European Journal of Obstetrics & Gynecology and Reproductive Biology 240 (2019) 103–108

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Ras association domain family protein 1a hypermethylation and PD-L1 expression in ovarian cancer: A retrospective study of 112 cases

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ARTICLE INFO

Article history:
Received 6 January 2019
Accepted 5 June 2019

Keywords:
RASSF1A
Promoter methylation
PD-L1
Ovarian cancer

ABSTRACT

Objective: This study assessed the interrelationships between Ras association domain family protein 1a (*RASSF1A*) gene hypermethylation, PD-L1 protein expression, and the clinicopathological characteristics of 112 ovarian cancer (OC) samples.
Methods: Formalin-fixed paraffin-embedded OC tissue samples from surgical resection were assessed. Bisulfite pyrosequencing and immunohistochemistry were applied to detect *RASSF1A* gene methylation and PD-L1 protein expression in tumor cells, respectively. *RASSF1A* gene methylation and PD-L1 protein expression levels were analyzed against clinicopathological features and prognosis through standard statistical methods.
Results: Of the 112 OC samples, 49.1% (55/112) exhibited *RASSF1A* gene hypermethylation. The frequency of *RASSF1A* hypermethylation was significantly higher in nonserous subtype (73.0%), early stage (66.7%), and nonrecurrent OC (62.9%, $p < 0.05$). Among all samples, 61.6% (69/112) were positive for PD-L1 protein expression in tumor cells. No significant differences in PD-L1 expression were identified for age, menstrual status, histological type, tumor location, grade, stage, lymph node metastasis, or prognosis ($p > 0.05$). *RASSF1A* methylation and PD-L1 expression were not correlated ($p > 0.05$).
Conclusions: This was the first study linking *RASSF1A* hypermethylation variability to PD-L1 expression and clinicopathological characteristics of OC. Epigenetic alteration of *RASSF1A* was closely associated with nonserous subtype, early stage, and nonrecurrent OC, indicating that *RASSF1A* hypermethylation may play a role in early detection of OC. Expression of PD-L1 had no relationship with the studied clinicopathological characteristics or *RASSF1A* hypermethylation in the 112 OC samples.

Introduction

Ovarian cancer (OC) is one of the most lethal gynecological cancers. Prompt diagnosis is challenging because of the nonspecific symptoms and signs exhibited in the early stage. Although most patients initially achieve remission following combined treatment of cytoreductive surgery and chemotherapy, many relapse within 16–18 months and die from the disease [1]. To improve OC outcomes, researchers have shifted their focus to exploration of new and effective biological markers that can contribute to early diagnosis and treatment. Accordingly, mutated and methylated genes and immune checkpoint proteins have received substantial research attention.

Accumulation of genomic mutations is considered the main driver of tumorigenesis and progression [2]. With developments in oncology and epigenetics, scientists have discovered that epigenetic alterations play critical roles in the complex events of cancers, including OC [3,4]. In contrast to genetic mutations, epigenetic changes are potentially reversible, and thus are promising biomarkers that may be used in OC prevention and treatment.

DNA hypermethylation is mediated by DNA methyltransferase (DNMT) enzymes. When the 5′ carbons of cytosines are methylated within the CpG island (CpGI) of a gene, the function of the gene is changed and the CpGI is described as hypermethylated [5]. Ras association domain family protein 1a (*RASSF1A*) is a tumor suppressor gene located on chromosomal area 3p21, spanning 11,000 bp, and containing two promoters and eight exons [6]. *RASSF1A* is vital in microtubule stability and is involved in cell cycle regulation and cell migration, apoptosis, and adhesion [7]. In OC,

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https://doi.org/10.1016/j.ejogrb.2019.06.015
0301-2115/

RASSF1A is one of the most frequently hypermethylated genes [8,9]. Although *RASSF1A* methylation has been discussed in numerous studies [10], no clear association between *RASSF1A* methylation and OC clinicopathological features has been reported.

Cancers escape a host's immune attack by utilizing various signaling pathways that constitute the cancer-immune escape system. Among these pathways, the most crucial is the immune checkpoint signal referred to as the programmed death-1 (PD-1) pathway. PD-1 protein contains two ligands: PD-L1 and PD-L2 [11]. PD-L1 is considered the primary ligand of PD-1, and is expressed in not only in activated cells such as T cells, B cells, natural killer cells, dendritic cells [12] but also human carcinomas of the lungs, ovary, and colon, as well as melanomas [13]. Immune escape processes contribute to tumor genesis and progression. Therefore, blocking PD-1 signaling may be a promising and valid treatment measure for several malignancies such as melanoma, non-small-cell lung cancer, and renal carcinoma [14]. Additionally, strong evidence suggests that OCs are immunogenic tumors [15]. However, few clinical trials of immunotherapy for OCs have been performed and few studies have investigated the relationship between PD-L1 expression and OC clinicopathological characteristics. In this study, we investigated the correlation between *RASSF1A* hypermethylation and PD-L1 protein expression and their links to clinicopathological features of OC.

Materials and methods

Patient samples

A total of 112 OC patients were retrospectively selected from January 1, 2009, to December 31, 2010 at the Department of Obstetrics and Gynecology, Peking Union Medical College Hospital. The inclusion criteria were as follows: primary operable OC, no family history for breast cancer or OC, no prior chemotherapies or radiotherapies before surgery, and surgical specimens with sufficient tumor tissue. Clinicopathological information was available for all cases, including age, menstrual status, tumor location, histological subtype, tumor grade, International Federation of Gynecology and Obstetrics (FIGO) stage, lymph node metastasis (LNM), and prognosis.

DNA isolation, extraction, and pyrosequencing

Genomic DNA extraction from 112 OC samples each with a minimum of 75% malignant tumor cells was performed using the DNA Isolation Kit according to the manufacturer's instructions (QIAGEN, China). DNA concentration was detected using a Nanodrop8000 Spectrophotometer (Thermo, USA) and sodium bisulfite conversion was performed using the Epitect Fast DNA Bisulfite Kit (QIAGEN, China). Quantitative DNA methylation detection was then conducted with pyrosequencing. Segments of the *RASSF1A* from the bisulfite-converted DNA were amplified and analyzed through polymerase chain reaction (PCR) with primers, as described in Table 1. To isolate single-stranded amplicon, the reverse primer was synthesized with a biotin moiety at the 5′ terminus. Following amplification, the PCR product was mixed with Streptavidin Sepharose High Performance beads (GE Healthcare, USA) and sequence analysis was performed through pyrosequencing on a PyroMark Q24 real-time quantitative pyrophosphate sequence analyzer (QIAGEN, China). Nine CpG loci were detected on the *RASSF1A* gene and the average methylation frequency at various loci was calculated to evaluate the gene's methylation level. Hypermethylation was defined as an index of $\geq$5% [16,17].

OC tissue immunohistochemistry assays

All 112 formalin-fixed paraffin-embedded OC samples were deparaffinized and subjected to antigen retrieval in a steam cooker for 1.5 min with edetate disodium of pH 9.0 (Maixin, China). Immunohistochemistry (IHC) staining was performed using anti-PD-L1 (clone SP142, Zsbio, China) primary monoclonal antibody at room temperature for 1.5 h. Universal secondary antibody (Daco, China) was applied for 15 min. Based on previously reported methods, tumor samples were scored as 0 (no positive staining), 1+ (weak cytoplasmic or membranous staining in <10% of the positive cells), 2+ (weak-to-moderate cytoplasmic or membranous staining in $\geq$10% of the positive cells), 3+ (strong cytoplasmic or membranous staining in $\geq$10% of the positive cells). Cases were considered PD-L1 positive (2+ and 3+) when more than 10% of tumor cells were positively IHC stained (Fig. 1) [18,19].

Statistical methods

Statistical analysis was performing using SPSS software (version 18.0 Chicago, IL, USA). The association between clinicopathological parameters and *RASSF1A* promoter hypermethylation and the level of PD-L1 expression in OC cells were analyzed using Pearson's chi-square test. Disease-free survival (DFS) was defined as the period from diagnosis until first recurrence or date of death from OC, and overall survival (OS) was defined as the period from diagnosis to date of death. Estimated DFS and OS were calculated using the Kaplan–Meier curve and multivariable Cox regression methods. Pearson correlation analysis was performed to investigate the correlation between *RASSF1A* methylation and PD-L1 expression in OC cells. All p values reported were two tailed and considered significant at 5% ($p < 0.05$).

Results

Clinicopathological features of the 112 OC samples

The clinicopathological parameters, *RASSF1A* promoter methylation, and PD-L1 expression status of the 112 OC samples are summarized in Table 2. The median age of the OC patients was 53.0 years (range: 19.0–75.0 years) and 49.1% (55/112) exhibited *RASSF1A* promoter methylation. Higher frequency of *RASSF1A* hypermethylation was significantly correlated with nonserous subtype (73.0% $p < 0.01$), early FIGO stage (FIGO I+II, 66.7%, $p < 0.01$), and nonrecurrent OC (62.9%, $p = 0.05$); this implied that *RASSF1A* hypermethylation may indicate early tumorigenesis in patients with OC. Of the 112 samples, 61.6% (69/112) cases were positive for PD-L1 expression and 38.4% (43/112) cases were

Table 1
Primers of *RASSF1A* used for pyrosequencing.

No.CG	PCR Amplification Primers		Sequencing primer
	Forward	Reverse-biotin	
9	AGTTTGGATTTTGGGGGAGG	CAACTCAATAAACTCAAACTCCCC**(5'biotin)**	GGGGTTAGTTTTGTGG

PCR, polymerase chain reaction.

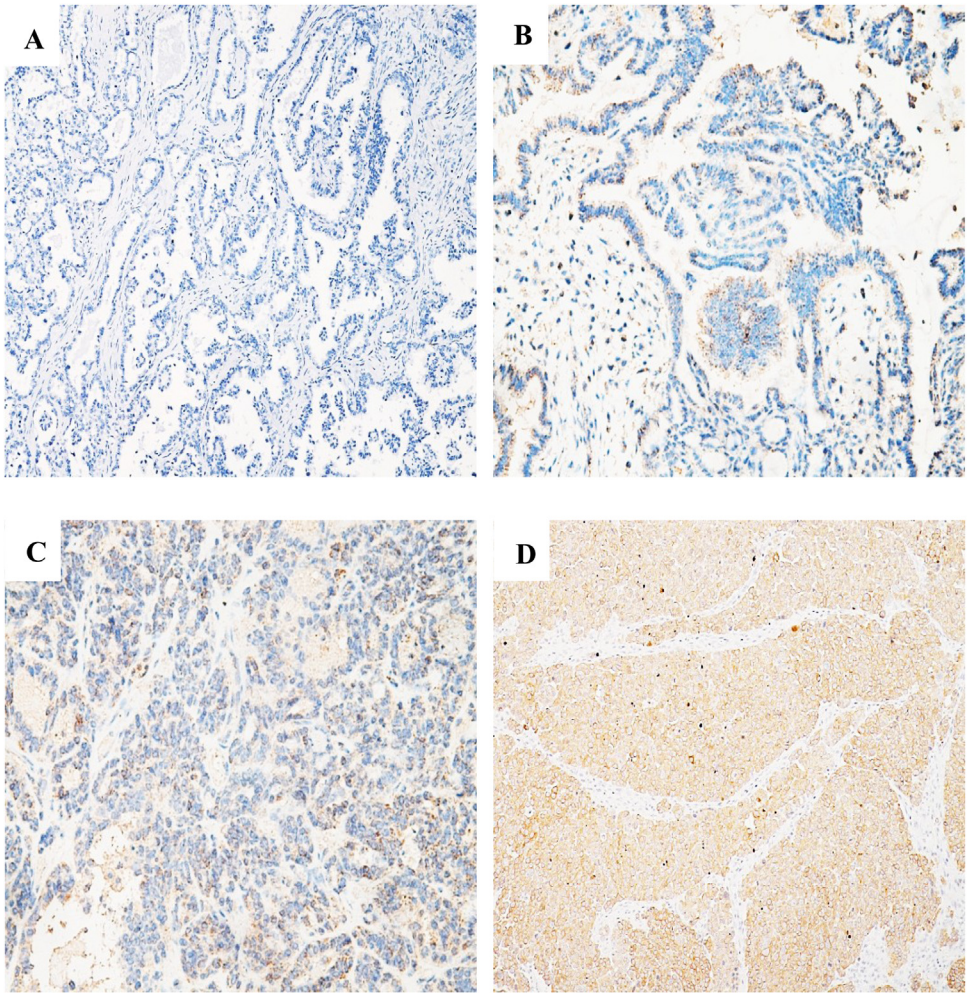

Fig. 1. Images of IHC staining of PD-L1 in OC. (A) PD-L1-negative expression in OC cells (0+, ×100); (B) PD-L1 expression scored as 1+ (×200); (C) PD-L1 expression scored as 2+ (×200); (D) PD-L1 expression scored as 3+ (×200).

negative. We did not observe a significant association between PD-L1 expression and any of the clinicopathological parameters of age, menstruation status, tumor location, histological type, tumor grade, FIGO stage, LNM, recurrence, or survival status ($p > 0.05$).

Survival analyses

Survival data was most recently updated on July 1, 2017, and median follow-up was 66.3 months (range: 0.53–101.43 months). *RASSF1A* methylation, tumor location, histological subtype, tumor grade, FIGO stage, and LNM were associated with DFS ($p < 0.05$; Table 3). Among the OC samples, low frequency of methylation, bilateral characteristics, serous subtype, high tumor grade, advanced FIGO stage, and LNM were associated with high likelihood of relapse. Moreover, advanced FIGO stage and LNM were associated with inferior OS ($p < 0.05$; Table 3). The total relapse rate of the 112 OC cases was 68.8% (77/112). The relapse rate for *RASSF1A* methylated OC was 42.9% (33/77), which was significantly lower than that for nonmethylated OC (57.1%, $p = 0.05$). According to the Kaplan–Meier analysis results presented in Fig. 2A, the median OS period for *RASSF1A* methylated cases (69.00 months) was longer than that for the nonmethylated OC cases (52.63 months); the corresponding p value was 0.054. Regarding PD-L1 protein expression, the median survival periods were 67.27 months in the PD-L1-positive group and 63.40 months in the PD-L1-negative group. No significant difference was identified between these two groups for DFS or OS ($p = 0.814$ and 0.750, respectively).

Multivariate analyses revealed that FIGO stage was independently associated with DFS ($p < 0.01$, 95% confidence interval [CI]: 3.506–17.848) and OS ($p < 0.01$, 95% CI: 4.069–17.564). *RASSF1A* methylation and PD-L1 expression in OC cells were not independent factors for the prognosis of OC ($p > 0.05$; Table 4).

Correlation between RASSF1A *promoter methylation and PD-L1 expression*

To investigate the correlation between *RASSF1A* methylation and PD-L1 expression in OC, Pearson correlation analysis was performed. The results did not indicate significant correlation ($p = 0.411$, $r = 0.078$).

Discussion

RASSF1A methylation is a common and early event in tumorigenesis that was first identified in OC samples in a study conducted in 2001 [20]. Studies have reported *RASSF1A* hypermethylation prevalence in OC as 10%–68% [20–22]. The histological heterogeneity of OC samples alongside variations in sample processing, detection methods, and cut-off values may account for the range in reported frequencies of *RASSF1A* methylation. Methylation-specific PCR (MSP) and pyrosequencing are the most common methods used to detect DNA methylation. In contrast to

Table 2
Tumor clinicopathological characteristics, *RASSF1A* methylation, and PD-L1 expression.

Clinicopathological characteristics	N	*RASSF1A* methylation N (%)		*p* value	PD-L1 expression N (%)		*p* value
		Methylated	Unmethylated		Positive	Negative	
Age (years, median)		54.0	53.0		53.0	54.0	
≤53	57	25 (43.9%)	32 (56.1%)		38 (66.7%)	19 (33.3%)	
>53	55	30 (54.5%)	25 (45.5%)	0.258	31 (56.4%)	24 (43.6%)	0.262
Menopause status							
Pre-menopause	49	25 (51.0%)	24 (49.0%)		30 (61.2%)	19 (38.8%)	
Post-menopause	63	30 (47.6%)	33 (52.4%)	0.721	39 (61.9%)	24 (38.1%)	0.941
Tumor location							
Single side	46	26 (56.5%)	20 (43.5%)		27 (58.7%)	19 (41.3%)	
Both sides	66	29 (43.9%)	37 (56.1%)	0.190	42 (63.6%)	24 (36.4%)	0.597
Histological type							
Serous	75	28 (37.3%)	47 (62.7%)		47 (62.7%)	28 (37.3%)	
Non-serous	37	27(73.0%)	10 (27.0%)	<0.01	22 (59.5%)	15 (40.5%)	0.743
Tumor grade							
1 + 2	80	33 (41.3%)	47 (58.8%)		48 (60.0%)	32 (40.0%)	
3	16	8 (50.0%)	8 (50.0%)	0.518	10 (62.5%)	6 (37.5%)	0.852
FIGO stage							
I + II	42	28 (66.7%)	14 (33.3%)		26 (61.9%)	16 (38.1%)	
III + IV	70	27 (38.6%)	43 (61.4%)	<0.01	43 (61.4%)	27 (38.6%)	0.960
LNM							
No	63	35 (55.6%)	28 (44.4%)		39 (61.9%)	24 (38.1%)	
Yes	30	13 (43.3%)	17 (56.7%)	0.270	20 (66.7%)	10 (33.3%)	0.656
Recurrence status							
Relapse	77	33 (42.9%)	44 (57.1%)		48 (62.3%)	29 (37.7%)	
No relapse	35	22 (62.9%)	13 (37.1%)	0.050	21 (60.0%)	14 (40.0%)	0.814
Survival status							
Survival	63	36 (57.1%)	27 (42.9%)		38 (60.3%)	25 (39.7%)	
Dead	49	19 (38.8%)	30 (61.2%)	0.054	31 (63.3%)	18 (36.7%)	0.750
Total (%)	112	55 (49.1%)	57 (50.9%)		69(61.6%)	43(38.4%)	

FIGO, International Federation of Gynecology and Obstetrics; LNM, lymph nodes metastasis.

Table 3
Univariate analysis of survival using the Cox proportional hazard model.

Parameters	Disease-free survival		Overall survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
RASSF1A methylation	0.613 (0.386-0.974)	0.038	0.570 (0.321-1.013)	0.055
Tumor location	1.832 (1.125-2.982)	0.015	1.466 (0.814-2.641)	0.202
Histological type	0.382 (0.219-0.667)	<0.01	0.542 (0.283-1.041)	0.066
Tumor grade	2.339 (1.065-5.137)	0.034	0.549 (0.216-1.394)	0.208
FIGO stage	6.215 (3.362-11.492)	<0.01	3.153 (1.571-6.326)	<0.01
LNM	2.809 (1.658-4.760)	<0.01	2.628 (1.326-5.208)	<0.01

CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; LNM, lymph node metastasis.

MSP, which involves qualitative methods and leads to high incidence of false-positive results, pyrosequencing enables quantification of DNA methylation [22,23]. In the present study, we applied a relatively simple and inexpensive pyrosequencing method to detect the prevalence of *RASSF1A* promoter methylation and adopted 5% as the cut-off value. The hypermethylation frequency among the samples was 49.1% (55/112).

The rate of *RASSF1A* hypermethylation was significantly higher in the early FIGO stage (66.7%, $p < 0.05$); this agreed with the findings of previous studies [20,24]. Promoter hypermethylation of *RASSF1A* may be a relatively early event in ovarian tumorigenesis before symptomatic or overt manifestations; thus, it may be a useful biomarker for evaluating the OC carcinogenic process in high-risk populations. In addition, *RASSF1A* hypermethylation was significantly related to nonserous subtype (73.0%) and nonrecurrent OC (62.9%, $p < 0.05$). We did not observe a significant relationship between *RASSF1A* hypermethylation and the OS of OC. To date, few studies have investigated *RASSF1A* hypermethylation and the prognosis of OC. Choi et al. [25] first investigated the correlation between *RASSF1A* methylation and OC prognosis but found no correlation. A study conducted in Vietnam reported that no significant association was observed between the methylation status of *RASSF1A* and the clinical and pathological parameters of tumors [22]; this suggests that ethnic variations may have accounted for these results. However, Matei et al. [26] revealed that demethylation of *RASSF1A* in OC positively correlated with progression-free survival ($p < 0.05$).

Tumor-infiltrating lymphocytes (TILs) in the OC microenvironment were first reported in 1991, and increasing evidence from subsequent studies has verified that OCs are immunogenic tumors [27]. Immune evasion is characteristic of tumorigenesis and progression. One of the evasion patterns exhibited by cancers is impairment of antitumor immune response via the PD-1 pathway. The predictive role of PD-L1 is unclear among OC patients. A study reported that PD-L1 expression was not significantly correlated with various clinicopathological factors such as age, primary tumor status, LNM, distant metastasis, histological type, residual tumor status, and chemotherapy in OC [28]. Similarly, our study demonstrated that 61.6% (69/112) of the 112 cases were positive for PD-L1 protein expression; no significant difference in PD-L1 expression was observed for age, menstrual status, histological type, tumor location, stage, or LNM ($p > 0.05$). In a study of breast

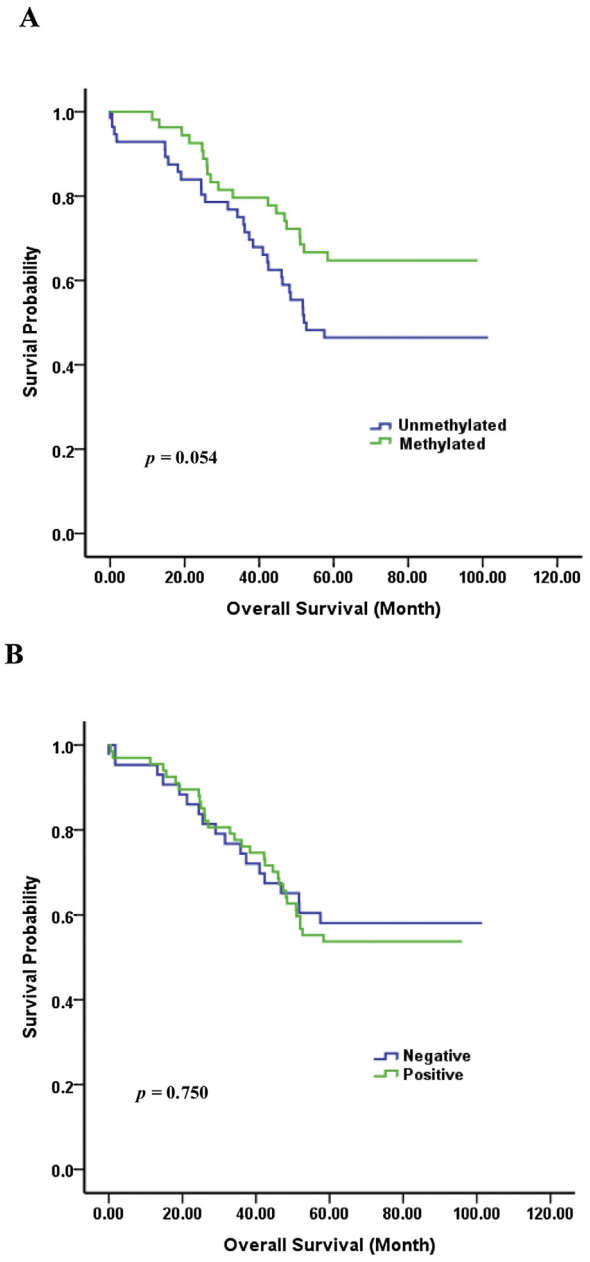

Fig. 2. Kaplan–Meier curves of OS for patients with OC with (A) *RASSF1A* gene methylation and (B) PD-L1 protein expression.

Table 4
Multivariate analysis of prognostic factors associated with DFS and OS.

Parameter	Disease-free survival		Overall survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
FIGO stage	7.910 (3.506-17.848)	<0.01	4.361 (1.802-10.554)	<0.01

CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio.

cancer tumors, PD-L1 messenger RNA level was associated with an increase in TILs and improved recurrence-free survival [29]. For patients with gastric cancer, PD-L1 expression was upregulated and associated with poor prognosis [30]. Several factors may account for the controversial diagnostic and predictive value of PD-L1. First, IHC-based evaluation of PD-L1 involves technical concerns; differences in staining patterns among studies can be caused by differences in the anti-PD-L1 antibodies, staining platforms, and staining protocols used. Second, scoring systems for IHC staining and cut-off values for positivity vary among studies. Third, the number of cases enrolled in our study was relatively small, and this may have limited statistical estimation.

Hypermethylated or mutated OCs exhibit higher antigen loads. High antigen loads are frequently found in drug resistance, and re-expression of silenced genes enables resensitization of drug-resistant OCs [7]. In contrast to genetic mutations, epigenetic changes are potentially reversible, which makes them promising targets for combating OC. Additionally, DNA methyltransferase inhibitors (DNMTIs) can improve tumor immunogenicity and immune response in cancers [31]. A study using mice models bearing human OC cells demonstrated that DNMTIs increased the number of TILs in tumor sites [32]. In the mice, treatment with PD-L1 blockade plus DNMTI reduced the tumor volume and increased the number of tumor-infiltrating CD8 + T cells and Th1-type chemokine expression. Thus, changes in the epigenetic program can augment the therapeutic efficacy of PD-L1 blockade therapy [32]. In the present study based on samples from OC patients, we evaluated the relationship between *RASSF1A* methylation and PD-L1 expression; although no significant correlation was observed between the two markers, our study suggested the new strategy of a combined treatment of DNMTIs with PD-L1 blockades for OC, especially drug-resistant OC. Furthermore, in future research, a panel of gene methylations rather than an individual gene should be assessed to increase sensitivity and specificity.

Formatting of funding sources

The study is supported by Scientific Research Seed Fund of Peking University First Hospital (no. 2019SF03).

Authors' contributions

Xinxin Zhu contributed to data collection, discussing content and writing. Huixia Yang revised the article. Jinghe Lang designed and reviewed the article. Yan Zhang reviewed and revised the manuscript.

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