PD-L1 overexpression was observed in primary germ cell tumors, with lower levels of PD-L1 expression in metastasized tumors[19].

Although studies have indicated that checkpoint inhibitors are effective for numerous cancer types, such as melanoma and lung cancer[20–22], the use of PD-L1/PD-1 inhibitors for germ cell tumors remains unknown. This study bridges this gap by exploring the role of predictive biomarkers of ovarian germ cell cancer for treatment response. This study is the first to investigate the role of PD-L1 expression in OVGCT microenvironment cells, including immune cells and cancer stem cells. These findings will likely improve our knowledge about the potential benefits of ovarian cancer immunotherapy in certain subsets of OVGCT patients.

Materials and methods

Patient cohort and sample collection

All experimental protocols in this study were performed in accordance with the King Fahad Specialist Hospital-Dammam guidelines and regulations and approved by the Institutional Review Board (IRB) under protocol number (ONC0340). Patient demographic and clinicopathological information was obtained from the hospital and clinical information system (MedicaPlus) and the local cancer registry. The archived formalin-fixed paraffin-embedded (FFPE) cancer tissues were collected from the pathology department at KFSH-D. The collected tumor tissues were resected from ovarian cancer patients before any neoadjuvant therapy. Among the ninety-five patients with ovarian tumors, thirty-four patients with germ cell tumors were eligible for this study. All hematoxylin and eosin-stained sections were investigated and classified by two specialized pathologists according to the World Health Organization (WHO) classification. Staging assessments followed the Tumor-Node-Metastasis (TNM) and the International Federation of Gynecology and Obstetrics (FIGO) staging[23]. FFPE tumor blocks were sectioned for immunohistochemistry and gene sequencing.

Immunohistochemistry (IHC)

IHC was performed using 4 µm-thick sections. For analysis of PD-L1, mouse monoclonal anti-PD-L1 primary antibody (Dako; PD-L1 IHC 22C3 pharmDx) was used with the Dako autostainer/PTLink in accordance with the manufacturer's instructions. CD44 was detected using a Ventana Benchmark Autostainer, an iVIEW DAB detection kit (Roche, Switzerland), and an anti-CD44 (SP37) rabbit monoclonal primary antibody as instructed.

Manual IHC experiments were performed to determine the expression of tumor-infiltrating lymphocytes and other stem cell markers. Briefly, sectioned tumors were deparaffinized using xylene and rehydrated with graded ethanol. The tumor sections were heated for antigen retrieval using PT LINK (DAKO) with the epitope retrieval solution PH9 (Leica Biosystems, Germany). Before the introduction of primary antibodies, the sections were first incubated with a peroxidase inhibitor and treated with Novocastra protein blocking solution (Leica Biosystems). Anti-CD8 (clone 4B11) and anti-CD4 (clone 4B12) mouse monoclonal antibodies (Leica Biosystems) were used at a 1:100 dilution to test for TILs. For cancer stem cell markers, anti-ALDH was used at a 1:200 dilution, and the anti-LGR5 antibody was used at a 1:150 dilution (Invitrogen, CA). An anti-OCT3 antibody (1:200 dilution) and an anti-CD117 antibody (CKIT) (1:100 dilution) were used (Leica Biosystems). A Novolink TM Max DAB (Polymer) kit (Leica Biosystems) was used to measure protein expression, and a Ventana Slide scanner (Roche) was used to visualize any immunoreactivity.

Immunohistochemical protein expression was defined as the degree to which a protein was expressed in the tissue relative to the whole tissue. For PD-L1 expression, only membranous staining was considered, and the tumor proportion score (TPS) was calculated as the percentage of tumor cells. PD-L1 expression in tumor tissue was determined according to the following values: $< 1\%$ (negative) and $> 1\%$ (positive). All sections presenting a percentage less than 1% were considered PD-L1 negative; therefore, any section with a percentage greater than 1% positivity was considered PD-L1 positive. CD8 and CD4 were considered positive (high) when they were expressed in more than 50% of the TILs and negative (low) when they were expressed in less than 50% of tumor cells. CD44 was also considered positive (high) when more than 50% of the tumor cells were positive and negative (low) when less than 50% of tumor cells were expressed CD44. In contrast, the positive threshold for the remaining cancer stem cells markers was 10% or greater expression in the tumor cells.

Next-generation sequencing (NGS)

The NGS experiments were performed following the manufacturer's guidelines and as described previously[24]. Briefly, DNA was extracted from the FFPE sections for OVGCT using an Ion AmpliSeq direct FFPE DNA kit (Thermo Fisher, MA). For library preparation, 10 ng of DNA from each sample was loaded into the Ion Chef System using the AmpliSeq Kit for Chef DL8 (Thermo Fisher, MA) to automatically construct the DNA. Real-time PCR was used to assess the quality of the DNA. DNA sequencing was performed using an Oncomine Comprehensive Assay v3 kit and an Ion GeneStudio S5 Plus System (Thermo Fisher, MA) following the manufacturers' guidelines. Variant alignment and variant calling were performed using Torrent Suite software. The variant annotations and filtrations were performed using Ion Reporter (Software 5.18. 4, Thermo Fisher, MA) and the open-source database Varsome (varsome.com).

The clinical and genetic data for 149 patients from the TGCT study were collected from The Cancer Genome Atlas (TCGA) through the Genomic Data Commons Data Portal and from cBioPortal. The data were analyzed using cBioportal (cBioportal.org) and Gene Expression Profiling Interactive Analysis (GEPIA, gepia.cancer-pku.cn/).

Statistical analyses

GraphPad Prism 9 Software and IBM SPSS Statistics (version 29.0) were used to perform the statistical analyses. Clinicopathologic variables were assessed using Pearson's chi-square test with Fisher's exact test for categorical variables. Overall survival (OS) was measured statistically using the Kaplan–Meier method. TCGA data for TGCTs were analyzed using the Kruskal–Wallis test to determine mutation count and tumor mutational burden (TMB). Gene expression correlations for stem cell biomarkers, TILs and PD-L1 were identified using GEPIA with Pearson correlation analysis and ANOVA for normalized gene expression.

Results

PD-L1 expression in germ cell ovarian cancer

To investigate the expression levels of PD-L1 (CD274) in germ cell tumors, we analyzed datasets for TGCTs and epithelial ovarian cancer (OV) from the TCGA dataset. We found that the germ cell tumor samples highly expressed PD-L1 compared with the epithelial ovarian cancer samples (Fig. 1A). To confirm our gene expression findings from the TCGA dataset, we performed immunohistochemistry experiments for all different types of ovarian cancer. We found that compared with other types of ovarian cancer, ovarian germ cell tumors present a very high percentage (70%) of PD-L1 (Fig. 1B).

To investigate which histological subtypes of germ cell tumors express the highest level of PD-L1, we performed immunohistochemistry on all the different types of ovarian germ cell tumors. Immunohistochemistry analysis revealed that dysgerminoma, immature teratoma, choriocarcinoma, and mixed germ cell tumors expressed PD-L1. Interestingly, dysgerminoma tissues significantly expressed PD-L1 compared with other subtypes, whereas neither yolk sac tumors nor mature teratomas expressed PD-L1 ($P<0.001$) (Fig. 2A and B). Although we found that a high percentage of mixed germ cell tumors were PD-L1 positive, the PD-L1 expression in mixed germ

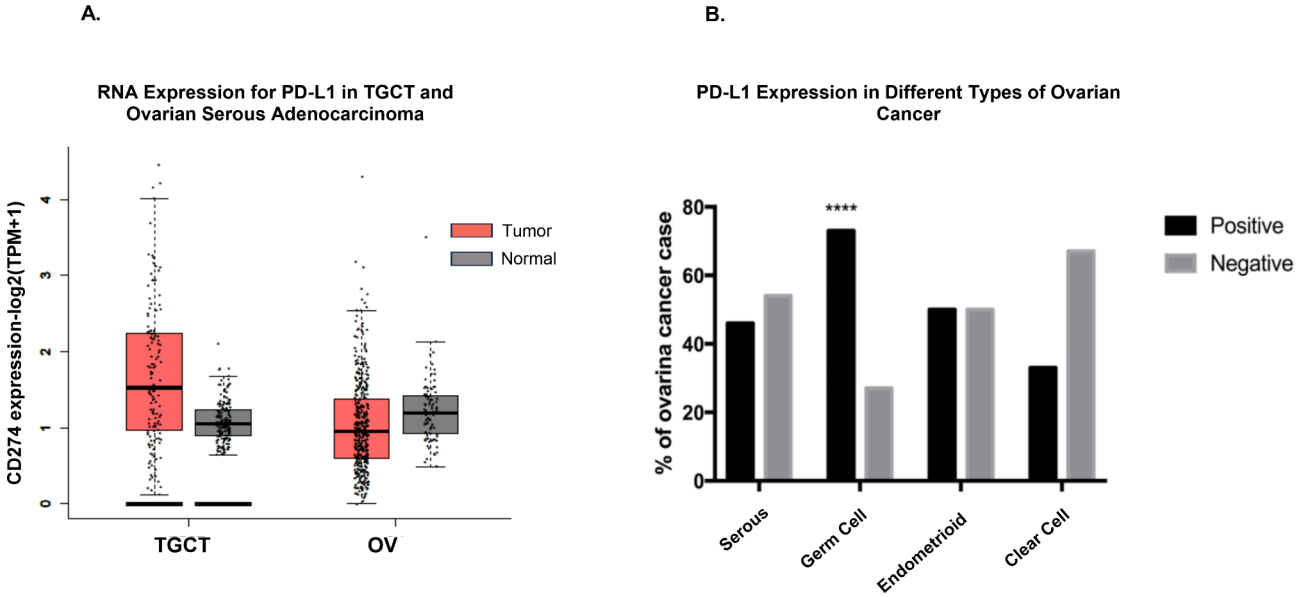

Fig. 1. PD-L1 expression in germ cell tumors. (**A**) A box plot showing the analysis of TCGA data showing the greater expression of PD-L1 in testicular germ cells (TGCTs) than in epithelial ovarian cells (OVs) for both normal and tumor tissues. (**B**) A bar chart showing high expression of PD-L1 in ovarian germ cells compared to other ovarian cancer subtypes. $P=0.001$. Transcripts per million (TPM).

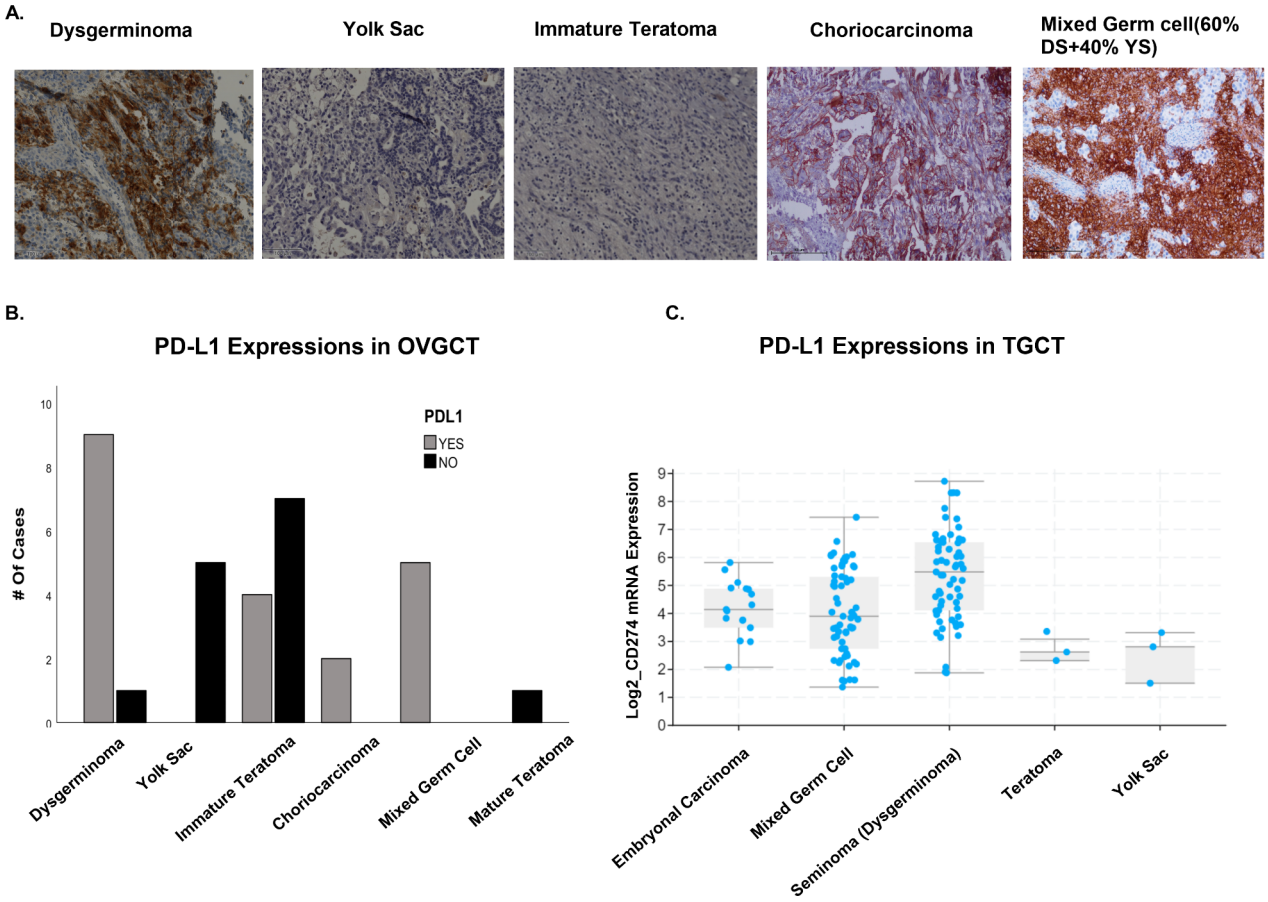

Fig. 2. PD-L1 expression in different types of germ cell tumors. (**A**) Immunohistochemical staining showing PD-L1 expression in different types of ovarian germ cell tumors. (**B**) Quantification of PD-L1 expression in each type of ovarian germ cell tumor. (**C**) TCGA data analysis of testicular germ cell tumors revealed high expression of the gene encoding PD-L1 in seminoma (dysgerminoma) and mixed germ cell tumors and low expression in the yolk sac.

cell tumors varied based on the tumor components. We found that only dysgerminoma and choriocarcinoma components in the mixed germ cells were positive for PD-L1, whereas the yolk sac was always negative in the mixed germ cell tumors. For immature teratomas, only one-third showed PD-L1 expression (Table 1).

To confirm our findings, we analyzed the TGCT dataset to investigate PD-L1 gene expression in all germ cell subtypes. This TGCT analysis was consistent with our findings and showed that seminoma in TGCT, the pathological counterpart of dysgerminoma in OVGCT, and mixed germ cells were the most common subtypes with high PD-L1 expression among the germ cell tumors (Fig. 2C).

PD-L1 expression and tumor-infiltrating lymphocytes

To determine whether the tumor microenvironment can affect the expression of PD-L1, we investigated tumor lymphocyte infiltration in ovarian germ cell cancers. Immunohistochemistry experiments were performed for lymphocytes that express CD8 and CD4 in PD-L1 tested tumor tissues. Our immunohistochemistry analysis of germ cell tumor sections revealed that PD-L1 expression is strongly associated with tumor-infiltrating lymphocytes, and compared with the non-dysgerminoma tissues, the dysgerminoma tissues exhibit dense lymphoid infiltration. Interestingly, PD-L1 was significantly associated with cytotoxic TILs expressing CD8 ($P < 0.001$) and was slightly more strongly associated with TILs expressing CD4 ($P = 0.07$) (Fig. 3A, B; Table 2). Interestingly, our RNA-seq analysis for TCGA data showed positive association between PD-L1 (CD274) gene and tumor-infiltrating lymphocytes genes for T cells (CD3) including cytotoxic T cells (CD8), T-helper cells (CD4), T-regulatory cells (FOXP3). However, PD-L1 (CD274) gene expression did not show significant association with lymphocytes that expressing B cell biomarkers (CD19, MS4A1), (Fig. 3C).

PD-L1 expression and stem cell biomarkers

To determine whether PD-L1 expression is associated with stem cell biomarkers, we investigated different stem cell biomarkers that are known to be associated with ovarian cancer. We performed immunohistochemistry experiments for embryonic stem cell biomarkers and adult cancer stem cell biomarkers. Immunohistochemical

	Sample *n* (%)	PD-L-1 *n* (%)		*p*-value
		Yes	No	
Total samples	34	20 (58.8)	14 (41.2)	
Age at diagnosis[1]				0.2
1–10	2 (8.6)	1 (3)	1 (3)	
11–20	14 (43.7)	11(34)	3 (9.3)	
21–30	12 (37.5)	6 (18.7)	6 (18.7)	
31–40	2 (6.2)	0 (0)	2 (6)	
41>	2 (6)	2 (12.5)	0 (0)	
Tumor stage [2]				0.2
Stage I	6 (42)	4 (28.5)	2(14)	
Stage II	2 (14)	2 (14)	0 (0)	
Stage III	4 (28.5)	2(14)	2 (14)	
Stage IV	2 (14)	0 (0)	2 (14)	
Tumor grade [3]				0.5
G1	4 (22)	2 (11)	2 (11)	
G2	6 (33)	4 (22)	2 (22)	
G3	8 (44)	3 (16)	5 (27)	
Histological type				
Dysgerminoma	10 (29.4)	9 (26.5)	1(2.9)	0.001*
Yolk Sac	5 (14.7)	0(0)	5 (14.7)	
Immature Teratoma	11 (32.3)	4(11.8)	7 (20.5)	
Choriocarcinoma	2 (5.9)	2 (5.9)	0 (0)	
Mixed Germ Cell Tumor	5 (14.7)	5 (14.7)	0 (0)	
Mature Teratoma	1 (2.9)	0(0)	1 (2.9)	
Total	34	20 (58.8)	14 (41.2)	

Table 1. The association of PD-L-1 expressions and the clinicopathological characteristics. [1]2 observations were missing. [2]20 observations were missing. [3]16 observations were missing.

analysis of PD-L1-positive dysgerminomas also revealed positive staining for the stem cell markers OCT3, CD44, LGR5, and CD117 (Fig. 4A and B).

Immunohistochemical analysis of stem cell biomarkers associated with all types of OVGCTs revealed that CD44 was expressed in 68% of the PD-L1-positive cancer tissues. Interestingly, all LGR5-positive tissues also expressed PD-L1 on the cells, and none of the LGR5-negative tissues expressed PD-L1. However, our statistical analysis revealed that PD-L1 expression was significantly associated with the embryonic stem cell biomarker OCT3/4 ($P<0.001$) but was not significantly associated with the adult stem cell biomarkers CD44 ($P=0.2$), LGR5 ($P=0.07$) and ALDH2 ($P=0.1$) (Table 3).

Due to the small number of samples and to confirm our results, we analyzed the gene expression of stem cell biomarkers from the TCGA dataset. The correlation between the gene expression of PD-L1 (CD274) and other stem cell markers showed that PD-L1 was positively associated with the most adult cancer stem cell markers including CD44, Aldh2, Kit (CD117), Pouf (OCT3) and Prom1 (CD133) (Fig. 4C).

Next-generation sequencing of germ cell tumors and somatic mutations

To confirm whether germ cell tumors with high PD-L1 expression also have high levels of genetic mutations, we analyzed the TCGA dataset for TGCTs. Our analysis showed that the mutation count and TMB are greater in seminomas (the testis counterpart of ovarian dysgerminoma) and mixed germ cells than in other subtypes. Furthermore, the microsatellite instability (MSI) score was higher in seminomas than in other germ cell subtypes (Fig. 5A).

To determine whether somatic mutations affect the protein expression of PD-L1, stem cell biomarkers, and TILS proteins in the tumor microenvironment, we performed next-generation sequencing of three different types of germ cells via the Oncomine panel. These include dysgerminomas with the highest PD-L1 expression, PD-L1-negative yolk sacs, and PD-L1-negative immature teratomas. We found that there was a greater percentage of somatic variants in dysgerminomas than in other types of PD-L1-negative germ cell cancers (Fig. 5B). This finding explains the high levels of lymphocyte infiltration. The variant predictive analysis showed that non-sense variants, such as variants in SETD2, CDK12, ATR, and NF1 and one splice variant in CREBBP, occurred in dysgerminomas. However, we found that the yolk sac and immature teratomas acquired variants in MTOR and NRAS with an unknown impact (Fig. 5B).

Survival and PD-L1 expression

The overall survival analysis for all subtypes of ovarian germ cells did not reveal a significant difference between PD-L1-positive and PD-L1-negative patients ($P=0.4$) (Fig. 6A). However, our analysis of patients with cancer

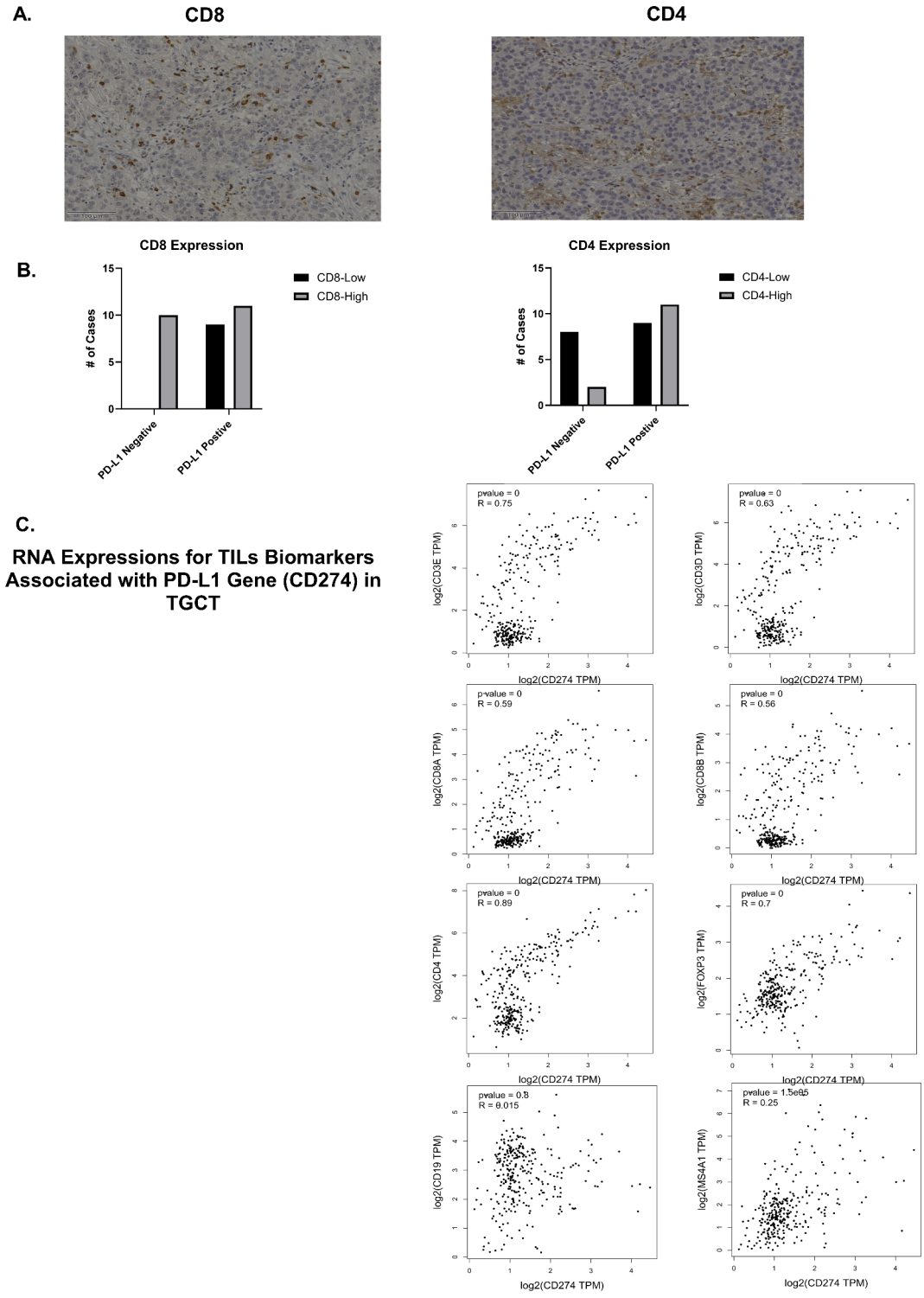

Fig. 3. Immunohistochemical staining and analysis of PD-L1-positive and PD-L1-negative TILs. (**A**) A bar chart and immunohistochemical staining showing high numbers of tumor-infiltrating lymphocytes expressing CD8 in PD-L1-positive ovarian germ cell tumors. (**B**) A bar chart and immunohistochemical staining showing high levels of tumor-infiltrating lymphocytes expressing CD4 in PD-L1-positive ovarian germ cell tumors compared to that in PD-L1-negative tumors. (**C**) RNA-seq data analysis of TCGA data showing the significant gene expression correlation between PD-L1 (CD274) and tumor-infiltrating lymphocytes markers including cytotoxic T cells (CD8), T-helper cells (CD4), T-regulatory cells (FOXP3) and not significant for B cells (CD19, MS4A1). Scale bar, 100 µm.

PD-L-1	CD8 *n* (%)			CD4 *n* (%)		
	30			30		
Total samples	Low	High	p-value	Low	High	p-value
PD-L1 Negative	0 (0)	10 (33)	0.003*	8(47%)	2(15.4%)	0.07
PD-L1 Positive	9 (30)	11 (36.6)		9 (53%)	11(64%)	

Table 2. Association of PD-L-1 with tumor infiltrating lymphocytes expressions.

and recurrence status revealed that 66.7% of the patients who were alive were PD-L1-positive, while 33.3% were PD-L1-negative (33.3%), and a high percentage of PD-L1-positive patients were tumor-free for ten years (66.7%) and recurrence-free (57%) (Fig. 6B).

Interestingly, our ten-year survival analysis for germ cell tumors subtype revealed that patients with seminoma (dysgerminoma), which is characterized by the highest expression of PD-L1, had better survival rate compared to non- seminoma (dysgerminoma) in TGCTs. However, because of the small sample size of OVGCTs, dysgerminoma tumors show only slight increase in survival compared to non- dysgerminoma in OVGCT (Fig. 6C and D).

Discussion

Although studies have shown that PD-L1/PD-1 inhibitors for immunotherapy are effective for numerous cancer types, such as melanoma and lung cancer[20,25,26], the use of PD-L1/PD-1 inhibitors for ovarian cancer treatment, particularly for germ cell tumors, remains controversial[11,17]. PD-L1 expression has been studied among different tumor types, including ovarian cancers; however, studies investigating PD-L1 and the tumor microenvironment in germ cell tumors are very rare[27,28].

Our previous study on epithelial ovarian cancer showed that PD-L1 expression was associated with favorable prognosis in patients with epithelial ovarian cancer[11]. However, another study noted increased survival in patients with high-grade serous ovarian carcinoma (HGSOC), which is associated with high levels of PD-L1 expression[29].

In this study, we investigated PD-L1 expression and the tumor microenvironment, including tumor-infiltrating lymphocytes, stem cell biomarkers, genetic alterations, and the correlation of PD-L1 with clinical outcomes in patients with ovarian germ cell tumors. Consistent with a previous study on testicular germ cell tumors[19], we demonstrated that PD-L1 was frequently expressed in dysgerminomas, choriocarcinomas, and in dysgerminoma areas of mixed germ cell tumors with an active tumor microenvironment. On the other hand, PD-L1 was not expressed in pure yolk sac tumors in any of the tested samples.

The high expression of PD-L1 in dysgerminoma and pure choriocarcinoma alone or in mixed germ cells suggests the possibility of anti-PD-L1 treatment for patients with chemotherapy-resistant tumors. The response has been described as variable in scattered case reports. Some case reports, for example, described a durable response of choriocarcinoma to anti-PD-L1 treatment[30,31], while others reported no response[32,33]. The limited antitumor activity of pembrolizumab in patients with advanced germ cell tumors was observed in clinical trials reported by Tsimberidou[32]. However, the generalizability of the results of that study is limited due to the small number of participants and the fact that the majority of the patients had mixed germ cell tumors that were treated with anti-PD-L1 regardless of its expression in the tumor cells. Nevertheless, we showed in our study that PD-L1 expression varies in each component of germ cell tumors with multiple histologic types.

In the tumor microenvironment, tumor-infiltrating lymphocytes, cancer stem cells and other factors all play important roles in the survival of cancer cells and evasion of the immune response[34–36]. The presence of CD8 + and CD4 + TILs is a prognostic factor for increased survival in many cancers, including ovarian cancer[34,37,38]. These cells aid in controlling the growth of solid tumors by recognizing cancer antigens or overexpressed self-antigens[39].

Our investigations of TILs revealed that OVGCTs that have high expression of PD-L1 also have high expression of CD8, which is expressed by cytotoxic infiltrating lymphocytes; in particular, dysgerminomas have shown enrichment of TILs expressing both CD8 and CD4. Similarly, the T-cell lymphocyte-rich microenvironment was previously documented in testicular seminomas, the counterpart of dysgerminomas[19,28].

The correlation between CD8 positivity and PD-L1 expression in the current study reflects the role of the microenvironment in regulating the expression of PD-L1. The cytokine-mediated upregulation of PD-L1 on tumor cells in inflammatory-rich tumors plays an important role in suppressing antitumor activity[40]. Furthermore, high expression of the TILs in cancer tumors indicates a good prognosis[41].

The other notable cell populations in germ cell tumors are stem cell populations. Stem cells may present different biomarkers in different tissues. Scientists are regularly investigating the role of new stem cell biomarkers that are associated with specific types of cancer[42]. We found that PD-L1 expression is associated with the cancer stem cell population in ovarian epithelial tumors[11]. Consistent with our findings, a study on breast cancers showed that high PD-L1 expression was associated with stem cells expressing CD44 and OCT3/4[43]. Although there was no significant difference in the expression of stem cell markers (CD44, LRG5, and ALDH2) or PD-L1 in our small study population of OVGCTs, the statistical analysis of TCGA data for 149 TGCT patients revealed significant differences in the expression of CD44 and LGR5 in PD-L1-positive patients. However, our investigation revealed that OCT3/4 expression was significantly associated with PD-L1 expression in patients with dysgerminoma. OCT 3/4 is known to be expressed in 100% of dysgerminomas and embryonal carcinomas[44], the more undifferentiated types of germ cell tumors, but not in yolk sac tumors[45]. OCT3/4 is a marker for normal

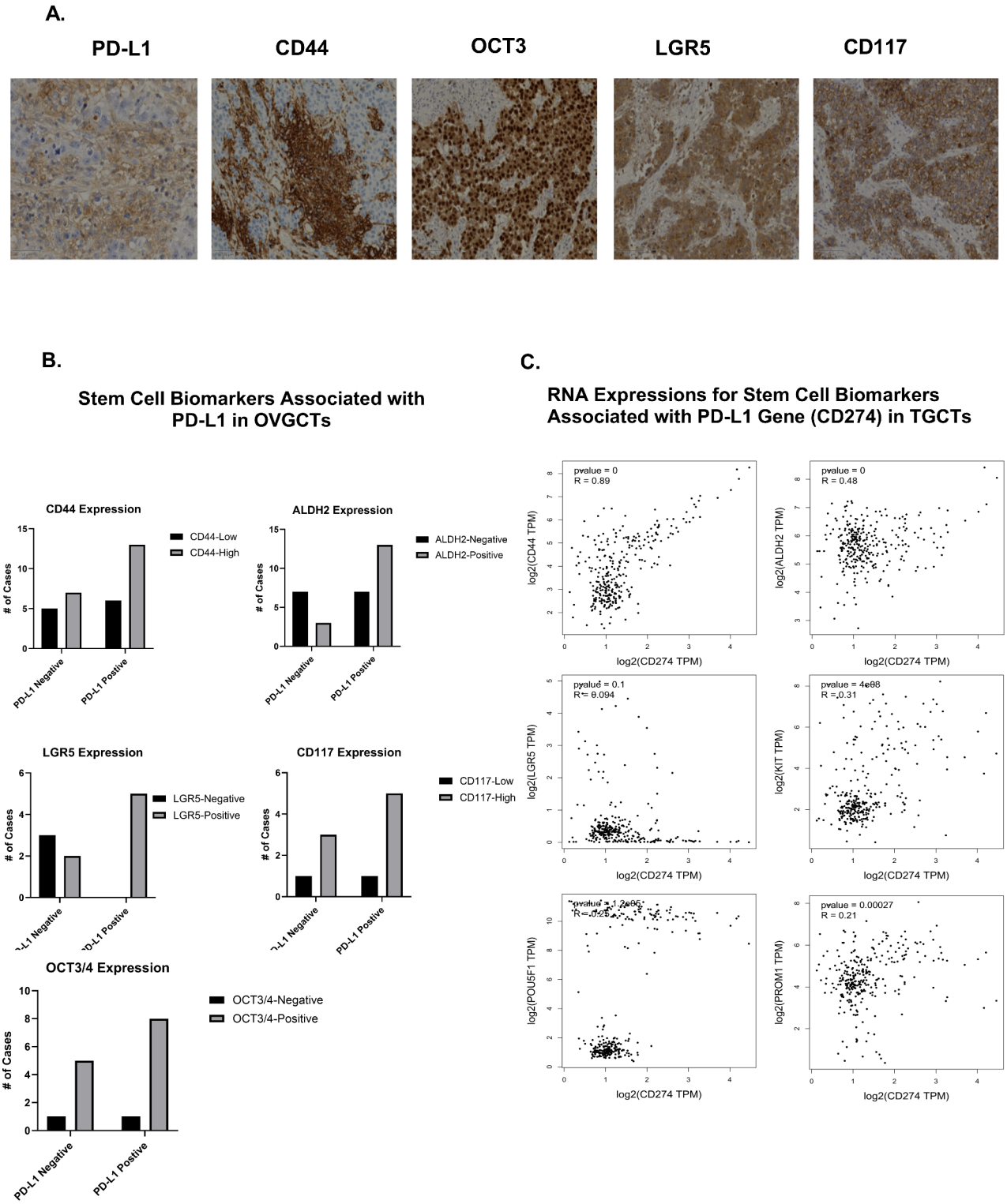

Fig. 4. PD-L1 expression associated with stem cell biomarkers. (**A**) Immunohistochemical staining of serial sections of dysgerminoma tissue showing high expression of PD-L1 and that stem cells express CD44, OCT3, LGR5 and CD117. (**B**) A bar chart shows high numbers of PD-L1-positive tissues expressing stem cell markers (CD44, ALDH2, LGR5, CD117, OCT3) compared to PD-L1-negative tissues. (**C**) RNA-seq data analysis of TCGA data showing the gene expression correlation between PD-L1 (CD274) and the stem cell markers CD44, Lgr5, Aldh2, Kit (CD117), Pou5fA (OCT3), and Prom1 (CD133). Transcripts per million (TPM), scale bar: 100 μm.

PD-L-1	CD44 *n* (%)			LGR5 *n* (%)			ALDH2 *n* (%)			OCT3/4 *n* (%)			CD117 *n* (%)		
	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value
Yes	15(46%)	5(15.6%)	0.2	5(45%)	0 (0.0%)	0.07	12(38.7%)	8 (25.8%)	0.2	9 (52%)	1 (5.8%)	0.004*	5 (45%)	1 (9%)	0.7
No	7(21.8%)	5(15.6%)		3(27%)	3(27%)		4 (12.9%)	7 (22.5%)		1 (5.8%)	6 (35%)		4 (36%)	1 (9%)	
Total	32			11			31			17			11		

Table 3. Association of PD-L-1 with stem cell expression.

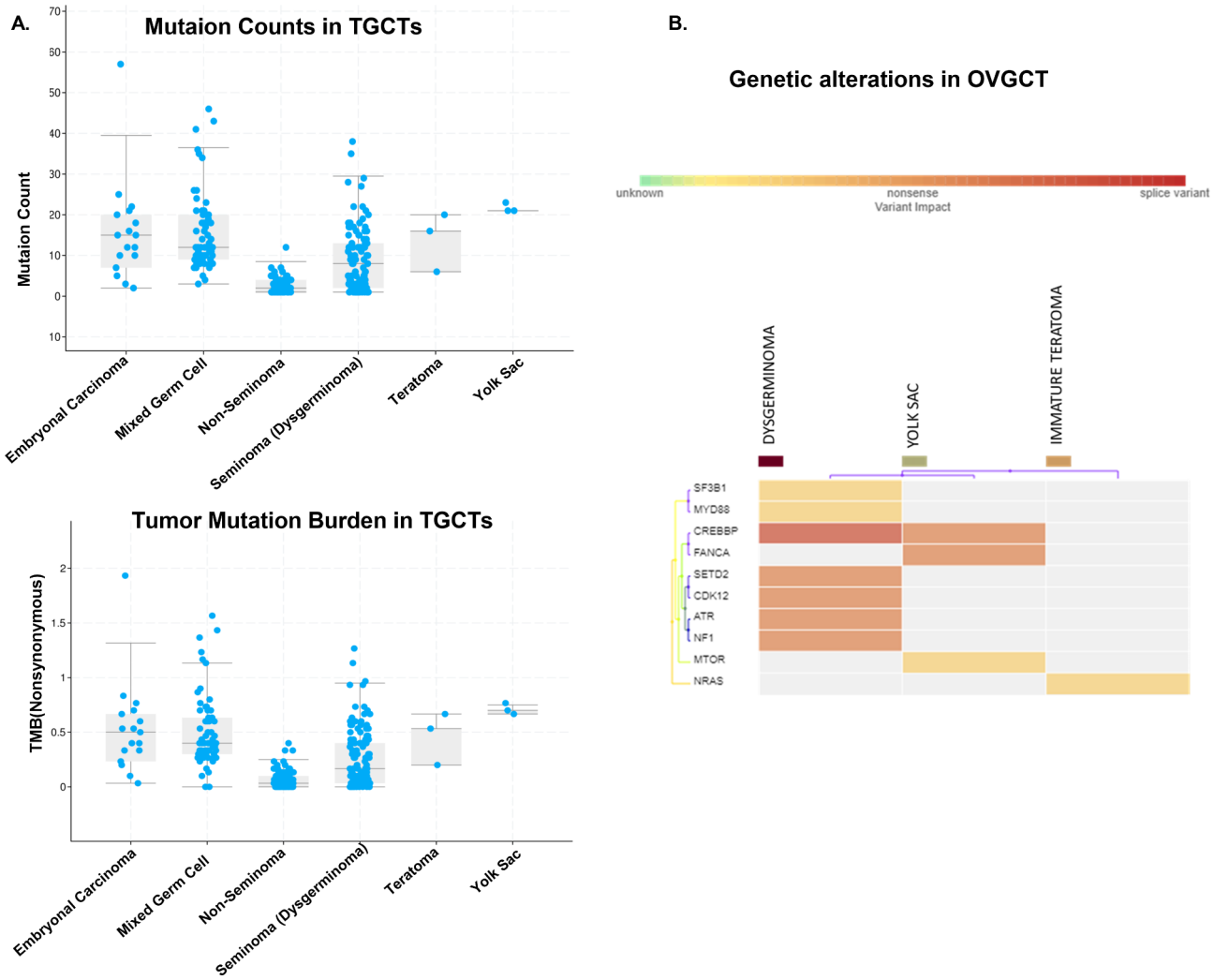

Fig. 5. Genetic alterations in PD-L1-positive and PD-L1-negative ovarian germ cell tumors. (**A**) TCGA data analysis of testicular germ cell tumors revealed high levels of genetic alterations and tumor mutational burdens in seminomas and mixed germ cell tumors. (**B**) NGS data analysis of ovarian germ cell tumors revealed higher DNA alterations in PD-L1-positive dysgerminomas than in PD-L1-negative yolk sac tumors and immature teratomas.

pluripotency of stem cells but also plays a role in maintaining the stemness of cancer stem cells[46]. A recent study revealed the role of PD-L1 in upregulating OCT3/4 in breast cancer through the PI3K/AKT signaling pathway, which indicates the role of PD-L1 in regulating stemness in cancer tissue[43]. Similarly, in TGCTs, particularly seminomas, CD44, CD117 and other cancer stem cell markers have been studied for their role in metastasis, tumor progression, and chemotherapy resistance[47]. C-KIT (CD117), which regulates germ cell differentiation, is well studied in TGCTs. Studies have shown that some OVGCTs, specifically dysgerminomas, harbor c-KIT even without mutations in the c-KIT gene. Stem cells expressing c-KIT, along with OCT3/4, might increase the survival of underdeveloped oocytes, leading to the formation of these tumors[48]. Furthermore, our study revealed that the expression of PD-L1 might be associated with stem cells in dysgerminomas. However, disruption of c-KIT pathways is an area of interest in developing new immunotherapies for germ cell tumors[49].

In addition, genetic studies were performed to link the expression of PD-L1 with genetic alterations in germ cell tumors. We found that patients with dysgerminoma, which is characterized by higher PD-L1 expression, presented a greater number of somatic genetic alterations than patients with yolk sac tumors. The infrequent expression of PD-L1 in yolk sac tumors can reflect the molecular events in yolk sac tumors, which have been reported to have a low tumor mutational burden. A molecular study of ten ovarian yolk sac tumors revealed that all the tumors were microsatellite stable and exhibited a low mutational burden[50,51]. Furthermore, our investigation of TCGA data for TGCTs showed that compared with other germ cell subtypes, dysgerminomas have the highest mutation counts and tumor mutational burden. Based on these findings, we anticipate that genetic alterations influence tumor lymphocyte infiltration and increase PD-L1 expression, which might positively affect treatment response.

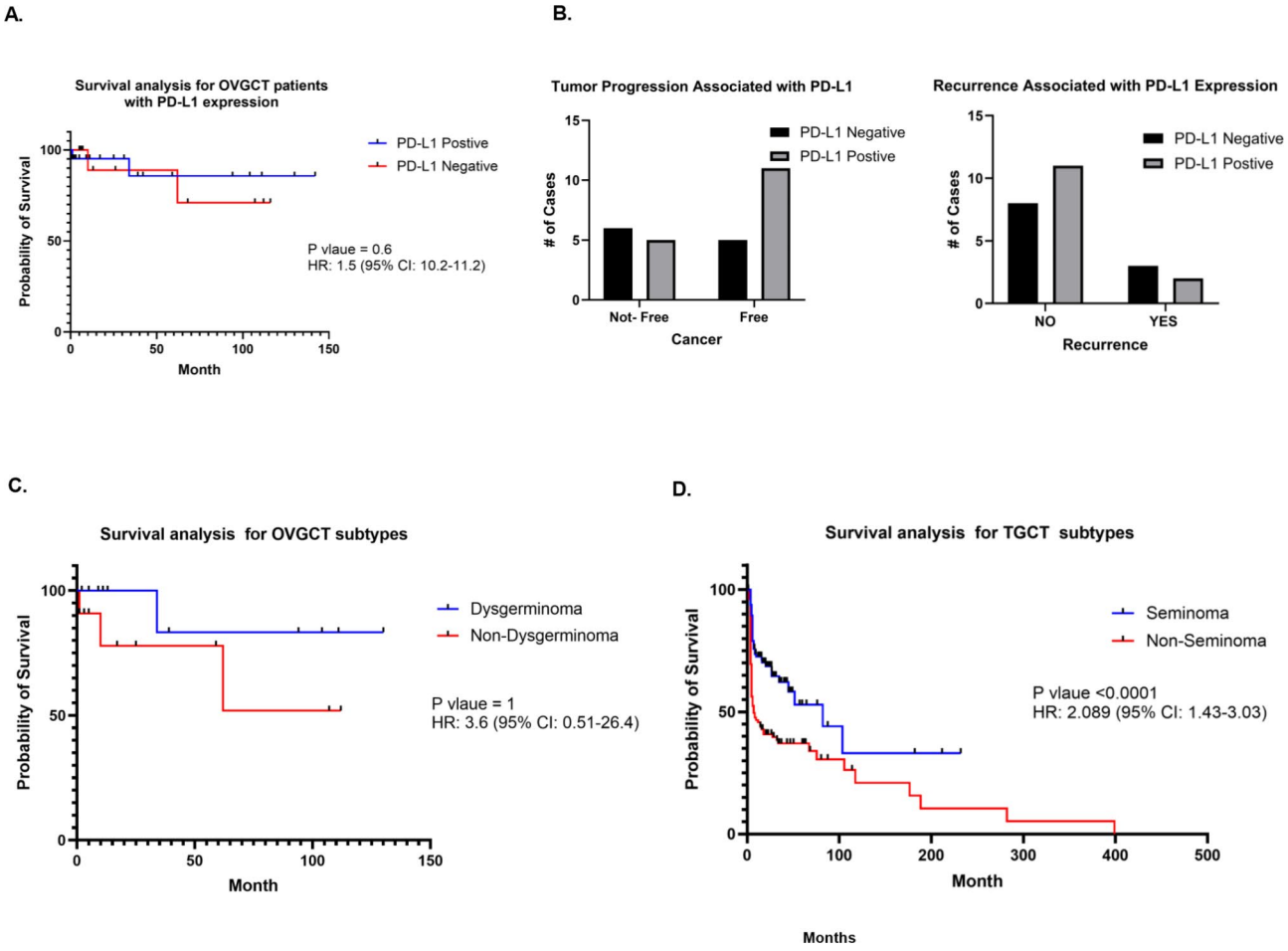

Fig. 6. Kaplan–Meier curve for the survival probability of patients with PD-L1-positive and PD-L1-negative ovarian germ cell tumors. (**A**) Survival probability curve for all types of ovarian germ cell tumors showing slight poor survival for patients with PD-L1-negative tumors. (**B**) Quantitative analysis of cancer status and recurrence in patients with and without PD-L1 expression revealed a high number of tumor-free patients and no recurrence-positive PD-L1 patients. (**C**) Survival probability for subtypes of ovarian germ cell tumors. (**D**) Survival probability for subtypes of testicular germ cell tumors show favorable survival for seminoma (dysgerminomas) vs. non- seminoma (non- dysgerminomas).

Although the statistical analysis of the overall survival of patients with PD-L1 expression in the small group was not definitive, our investigation showed that patients with dysgerminoma had the highest survival rate and lower recurrence which is consistent with the findings of a previous study[52]. In contrast, patients with non-seminomas (non-dysgerminomas) have the worst survival rate.

Like other studies, some limitations were encountered in this study. The main limitation of our study is the low number of tested samples of ovarian germ cell tumors, which is expected in rare diseases[53]. Although patients with dysgerminoma are known to have a better prognosis than patients with yolk sac, our analysis of a small population did not reveal a significant difference in 10-year survival in OVGCT. Therefore, the association between high expression of PD-L1 and patient survival in patients with dysgerminoma was not analyzed in OVGCT patients. However, our survival analysis conclusion was supported by our findings from testicular germ cell tumor data. Large populations of patients with dysgerminomas and yolk sac tumors should be studied in the future to investigate survival with and without PD-L1 expression. Furthermore, molecular genetic studies are needed in larger numbers of ovarian germ cell tumors to investigate the associations of genetic biomarkers and tumor mutational burden with the immunotherapy response in ovarian germ cell tumors.

Conclusion

PD-L1 is highly expressed in dysgerminomas and mixed cells, which have a high percentage of dysgerminomas compared to the yolk sac. PD-L1 expression is associated with high lymphocyte infiltration and cancer stem cell populations. In addition, testicular and ovarian germ cell cancer patients with high PD-L1 expression in seminomas (dysgerminomas) presented high genetic alterations. In contrast, yolk sac tumors were negative for PD-L1 expression and were associated with poor prognosis.

The PD-L1 expression, microenvironment characteristics and genetic status of germ cell tumors helped us to identify which germ cell tumor subtypes were associated with a better prognosis. Therefore, our research findings provide a set of biomarkers that are associated with PD-L1 expression and clinical outcomes in patients with ovarian germ cell tumors that can be used for risk assessment and treatment response prediction.

Data availability

All data will be available upon request from the corresponding author K.A. The archived genetic datasets that were analyzed during the current study are available through the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/projects/TCGA-TGCT) and/or cBioportal for Cancer Genomics (http://cbioportal.org).

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Author contributions
All authors have read and approved the manuscript. Conceptualization: K.A. and Z.A.; methodology, K.A., W.A. and R.A.; software, F.M.A.; validation, H.A., A.A. and K.A.; formal analysis, K.A. and F.M.A.; investigation, K.A., Z.A., W.S., F.A. and M.M.; resources, W.S., F.A. and M.M. and A.A.; data curation, Z.A., F.A., M.M. and A.A.; writing—original draft preparation, K.A.; Z.A. and R.A.; writing—review and editing, K.A. and H.A.; visualization, M.M.; supervision, K.A.; project administration, H.A.; funding acquisition, K.A. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval and consent to participate
This retrospective study was approved by the Institutional Review Board (IRB) of the King Fahad Specialist Hospital-Dammam with IRB# ONC0340. The need to consent to participate was waived by the IRB due to the use of old and archived samples.

Consent for publication
Consent for publication is not applicable.

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