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ORIGINAL ARTICLE

Distinct immunological properties of the two histological subtypes of adenocarcinoma of the ampulla of Vater

Min Hwan Kim[1,2] · Mi Jang[3] · Hoguen Kim[3] · Woo Jung Lee[4] · Chang Moo Kang[2,4] · Hye Jin Choi[1,2]

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Abstract

Adenocarcinoma of the ampulla of Vater (AOV) is classified into intestinal type (IT) and pancreatobiliary type (PB); however, the immunological properties of these subtypes remain to be characterized. Here, we evaluated the clinical implications of PD-L1 expression and $CD8^+$ T lymphocyte density in adenocarcinomas of the AOV and their potential association with Yes-associated protein (YAP). We analyzed 123 adenocarcinoma-of-the-AOV patients who underwent surgical resection, and tumors were classified into IT or PB type. Tumor or inflammatory cell PD-L1 expression, $CD8^+$ T lymphocyte density in the cancer cell nest (intratumoral) or in the adjacent stroma, and YAP localization and intensity were analyzed using immunohistochemical staining. PB-type tumors showed higher tumoral PD-L1 expression than IT-type tumors, and tumoral PD-L1 expression was associated with a shorter disease-free survival (DFS) [hazard ratio (HR), 1.77; $p=0.045$] and overall survival (OS) (HR 1.99; $p=0.030$). Intratumoral $CD8^+$ T lymphocyte density was higher in IT type than in PB type and was associated with a favorable DFS (HR 0.47; $p=0.022$). The nuclear staining pattern of YAP in tumor cells, compared to non-nuclear staining patterns, was more frequently associated with PB type and increased tumoral PD-L1 expression. Nuclear YAP staining was a significant prognostic factor for OS (HR 2.21; $p=0.022$). These results show that the two subtypes of adenocarcinoma of the AOV exhibit significant differences in tumoral PD-L1 expression and intratumoral $CD8^+$ T lymphocyte density, which might contribute to their distinct clinical features.

Keywords Adenocarcinoma of the ampulla of Vater · PD-L1 · CD8 T lymphocytes · YAP · Prognosis · Immunohistochemistry

Abbreviations

AJCC	American Joint Committee on Cancer
AOV	Ampulla of Vater
DFS	Disease-free survival
IHC	Immunohistochemical
IT	Intestinal
MDSC	Myeloid-derived suppressor cells
OS	Overall survival
PB	Pancreatobiliary
YAP	Yes-associated protein

Introduction

Adenocarcinomas of the ampulla of Vater (AOV) have cellular origins from three conjunctive epithelia of the periampullary regions: the biliary duct, pancreatic duct, and duodenal epithelium. This complex repertoire of origins results in the heterogeneous clinical attributes of adenocarcinoma of the AOV, with a wide range of survival outcomes in patients.

Parts of this paper, including Figures 1–3, Supplementary Figures 1–4, Tables 1–3, and Supplementary Tables 2, 4, and 6 were published before as a poster at the "30th EORTC-AACR-NCI symposium, molecular targets and cancer therapeutics" (EORTC-NCI-AACR 2018), 14th November 2018, Dublin, Ireland (Poster number 217, see European Journal of Cancer, November 2018, Volume 103, Supplement 1, e77).

Min Hwan Kim and Mi Jang have contributed equally to this work.

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✉ Chang Moo Kang
cmkang@yuhs.ac

✉ Hye Jin Choi
choihj@yuhs.ac

Extended author information available on the last page of the article

The previous studies have classified adenocarcinoma of the AOV into two distinct histological subtypes: intestinal (IT) subtype and pancreatobiliary (PB) subtype [1, 2]. Studies of the clinicopathological characteristics of the two subtypes have consistently reported the distinct features of each [3–5]. The IT subtype exhibits high CDX2 and CK20 expression and favorable clinical outcomes. In contrast, the PB subtype lacks CDX2 and CD20 expression, and is positive for MUC1 and CK7 expression, displaying aggressive tumor behavior and poor prognosis [3, 4]. However, due to the low incidence of this type of cancer, little research has been done on the molecular pathogenesis of adenocarcinoma of the AOV, and patient survival is still poor in cases of advanced disease.

Recent advances in tumor immunotherapy underscore the importance of antitumor immunity in cancer treatment. The infiltration of $CD8^+$ cytotoxic T lymphocytes into the tumor microenvironment is essential for tumor cell clearance [6], and intratumoral $CD8^+$ tumor-infiltrating T cells have been associated with a favorable prognosis in many cancer types [7–9]. Despite this, a considerable subset of tumors lacks T-cell infiltration due to impairment in tumor antigen presentation or T lymphocyte recruitment [10]. Moreover, aberrant expression of PD-L1 in tumor cells enables the evasion of T-cell immune responses by inducing T-cell exhaustion [11]. The ligation of PD-1 to exhausted T cells with PD-L1 suppresses proliferation and cytokine production of T cells, and tumoral PD-L1 expression predicts patient prognosis in many cancer types [12–14]. On the other hand, anti-PD-1 blockade treatment can reinvigorate the antitumor T-cell response, and high PD-L1 expression predicts favorable therapy responses in anti-PD1 blockade therapy [15, 16]. Therefore, $CD8^+$ tumor-infiltrating T-cell density and tumoral PD-L1 expression serve as crucial prognostic markers for patient survival as well as predictive biomarkers for tumor immunotherapy responses.

The factors that mediate PD-L1 expression and $CD8^+$ T-cell recruitment to the tumor microenvironment are diverse, and the recent studies indicated Yes-associated protein (YAP) activation as an important mediator of the immune evasion processes of malignant cells [17, 18]. When upstream Hippo pathway activity is suppressed, YAP translocates into the nucleus to induce the transcription of genes that support tumorigenesis [19, 20]. YAP overexpression or nuclear localization in human tumors has been associated with poor survival outcomes and aggressive behavior of tumors [21, 22]. Moreover, the recent evidence suggests that YAP activation in tumor cells recruits myeloid-derived suppressor cells (MDSCs) and induces PD-L1 expression to suppress the antitumor immune response [23–25]. Since the previous studies have reported significant YAP activation in cholangiocarcinoma [26] and pancreatic cancer [27], we suggest that YAP may also play a crucial role in the tumor immune response in adenocarcinomas of the AOV.

The previous studies have reported intratumoral $CD8^+$ T lymphocytes and PD-L1 expression in biliary tract cancer and pancreatic cancer tumors [28, 29]. However, PD-L1 expression and $CD8^+$ lymphocyte infiltration status in adenocarcinoma-of-the-AOV patients, according to tumor subtype and their implication in patient prognosis, have not been studied before. In addition, the involvement of YAP in the antitumor immune response has not been studied in adenocarcinoma of the AOV. In this study, we comprehensively analyzed PD-L1 expression and $CD8^+$ T lymphocyte density as well as their relationship with the YAP staining pattern in surgically resected adenocarcinoma-of-the-AOV tumors by immunohistochemical (IHC) staining. We demonstrate the distinct immunological character and YAP activity of the two subtypes of adenocarcinoma of the AOV; these may serve as useful biomarkers for patient treatment during surgery, systemic chemotherapy, and immune checkpoint blockades.

Materials and methods

Study population

This study analyzed a consecutive cohort of adenocarcinoma-of-the-AOV patients who underwent surgical resection for primary tumors with curative intent at Severance Hospital between January 2005 and November 2012. A total of 130 patients with carcinoma of the AOV were recruited in the study cohort. We included only tumors with their epicenter located in the "ampulla of Vater" (defined as the junction of duodenal and ampullary mucosa) on microscopic examination [30, 31]. Other periampullary cancers, distal common biliary duct cancer, pancreatic head cancer, and duodenal cancer were excluded from this study. After histological review of their tumors, only patients with a confirmed pathologic diagnosis of adenocarcinoma were selected, resulting in the exclusion of seven patients who received an alternative pathologic diagnosis. Therefore, the final analysis cohort included 123 adenocarcinoma-of-the-AOV patients with clinical follow-up data and tumor tissue for IHC analysis. The clinicopathologic and survival outcome data of the patients were obtained from their diagnosis at Severance Hospital. Tumors were staged according to the criteria of the Seventh American Joint Committee on Cancer (AJCC) staging system.

Pathology review

We retrieved formalin-fixed paraffin-embedded tissue of surgically resected primary tumors for each patient within the cohort; 4-μm-thick tissue sections were obtained on adhesive slides using a microtome. Hematoxylin-and-eosin staining

was performed for pathological review. The diagnosis of adenocarcinoma of the AOV was verified in all the cases, and tumors were further classified as either PB type or IT type based on an examination of the hematoxylin-and-eosin-stained sections. In this study, all pathological examinations were performed using tumor sections generated by complete paraffin cut, not using tissue microarray, for reliable classification of subtypes in tumors with mixed features. IT type was defined by the dominance of columnar epithelial cells with elongated pseudostratified nuclei, and PB type was defined by cuboidal to low columnar cells with round nuclei which formed a single layer of glands surrounded by desmoplastic stroma. For tumors with mixed features, the proportion of IT type and PB type in the tumor was measured, and the subtype was determined according to the predominant pathologic feature. Tumor gross findings, differentiation grade, and invasion pattern were thoroughly reviewed in all cases. All pathologic reviews and analyses of IHC staining were performed by those blinded to the clinical data, and a consensus was reached for every discordant interpretation by discussion between the evaluators.

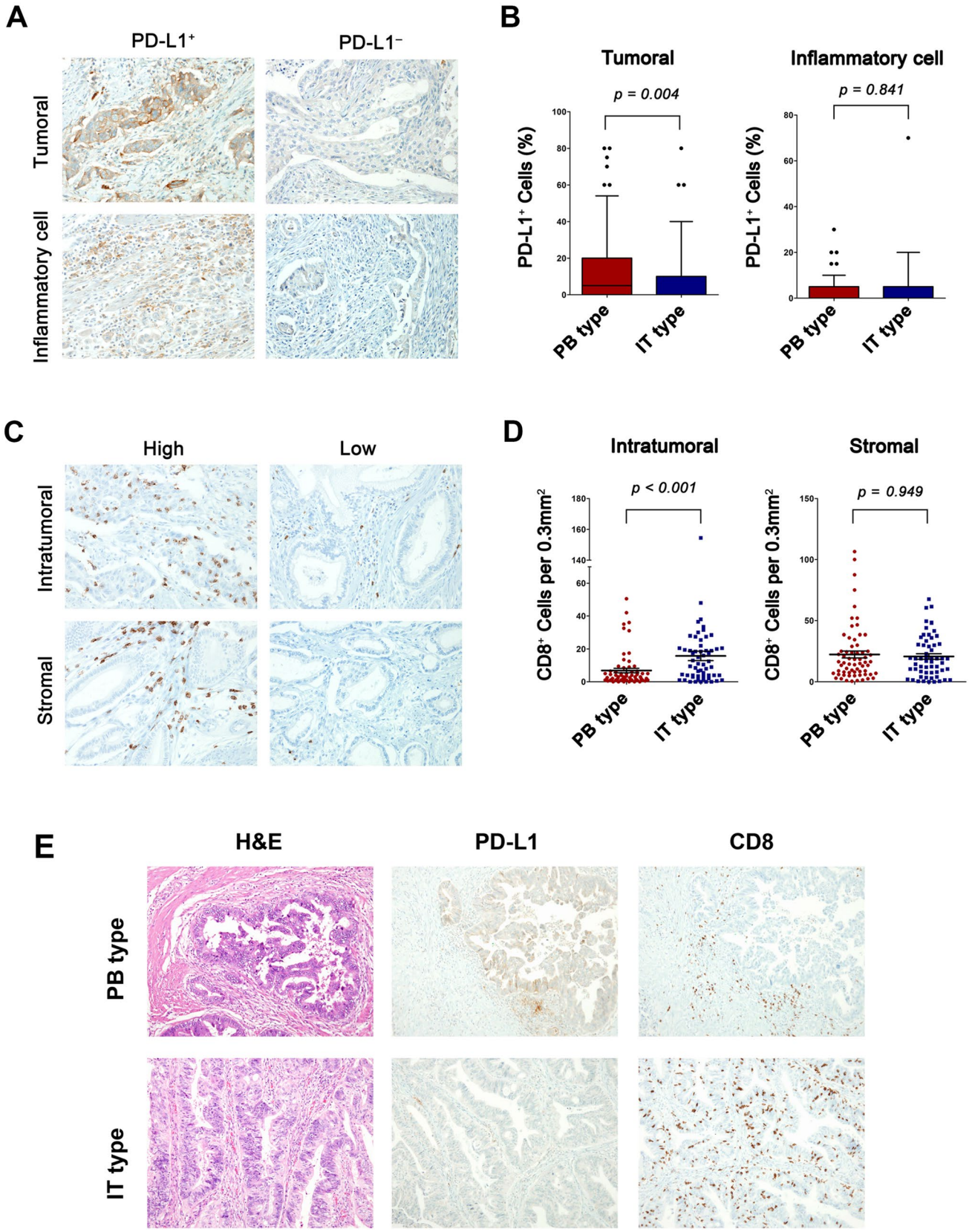
IHC analysis

Four-micrometer-thick sections of tissue blocks were deparaffinized and rehydrated with a xylene and alcohol solution. IHC staining was performed using the Ventana Benchmark XT automated staining system (Ventana Medical Systems) or Dako Omnis (Dako, Agilent Technologies) according to the manufacturer's instructions. Antigen retrieval was performed using Cell Conditioning Solution (CC1; Ventana Medical Systems) or EnVision FLEX Target Retrieval Solution, High pH (Dako, Agilent Technologies). Tissue sections were subsequently incubated with primary antibodies against CDX2 (1:400; clone EPR2764Y; Cell Marque), MUC1 (1:200; clone E29; Dako), CK7 (1:200; clone M7018, Dako), CK20 (1:200; clone M7019, Dako), PD-L1 (1:100; clone E1L3N; Cell Signaling), CD8 (prediluted; clone C8/144B; Dako), CD3 (prediluted; clone IR503; Dako), and YAP (1:200; clone 63.7; Santa Cruz). After the chromogenic visualization step using the ultraView Universal DAB Detection Kit (Ventana Medical Systems) or EnVision FLEX /HRP (Dako, Agilent Technologies), slides were counterstained with hematoxylin and coverslipped. Appropriate positive and negative controls were concurrently stained to validate the staining procedure.

For validation of the histological classification, CDX2, MUC1, CK7, and CK20 IHC staining were performed. The staining intensity was scored 0–3+, and the percentage of positive cells was measured. Consistent with a previous study [4], the tumors were classified as CDX2-positive with a cut-off H score (staining intensity x positive cell percentage) > 35, and MUC1-positive if there were any positive membranous and cytoplasmic reactivity (staining intensity ≥ 1+). CK7 positivity was determined as an H score ≥ 150 and CK20 positivity was defined as ≥ 10% of stained cells. We adopted subtype classification criteria from a previous study [32] and modified it: (1) we removed MUC2 IHC from the criteria (2) and determined MUC1(+)CDX2(+)CK7(+)CK20(−) as PB type, MUC1(−)CDX2(−)CK7(+)CK20(−) as PB type, and MUC1(−)CDX2(−)CK7(−)CK20(+) as IT type (Supplementary Table 1). PD-L1 expression in tumor cells and intercalated mononuclear inflammatory cells was independently examined, and tumors with ≥ 5% of membranous PD-L1 staining, which is a widely used cut-off of PD-L1 staining [33, 34], in each type of target cell were determined to be positive for PD-L1 expression. To measure the $CD8^+$ T lymphocyte density, $CD8^+$ stained slides were scanned (magnification 200×) with a Ventana iScan HT slide scanner (Ventana Medical Systems). The number of $CD8^+$ T cells was counted in an area of 0.3 mm^2. The $CD8^+$ T lymphocyte densities were measured independently in cancer cell nests (intratumoral) and in the adjacent stromal regions (stromal). Patient tumors were dichotomized according to intratumoral and stromal $CD8^+$ T lymphocyte density using a cut-off of ≥ 10 cells/0.3 mm^2. YAP staining was scored for YAP localization and staining intensity: localization was classified as negative, cytoplasmic, nucleo-cytoplasmic, or nuclear; intensity was scored as negative (−), one-positive (1+), and two-positive (2+).

Statistical analysis

The clinicopathologic characteristics and IHC staining scores were compared using the Chi-square or Fisher's exact test for categorical variables, Student's *t* test for continuous variables with a normal distribution, and the Mann–Whitney *U* test for continuous variables that were not normally distributed. The survival outcomes of patients were defined by calculating disease-free survival (DFS) and overall survival (OS) from their primary surgical resection. The Kaplan–Meier method and the log-rank test were used for the comparison of DFS and OS of patients. Univariate and multivariate Cox regression models adjusted for patient age, sex, and AJCC stage were estimated to test the prognostic impact of biomarkers on the survival outcomes of patients. Statistical significance was defined as a p value < 0.05 in a two-sided test. All data were analyzed using SPSS for Windows, version 24.0 (SPSS Inc). The graphs were created using GraphPad Prism (GraphPad Software).


A
PD-L1+
PD-L1−
Tumoral
Inflammatory cell
B
Tumoral
Inflammatory cell
p = 0.004
p = 0.841
PD-L1+ Cells (%)
PB type
IT type
C
High
Low
Intratumoral
Stromal
D
Intratumoral
Stromal
p < 0.001
p = 0.949
CD8+ Cells per 0.3mm2
PB type
IT type
E
H&E
PD-L1
CD8
PB type
IT type

◂**Fig. 1** PD-L1 expression and CD8$^+$ T lymphocyte density in PB-subtype and IT-subtype adenocarcinoma-of-the-AOV tumors. **a** Immunohistochemical staining for PD-L1 (×200). Tumoral PD-L1-positive and -negative tumors (upper panel) and inflammatory cell PD-L1-positive and -negative tumors (lower panel). **b** Box-and-whisker (10–90th percentile) plots of tumoral and inflammatory cell PD-L1$^+$ percentage in PB type vs. IT-type tumors. *p* values were calculated using the Mann–Whitney *U* test. **c** Immunohistochemical staining for CD8$^+$ T lymphocytes. High and low CD8$^+$ T lymphocyte density in intratumoral (upper panel) and stromal (lower panel) regions of tumors. **d** Dot plots of intratumoral and stromal CD8$^+$ T lymphocyte numbers per 0.3 mm^2 in PB-type vs. IT-type tumors. *p* values were calculated using the Mann–Whitney *U* test. **e** Representative images for the comparison of PD-L1 staining and CD8 T lymphocyte density in PB-type and IT-type tumors. PB type shows high tumoral PD-L1 expression and low intratumoral CD8$^+$ T lymphocyte density, whereas IT type shows high levels of CD8$^+$ T-cell infiltration without tumoral PD-L1 expression

Results

Patient baseline characteristics according to tumor histological subtype

A total of 123 adenocarcinoma-of-the-AOV patients who underwent primary resection of their tumors were analyzed in this study. The patients received pylorus-preserving pancreaticoduodenectomy (95.9%) or radical pancreaticoduodenectomy (4.1%), followed by adjuvant chemotherapy (37.4%), chemoradiation therapy (5.7%), or observation only (56.9%). The median follow-up duration was 71.3 months. The tumors were classified into PB type ($n=65$, 52.8%) and IT type ($n=58$, 47.2%) by histological classification (Supplementary Fig. 1 and Supplementary Table 2). The PB-type patients showed a significantly higher pathologic lymph-node stage ($p=0.036$) and poorer tumor differentiation status ($p=0.006$). The proportion of infiltrative invasion pattern was higher in PB type, whereas the proportion of expansional invasion pattern was higher in IT type ($p<0.001$). PB-type patients had significantly poorer DFS ($p<0.001$) and OS ($p<0.001$) than IT-type patients (Supplementary Fig. 2). The overall survival rates at 12, 36, and 60 months after surgery were 92.1%, 59.1%, and 46.7% in PB type, and 98.2%, 88.9%, and 79.3% in IT type, respectively.

Validation of histological subtypes by four IHC markers

CDX2, MUC1, CK7, and CK20 IHC staining were performed to validate the histological classification. Using the IHC classification criteria of tumor subtypes, the IHC subtype was determined as PB type or IT type in 119 out of 123 patients (96.7%). The IHC subtyping showed high sensitivity (95.2%) and specificity (96.5%, Supplementary Table 3) for predicting histological subtypes after the exclusion of four undetermined-type cases. For further analysis, we used tumor subtypes determined by histologic classification that were validated by IHC marker classification.

PD-L1 expression in tumor and inflammatory cells

We separately examined PD-L1 expression in tumor cells (tumoral PD-L1) and intercalated inflammatory cells (inflammatory cell PD-L1) of tumor sections. Fifty-nine (48.0%) patients were positive for tumoral PD-L1, and 47 (38.2%) patients were positive for inflammatory cell PD-L1 (Fig. 1a; Table 1). Of note, the proportion of tumoral PD-L1$^+$ tumors was significantly higher in PB-type tumors than in IT-type tumors (60% vs. 34.5%; $p=0.007$). The percentage of PD-L1$^+$ tumor cells was higher in PB-type tumors than in IT-type tumors ($p=0.004$, Fig. 1b). In contrast, inflammatory cell PD-L1 expression was not different between PB type and IT type. When tumors were classified according to CDX2 (IT-type marker) and MUC1 (PB type marker) expression, tumoral PD-L1 expression was higher in CDX2(−)MUC1(+) or CDX2(+)MUC1(+) tumors than in CDX2(+)MUC1(−) tumors (Supplementary Fig. 3a). In the subgroup analysis of PB type and IT type, the proportion of PD-L1$^+$ cells was significantly higher in CDX2(+)MUC1(+) tumors compared to CDX2(+)MUC1(−) in IT-type tumors (Supplementary Fig. 3b).

CD8$^+$ T lymphocyte density and immunological classification

The density of infiltrating CD8$^+$ T lymphocytes in the tumoral region was significantly higher in IT-type tumors than in PB-type tumors ($p<0.001$, Fig. 1c, d). In contrast, there was no difference in stromal CD8$^+$ T-cell lymphocyte density between the two subtypes. Consistent with the histologic classification, intratumoral CD8$^+$ T lymphocyte density was significantly higher in CDX2(+)MUC1(−) or CDX2(+)MUC1(+) tumors than in CDX2(−)MUC1(+) tumors (Supplementary Fig. 3c). The intratumoral CD8$^+$ T lymphocyte density was not different according to CDX2 or MUC1 expression in the subgroup analysis of PB type and IT type (Supplementary Fig. 3d). High intratumoral and stromal CD8$^+$ T lymphocyte density (cut-off: ≥ 10 cells/0.3 mm^2, CD8^{+High}) were associated with a lower T stage (tumoral, $p=0.090$; stromal, $p=0.013$), better tumor differentiation (tumoral, $p=0.016$; stromal, $p=0.059$), and a higher expansional invasion pattern proportion (tumoral, $p=0.010$; stromal, $p=0.686$) than CD8^{+Low} tumors (Table 2). In addition, the CD3$^+$ T lymphocyte density correlated well with the CD8$^+$ lymphocyte density in the tumors of patients (Supplementary Fig. 4a). Intratumoral CD3$^+$ T lymphocyte density was significantly higher in IT-type tumors than in PB-type tumors (Supplementary Fig. 4b).

Table 1 Baseline characteristics of adenocarcinoma-of-the-AOV patients according to PD-L1 expression

	Total ($n=123$)	Tumoral PD-L1			Inflammatory cell PD-L1		
		Negative ($n=64$)	Positive ($n=59$)	p value	Negative ($n=76$)	Positive ($n=47$)	p value
Age	61.15 ± 10.20	61.4 ± 9.1	60.9 ± 11.3	0.790	62.0 ± 9.6	59.8 ± 11.1	0.251
Subtype							
IT type	58 (47.2%)	38 (59.4%)	20 (33.9%)	**0.007**	37 (48.7%)	21 (44.7%)	0.713
PB type	65 (52.8%)	26 (40.6%)	39 (66.1%)		39 (51.3%)	26 (55.3%)	
Sex							
Male	67 (54.5%)	35 (54.7%)	32 (54.2%)	1.000	44 (57.9%)	23 (48.9%)	0.357
Female	56 (45.5%)	29 (45.3%)	27 (45.8%)		32 (42.1%)	24 (51.1%)	
Differentiation							
Well	46 (37.4%)	29 (45.3%)	17 (28.8%)	0.144	26 (34.2%)	20 (42.6%)	0.699
Moderate	71 (57.7%)	32 (50%)	39 (66.1%)		46 (60.5%)	25 (53.2%)	
Poor	6 (4.9%)	3 (4.7%)	3 (5.1%)		4 (5.3%)	2 (4.3%)	
Pathologic T[a]							
Tis	2 (1.6%)	1 (1.6%)	1 (1.7%)	0.395	1 (1.3%)	1 (2.1%)	0.846
T1	22 (17.9%)	15 (23.4%)	7 (11.9%)		13 (17.1%)	9 (19.1%)	
T2	44 (35.8%)	23 (35.9%)	21 (35.6%)		25 (32.9%)	19 (40.4%)	
T3	52 (42.3%)	23 (35.9%)	29 (49.2%)		35 (46.1%)	17 (36.2%)	
T4	3 (2.4%)	2 (3.1%)	1 (1.7%)		2 (2.6%)	1 (2.1%)	
Pathologic N[a]							
pN−	81 (65.9%)	46 (71.9%)	35 (59.3%)	0.183	51 (67.1%)	30 (63.8%)	0.845
pN+	42 (34.1%)	18 (28.1%)	24 (40.7%)		25 (32.9%)	17 (36.2%)	
Pathologic M[a]							
pM−	120 (97.6%)	62 (96.9%)	58 (98.3%)	1.000	75 (98.7%)	45 (95.7%)	0.557
pM+	3 (2.4%)	2 (3.1%)	1 (1.7%)		1 (1.3%)	2 (4.3%)	
Invasion pattern							
Infiltrative	53 (43.1%)	20 (31.3%)	33 (55.9%)	**0.003**	35 (46.1%)	18 (38.3%)	0.576
Expansion	66 (53.7%)	43 (67.2%)	23 (39%)		38 (50%)	28 (59.6%)	
Mixed	4 (3.3%)	1 (1.6%)	3 (5.1%)		3 (3.9%)	1 (2.1%)	

Chi-square or Fisher's exact test was used for p value calculation. For continuous variables, the data were presented as mean ± standard deviation and the Student's t test was used for p value calculation

Statistically significant values are in bold (p value < 0.05)

[a]Tumors were staged according to criteria of the Seventh American Joint Committee on Cancer (AJCC) staging system

Recent studies have proposed an immunological classification of tumors based on tumoral PD-L1 expression and intratumoral T lymphocyte infiltration [35], and the patient tumors were divided into four subgroups, accordingly. IT type presented a high proportion of PD-L1^{-}CD8^{+High} and PD-L1^{-}CD8^{+Low} (32.8% in both), and PB-type tumors showed enrichment in PD-L1^{+}CD8^{+Low} (50.8%, Fig. 1e and Supplementary Table 4).

PD-L1 expression and CD8^{+} T lymphocyte density predict patient prognosis

The tumoral PD-L1^{+} patients had significantly poorer DFS ($p=0.032$) and OS ($p=0.024$) than tumoral PD-L1^{-} patients (Fig. 2a). However, inflammatory cell PD-L1 expression did not affect the DFS and OS of patients. Regarding CD8^{+} T lymphocyte density, intratumoral CD8^{+High} patients showed significantly longer DFS ($p=0.044$), but not OS ($p=0.254$), than intratumoral CD8^{+Low} patients (Fig. 2b). A high stromal CD8^{+} T lymphocyte density was also associated with a longer DFS ($p=0.097$) and OS ($p=0.077$); however, this was not statistically significant. Univariate Cox regression analysis also revealed that tumoral PD-L1 expression and intratumoral CD8^{+} T lymphocyte density are significant prognostic factors for DFS and OS (Supplementary Table 5). In multivariate Cox regression analysis adjusted for patient age, sex, and AJCC stage, tumoral PD-L1 expression was an independent prognostic factor for DFS [Table 3; hazard ratio (HR) 1.77; 95% confidence interval (CI) 1.01–3.07; $p=0.045$] and OS (HR 1.99; 95% CI 1.07–3.72; $p=0.030$), and high intratumoral CD8^{+} T

Table 2 Baseline characteristics of adenocarcinoma-of-the-AOV patients according to $CD8^+$ T lymphocyte density

	Tumoral $CD8^+$ T lymphocytes			Stromal $CD8^+$ T lymphocytes		
	Low ($n=83$)	High ($n=40$)	p value	Low ($n=42$)	High ($n=81$)	p value
Age	61.7 ± 9.6	60.1 ± 11.4	0.407	60.7 ± 10.3	61.4 ± 10.2	0.899
Subtype						
IT type	28 (33.7%)	30 (75%)	**< 0.001**	18 (42.9%)	40 (49.4%)	0.569
PB type	55 (66.3%)	10 (25%)		24 (57.1%)	41 (50.6%)	
Sex						
Male	43 (51.8%)	24 (60%)	0.443	23 (54.8%)	44 (54.3%)	1.000
Female	40 (48.2%)	16 (40%)		19 (45.2%)	37 (45.7%)	
Differentiation						
Well	25 (30.1%)	21 (52.5%)	0.016	10 (23.8%)	36 (44.4%)	0.059
Moderate	55 (66.3%)	16 (40%)		29 (69%)	42 (51.9%)	
Poor	3 (3.6%)	3 (7.5%)		3 (7.1%)	3 (3.7%)	
Pathologic T[a]						
Tis	0 (0%)	2 (5%)	0.090	0 (0%)	2 (2.5%)	**0.013**
T1	15 (18.1%)	7 (17.5%)		7 (16.7%)	15 (18.5%)	
T2	26 (31.3%)	18 (45%)		9 (21.4%)	35 (43.2%)	
T3	40 (48.2%)	12 (30%)		26 (61.9%)	26 (32.1%)	
T4	2 (2.4%)	1 (2.5%)		0 (0%)	3 (3.7%)	
Pathologic N[a]						
pN−	56 (67.5%)	25 (62.5%)	0.685	27 (64.3%)	54 (66.7%)	0.842
pN+	27 (32.5%)	15 (37.5%)		15 (35.7%)	27 (33.3%)	
Pathologic M[a]						
pM−	82 (98.8%)	38 (95%)	0.247	41 (97.6%)	79 (97.5%)	1.000
pM+	1 (1.2%)	2 (5%)		1 (2.4%)	2 (2.5%)	
Invasion pattern						
Infiltrative	43 (51.8%)	10 (25%)	**0.010**	19 (45.2%)	34 (42%)	0.686
Expansion	37 (44.6%)	29 (72.5%)		21 (50%)	45 (55.6%)	
Mixed	3 (3.6%)	1 (2.5%)		2 (4.8%)	2 (2.5%)	

Chi-square or Fisher's exact test was used for p value calculation. For continuous variables, the data were presented as mean ± standard deviation and the Student's t test was used for p value calculation

Statistically significant values are in bold (p value < 0.05)

[a]Tumors were staged according to criteria of the Seventh American Joint Committee on Cancer (AJCC) staging system

lymphocyte density was predictive for DFS (HR 0.47; 95% CI 0.24–0.89; $p=0.022$) but not OS (HR 0.58; 95% CI 0.29–1.16; $p=0.125$).

YAP nuclear localization is associated with high PD-L1 expression and worse patient survival

The tumors with nuclear YAP staining presented a higher proportion of PB type than those with non-nuclear (negative, cytoplasmic, and nucleo-cytoplasmic) staining patterns (70% vs. 47.3%, $p=0.030$; Fig. 3a and Supplementary Table 6). Moreover, tumoral PD-L1 expression was significantly higher in adenocarcinoma-of-the-AOV tumors with nuclear YAP staining (63.3%, $p=0.038$, Fig. 3b and Supplementary Table 6), and tumors with negative YAP staining showed the lowest PD-L1 expression (31.9%). The nuclear YAP staining pattern was associated with a worse DFS ($p=0.081$) and OS ($p=0.017$) for adenocarcinoma-of-the-AOV patients (Fig. 3c, d); multivariate Cox regression analysis also showed the independent prognostic impact of nuclear YAP staining on the OS of adenocarcinoma-of-the-AOV patients with adjustments for age, sex, and tumor stage (Supplementary Table 7; HR 2.21; 95% CI 1.12–4.37, $p=0.022$). Tumor subtype, tumoral PD-L1 expression, $CD8^+$ T lymphocyte density, and patient prognosis were not affected by YAP staining intensity.

Discussion

The immunological properties of human malignancies can vary greatly according to tumor origin and histological type, and often display diverse immune cell recruitment and T

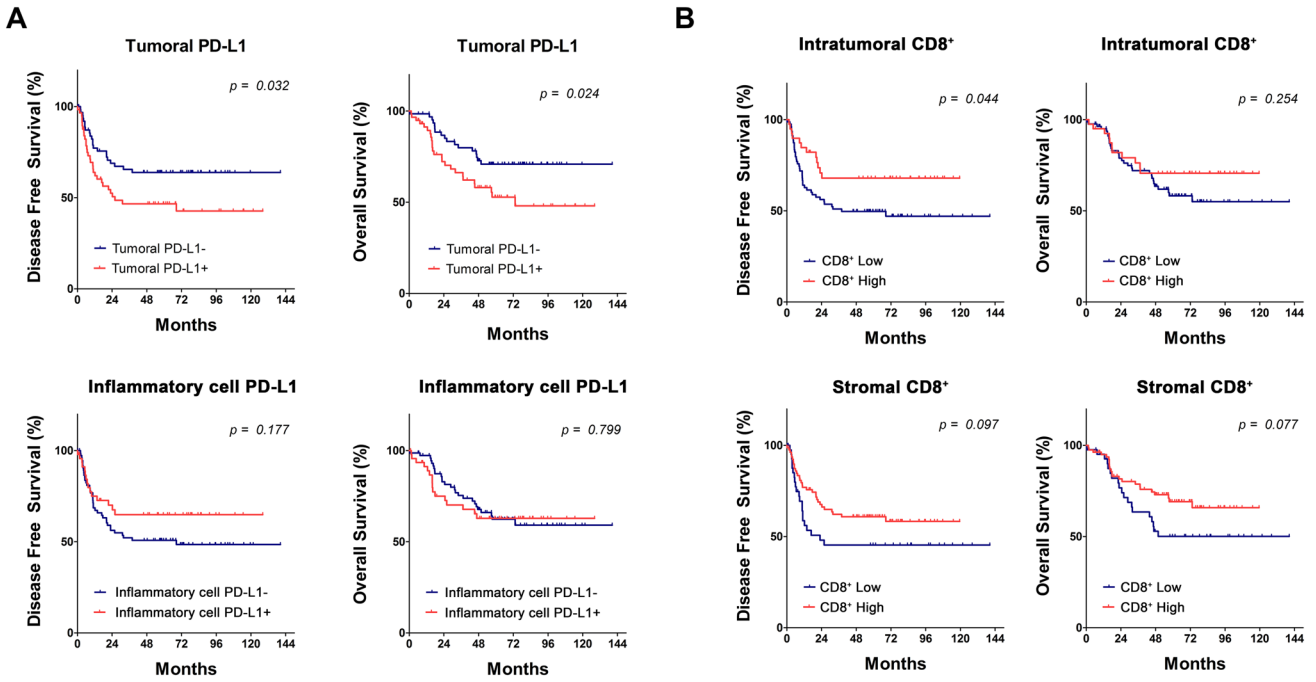


Fig. 2 Comparison of DFS and OS according to PD-L1 expression and $CD8^+$ T lymphocyte density in adenocarcinoma-of-the-AOV patients. Kaplan–Meier survival curves for DFS and OS according to **a** tumoral and inflammatory cell PD-L1 expression and **b** intratumoral and stromal $CD8^+$ T lymphocyte density. Survival was compared using the log-rank test

Table 3 Multivariate Cox regression analysis for DFS and OS of adenocarcinoma-of-the-AOV patients

Variable	Category	DFS			OS		
		HR	95% CI	*p* value	HR	95% CI	*p* value
Model I							
Age, years	Continuous variable	1.01	0.98–1.03	0.717	1.01	0.98–1.05	0.368
Sex	Female vs. male (Ref.)	1.04	0.6–1.81	0.876	0.8	0.43–1.48	0.483
AJCC stage[a]	IIB ~ IV vs. IA ~ IIA (Ref.)	5.05	2.78–9.17	**< 0.001**	5.3	2.73–10.3	**< 0.001**
Tumoral PD-L1	Positive vs. negative (Ref.)	1.77	1.01–3.07	**0.045**	1.99	1.07–3.72	**0.030**
Model II							
Age, years	Continuous variable	1.01	0.98–1.04	0.535	1.02	0.99–1.06	0.198
Sex	Female vs. male (Ref.)	1.08	0.63–1.88	0.772	0.85	0.46–1.58	0.608
AJCC stage[a]	IIB ~ IV vs. IA ~ IIA (Ref.)	5.27	2.92–9.48	**< 0.001**	5.39	2.8–10.36	**< 0.001**
Intratumoral $CD8^+$	High vs. low (Ref.)	0.47	0.24–0.89	**0.022**	0.58	0.29–1.16	0.125

Statistically significant values are in bold (p value < 0.05)

AJCC American Joint Committee on Cancer, *DFS* disease-free survival, *OS* overall survival

[a]Tumors were staged according to criteria of the Seventh American Joint Committee on Cancer (AJCC) staging system

lymphocyte marker expression patterns. This study is the first to reveal remarkable differences in PD-L1 expression and intratumoral $CD8^+$ T lymphocyte density between the two subtypes of adenocarcinoma of the AOV, which are intimately related to their tumor biology and clinical outcomes. We found significantly high tumoral PD-L1 expression and low intratumoral $CD8^+$ T lymphocyte density in PB-type adenocarcinoma of the AOV, whereas IT type showed high levels of $CD8^+$ T-cell infiltration. Tumoral PD-L1 expression and intratumoral $CD8^+$ T lymphocyte density were independent prognostic factors for patient survival, confirming the crucial roles of these markers in the pathogenesis of adenocarcinoma of the AOV. In addition, we found higher YAP nuclear localization in the PB subtype. There was a significant association between YAP nuclear localization and tumoral PD-L1 expression, suggesting that high YAP

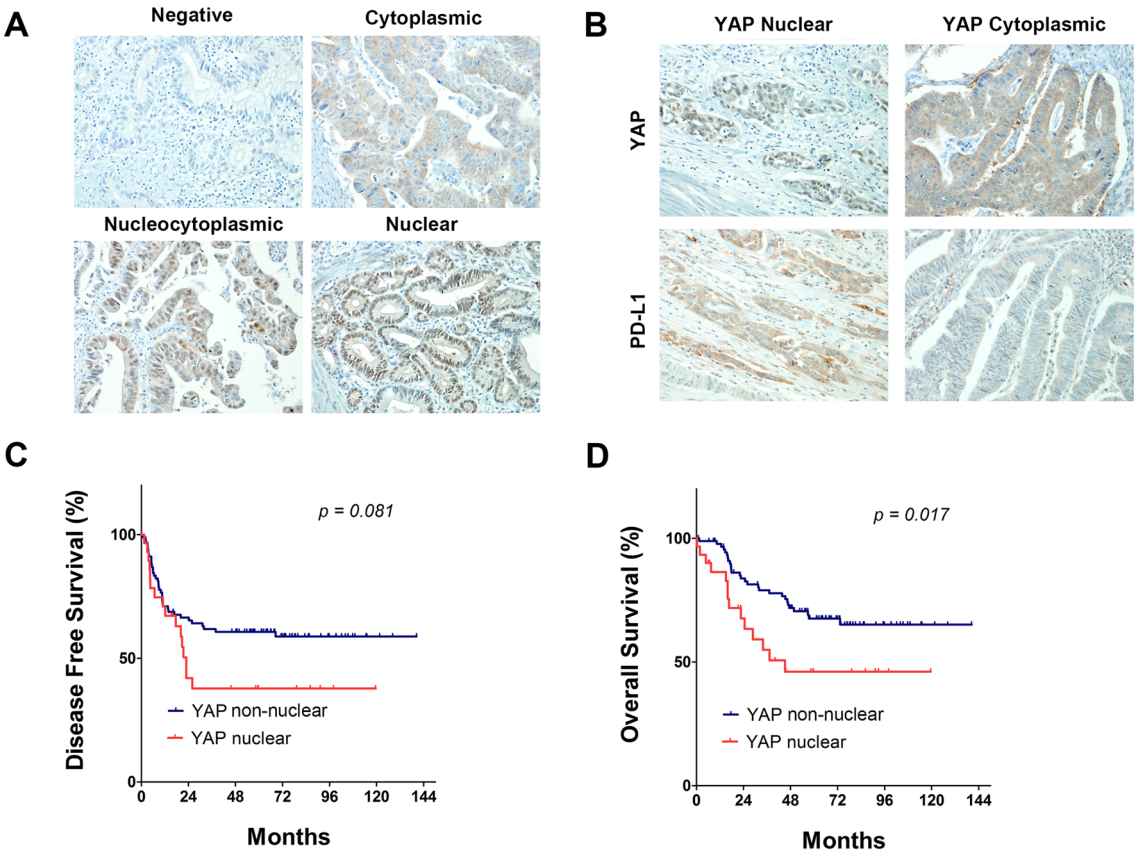


Fig. 3 Immunohistochemical staining for YAP and correlation of nuclear YAP staining pattern with PD-L1 expression and patient survival. **a** Negative, cytoplasmic, nucleo-cytoplasmic, and nuclear staining pattern of YAP in tumor cells (×200). **b** Tumoral PD-L1 expression in PB type tumors with nuclear YAP staining (left panel) and cytoplasmic YAP staining (right panel, ×200). **c** Kaplan–Meier survival curves for DFS in tumors with nuclear YAP vs. non-nuclear (negative, cytoplasmic, and nucleo-cytoplasmic) staining patterns. Survival was compared using the log-rank test. **d** Kaplan–Meier survival curves for OS according to the YAP staining pattern. Survival was compared using the log-rank test

activity in PB-subtype tumors contributes to their PD-L1 expression. Collectively, these findings provide an important clue for deciphering the distinct clinical features of the two subtypes in patients with these tumors.

In this study, we found high intratumoral $CD8^+$ T lymphocyte density in the IT subtype. This result suggests that IT-type patients are suitable candidates for tumor immunotherapy with abundant $CD8^+$ T lymphocyte recruitment in the tumor microenvironment. In contrast, low levels of $CD8^+$ T-cell infiltration in the PB subtype suggest that this subtype has features of non-immunogenic 'cold' tumors, in accordance with the previous reports on the poor immunogenicity of pancreatic cancers defined by the lack of tumor-infiltrating lymphocytes and a poor response to immunotherapy [36, 37]. The previous studies suggested that tumoral PD-L1 expression is mainly induced by interferon gamma which is secreted by tumor-infiltrating T lymphocytes. However, the PB subtype showed high PD-L1 expression despite its low level of T lymphocyte infiltration. Alternatively, tumoral PD-L1 expression can be intrinsically induced by oncogenic pathways, such as EGFR, AKT, and YAP [23, 25, 38]. Given the low level of $CD8^+$ T-cell infiltration in PB-type tumors, we suggest that PD-L1 expression in the PB subtype might be intrinsically induced by autonomous oncogene activation in tumor cells, including YAP activation. These results imply that the cellular origin has a large effect on the immunological character of adenocarcinoma of the AOV, even if they originated from the same anatomical locations. In addition, tumoral PD-L1 expression was significantly higher in CDX2(+)MUC1(+) tumors than in CDX2(+)MUC1(−) in the IT type, suggesting possible interactions between MUC1 and PD-L1 expression. However, this result should be carefully interpreted because of the limited sample size used for the subgroup analysis.

Recent clinical trials have demonstrated the antitumor activity of anti-PD-1/PD-L1 and anti-CTLA4 immune checkpoint blockades against various tumor types [16, 39]. However, the proportion of tumors responsive to the immune checkpoint blockade is limited in many cancer types, and patient selection based on reliable biomarkers

is essential. Recent studies have identified high PD-L1 expression, high $CD8^+$ T-cell infiltration, and high non-synonymous mutation burden as predictive markers for favorable anti-PD1 therapy response [15, 40]. In our study, 13.8% of adenocarcinoma-of-the-AOV patients were classified in the PD-$L1^+CD8^{+High}$ subset, and this proportion was higher in the IT type than in the PB type (19% vs. 9.2%). This result shows that a considerable portion of adenocarcinoma-of-the-AOV patients has favorable predictive markers for immune checkpoint blockade therapy. Moreover, tailored targeting strategies can be designed to enhance the efficacy of immunotherapy according to the immunological properties of the two subtypes. Since many PB-type tumors lack $CD8^+$ T-cell infiltration, it is necessary to intensify the T-cell recruitment process by T-cell adoptive transfer or combinatory cytokine treatment. For IT-type tumors, treatment needs to focus on the enhancement of the recruited effector T lymphocyte function through a combination blockade of immune checkpoint receptors (TIM-3, LAG-3, or TIGIT) or by targeting immunosuppressive cells, such as MDSCs and regulatory T cells.

Recent studies have observed that YAP and its paralog TAZ promote evasion from the T-cell immune response by recruiting type II macrophages [17] and MDSCs [18], and by inducing PD-L1 expression in cancer cells [23–25]. Our study also indicated that nuclear YAP localization correlates with increased PD-L1 expression and poor survival outcome in adenocarcinoma-of-the-AOV patients. Nuclear YAP staining was more common in PB-type than IT-type tumors, and we suggest that YAP activity plays a particularly important role in PB-type tumors inducing high PD-L1 expression. Therefore, targeting oncogenic YAP activation needs to be considered in adenocarcinoma-of-the-AOV patients to inhibit YAP-induced immune evasion and other tumorigenic processes.

Due to its rare incidence and heterogeneity, little has been revealed regarding the treatment of advanced adenocarcinoma of the AOV. Anti-PD-1 therapy has not been effective in the majority of pancreatic cancers, cholangiocarcinomas, and microsatellite stable colorectal cancers [36, 41, 42]. However, considerable PD-L1 expression and $CD8^+$ T-cell infiltration have been noted in adenocarcinoma-of-the-AOV patients by our study, and we expect that tumor immunotherapy approaches, including immune checkpoint blockade and adoptive T-cell transfer in combination with the other agents, could potently improve the survival outcomes.

This study has some limitations because of its retrospective nature. Moreover, it was conducted in a single institution with a limited patient sample size ($n = 123$). In addition, the survival outcome of patients may have been influenced by the heterogeneous adjuvant treatment modalities after surgical resection, including chemotherapy (37.4%), chemoradiation therapy (5.7%), and observation only (56.9%). Future prospective studies with larger sample sizes from multiple centers are required to validate the conclusions of this study.

In summary, we comprehensively analyzed PD-L1 expression and $CD8^+$ T-cell infiltration in a surgically resected adenocarcinoma-of-the-AOV patient cohort and found distinct immunological properties for the two subtypes. We believe our findings which enhance the current understanding of the immunological characters of the two subtypes of adenocarcinoma of the AOV and their clinical attributes. These results will be instrumental for designing optimal strategies for tumor immunotherapy in patients with this form of cancer.

Author contributions MHK, MJ, HK, CMK, and HJC conceived the study. MJ and HK performed and interpreted the pathologic review. Clinical data collection and interpretation were done by MHK, CMK, WJL, and HJC. MHK, MJ, CMK, and HJC wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

Ethical approval This study was reviewed and approved by the Institutional Review Board of Severance Hospital, Seoul, Korea (approval number: 4-2017-0542).

Informed consent Informed consent was obtained from all patients at the time of surgery for the analysis of tumor specimens used for investigations. The requirement for informed consent for the retrospective study was waived by the Institutional Review Board, because this study was performed more than 5 years after the surgery and acquisition of the tumor tissues.

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Affiliations

Min Hwan Kim[1,2] · Mi Jang[3] · Hoguen Kim[3] · Woo Jung Lee[4] · Chang Moo Kang[2,4] · Hye Jin Choi[1,2]

[1] Division of Medical Oncology, Department of Internal Medicine, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

[2] Pancreaticobiliary Cancer Clinic, Yonsei Cancer Center, Severance Hospital, Seoul, Republic of Korea

[3] Department of Pathology, Yonsei University College of Medicine, Seoul, Republic of Korea

[4] Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea