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ARTICLE

Expression of B7 family checkpoint proteins in cervical cancer

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The role of programmed cell death-ligand 1 (PD-L1) in cervical cancer has been widely investigated; however, the influences of other inhibitory B7 family members are poorly understood. We investigated the expression of PD-L1, B7 homolog 3 (B7-H3), B7-H4, and V-domain Ig suppressor of T-cell activation (VISTA) and their association with the clinicopathological features and outcomes of a large cohort of 673 patients with squamous cell carcinoma or adenocarcinoma of the uterine cervix. The positivity rates for PD-L1 (combined positive score ≥1), B7-H3 in tumor cells (TCs), B7-H4 (exclusively in TCs), VISTA in immune cells (ICs), and VISTA in TCs were 57.9%, 62.8%, 44.8%, 92.6%, and 4.8%, respectively, in 606 primary cervical cancer samples. Co-expression of PD-L1 with B7-H3 in TCs and with B7-H4 and VISTA in ICs was observed in 38.8%, 25.4%, and 57.9% of samples, respectively. B7-H3 in TCs and B7-H4 and VISTA in ICs were observed in 58.1%, 46.6%, and 83.1% of PD-L1-negative samples, respectively. These proteins were observed more frequently in squamous cell carcinomas and in moderately to poorly differentiated carcinomas. VISTA (in ICs) and B7-H4 were more frequent in primary tumors than in recurrent counterparts and correlated with improved survival; in contrast, B7-H3 positivity in TCs was less frequent in primary tumors and correlated with short disease-specific survival. Co-expression of B7-H4 and VISTA in ICs was an independent predictor of favorable outcomes overall and among patients with PD-L1-negative tumors. These data indicate that B7 family proteins exhibit differing expression patterns, distributions, and prognostic implications in cervical cancer. Furthermore, the co-expression of PD-L1 with other checkpoint proteins suggests that PD-1/PD-L1 blockade combined with modulating other immune checkpoints may present a novel therapeutic approach for cervical cancer. Future studies are needed to validate prognostic values of B7 family proteins and explore their biological roles in this malignancy.

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INTRODUCTION

Cervical cancer is diagnosed in about 500,000 women and causes over 265,000 deaths annually worldwide[1]. Despite the availability of screening programs, 5–15% of patients are diagnosed at advanced stages and have metastatic disease. Even among women with earlier disease stages, 15–61% will experience metastasis or recurrence[2]. The prognoses of patients with advanced or recurrent cervical cancer are poor, with response rates ranging from 13% to 36% using currently available therapeutic options[3]. Inhibiting immune checkpoint proteins such as programmed cell death 1 (PD-1) or its ligand PD-L1 improves the outcomes of patients with several types of malignancies[4,5]. The PD-1 inhibitor pembrolizumab has been approved by the United States Food and Drug Administration for patients with advanced PD-L1-positive (defined as a combined positive score [CPS] ≥ 1) cervical cancer that progresses during or after chemotherapy. However, the objective response rate is relatively low (12% and 17% in the KEYNOTE-028 and KEYNOTE-158 studies, respectively)[6,7]. Therefore, there is an unmet need for novel therapeutic strategies that increase durable response rates, utilize more effective agents, and improve lives and outcomes of patients with this disease.

The B7 family includes 10 immune checkpoint proteins: B7-1 (CD80), B7-2 (CD86), B7 homolog 1 (B7-H1, PD-L1), B7-DC (PD-L2), B7-H2, B7-H3, B7-H4, B7-H5 (V-domain Ig suppressor of T-cell activation, VISTA), B7-H6, and B7-H7[8,9]. These proteins function as important secondary signals that either stimulate and support T-cell action or suppress T-cell responses by selectively binding to CD28 family members such as PD-1. They also play an important role in maintaining self-tolerance and antitumor immunity. Like their well-studied family member PD-L1, other B7 proteins exhibit immunomodulatory behaviors and are correlated with survival in patients with cancer[9]. Such previously published data prompted us to investigate whether B7 family proteins can serve as prognostic biomarkers and/or potential therapeutic targets in patients with cervical cancer.

The expression of PD-L1, B7-H3, and B7-H4 in cervical cancer has been previously described;[10–16] however, VISTA expression status and the interactions among these immune checkpoint proteins remain unknown. Additionally, the sample sizes in previous studies were small, rendering their conclusions not very persuasive. We previously investigated the expression of B7 family proteins in clear cell carcinoma of the uterine cervix[17]. In the present study, we investigated the expression profiles of the inhibitory B7 family checkpoint proteins PD-L1, B7-H3, B7-H4, and VISTA, examined their potential associations, and searched for any correlations with clinicopathological features and outcomes in a large cohort of 673 patients with cervical cancer.

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MATERIALS AND METHODS

Study cohort and tissue microarray (TMA) construction

This retrospective study included 606 patients with primary cervical cancer who underwent cervical conization ($N = 16$) or hysterectomy (including radical hysterectomy) ($N = 590$), as well as 67 patients with recurrent cervical cancer who underwent pelvic exenteration or metastasectomy. All patients were treated between January 2004 and December 2018 at the Peking Union Medical College Hospital in Beijing, China. Only patients diagnosed with squamous cell carcinoma (SCC) and usual-type adenocarcinoma were included in this study; we excluded those with adenosquamous carcinoma, those with non-usual types of endocervical adenocarcinoma (i.e., mucinous gastric/intestinal, adenoma malignum, serous, clear cell, or endometrioid carcinoma), those with neuroendocrine carcinoma, or those with inadequate formalin-fixed and paraffin-embedded tissue blocks for TMAs. To determine the depth of stromal invasion, lymphovascular space invasion (LVSI), lymph node metastasis, histological type, tumor differentiation, hematoxylin and eosin-stained slides were reviewed by 2 gynecologic subspecialty pathologists (L. Zong and S. Yu) who were blinded to the original pathology reports and to each other's interpretations. In case of disagreement, a third expert pathologist (J. Chen) was consulted to arrive at a final decision. Representative tumor tissue areas exhibiting epithelial tumor tissue mixed with tumor-related stroma were marked on hematoxylin and eosin-stained slides and sampled for TMA blocks. TMAs with single 2-mm core per case were constructed using a tissue arrayer (MiniCore, Mitogen, Hertford, UK). Clinical data such as age at diagnosis, tumor size, neoadjuvant chemotherapy, postoperative adjuvant treatment (chemotherapy and/or radiotherapy), date of recurrence or last follow-up, and patient status at the last follow-up were collected from medical records. All patients were staged using the 2009 International Federation of Gynecology and Obstetrics (FIGO) staging system for carcinoma of the cervix. This study conformed to the ethical standards set forth in the Declaration of Helsinki and in national and international guidelines and was approved by the Institutional Review Board of Peking Union Medical College Hospital (SK-995). All patients signed consent forms upon admission to the hospital allowing the review of their medical records, use of their tissue samples, and possible publication of associated reports. None of the patients can be identified based on their clinical information or images in this study, and informed consent to determine B7 family proteins was not required owing to the retrospective nature of the analysis.

Immunohistochemistry

Immunohistochemistry was performed using our laboratory protocol as described previously[17–21]. Briefly, 4 μm TMA serial sections were deparaffinized and subjected to heat-induced epitope retrieval with 10 mM sodium citrate (pH 6.0) at 95°C for 20 min. The endogenous peroxidase activity was quenched using a 0.3% hydrogen peroxide solution. TMA sections were incubated with primary antibodies against PD-L1 (dilution 1:200, clone E1L3N, Cell Signaling Technology [CST], Danvers, USA), B7-H3 (dilution 1:200, clone D9M2L, CST), B7-H4 (dilution 1:200, clone D1M8I, CST), and VISTA (dilution 1:200, clone D1L2G, CST). Human tonsil tissues treated with primary antibodies were used as positive controls, while the same tissues treated with isotype-matched antibodies comprised the negative controls. All slides were stained using an automatic immunohistochemistry staining instrument (BOND-III; Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions.

Assessment of PD-L1, B7-H3, B7-H4, and VISTA

PD-L1 was evaluated based on the CPS, which was calculated as the sum of the number of PD-L1-stained cells (tumor cells [TCs], lymphocytes, and macrophages) divided by the total number of viable TCs, with the quotient multiplied by 100. A CPS ≥ 1 denoted positive PD-L1 expression. Samples were considered positive for B7-H3/B7-H4 when ≥5% of TCs expressed these proteins at any intensity, as described in a previous study[22]. Per previously published guidance, B7-H3 was considered positive when observed in stromal cells (predominately in fibroblasts)[23,24]. VISTA expression was evaluated in TCs and immune cells (ICs) separately. TCs were considered VISTA positive if at least 1% of cells per TMA core had membranous and/or cytoplasmic staining. The proportions of VISTA-expressing tumor-infiltrated and stromal (i.e., tumor-adjacent) ICs to the total tumor-infiltrated and stromal ICs per TMA core, respectively, were determined; these values were considered continuous variables. ICs or TCs with ≥1% VISTA staining were defined as VISTA-positive, as described in our studies in ovarian cancer, invasive ductal carcinoma of the breast, colorectal cancer, and endometrial cancer[18,19,21,25].

Statistical analysis

The χ^2 test was used to determine associations among categorical variables, and Spearman's rank correlation coefficients were used to determine correlations among continuous variables. Relapse-free survival (RFS) was defined as the interval between the date of surgery and that of the detection of the first relapse. Disease-specific survival (DSS) was defined as the interval between the date of surgery and that of death owing to cervical cancer. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. To identify prognostic predictors, univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model, and hazard ratios with 95% confidence intervals for recurrence and death were calculated. All statistical analyses were conducted using the Statistical Package for the Social Sciences (version 20.0; IBM Corp., Armonk, NY, USA). A 2-sided P-value < 0.05 was considered statistically significant.

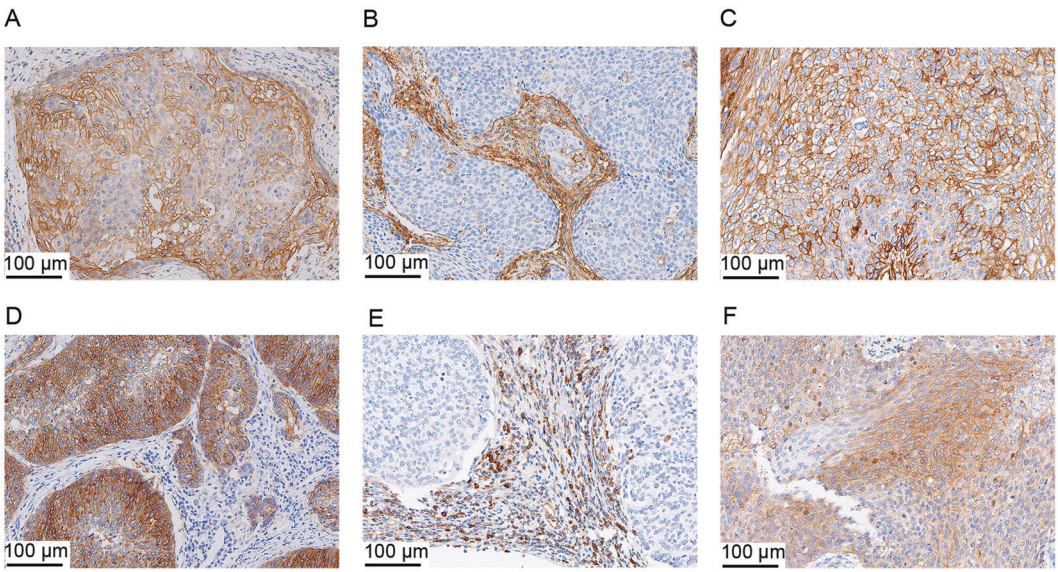

Fig. 1 Immunohistochemical staining for B7 family checkpoint proteins in squamous cell carcinoma of the uterine cervix. A programmed cell death-ligand 1, **B** B7 homolog 3 (B7-H3) in stromal cells, **C** B7-H3 in tumor cells, **D** B7 homolog 4 in tumor cells, **E** V-domain Ig suppressor of T-cell activation (VISTA) in immune cells, **F** VISTA in tumor cells.

RESULTS

Expression of B7 family proteins in primary and recurrent cervical cancer

The median age of patients with primary cervical cancer was 44 years (range, 23–74 years). Among the 606 samples, 491 (81.0%) and 115 (19.0%) were diagnosed as SCC and adenocarcinoma, respectively; the clinicopathological characteristics of the patients are summarized in Supplementary Table S1. One hundred and twenty-eight patients (21.1%) received neoadjuvant chemotherapy before undergoing surgery. Adjuvant treatment, when required, included radiotherapy (pelvic external beam radiotherapy and/or vaginal brachytherapy) with or without platinum-based chemotherapy. None of the patients were administered immune checkpoint inhibitors or other immunotherapeutic agents.

PD-L1 and VISTA were detected in both TCs and ICs, while B7-H3 was detected in both TCs and stromal cells (predominately in fibroblasts). In contrast, B7-H4 was expressed exclusively in TCs and exhibited a cytoplasmic/membranous staining pattern (Fig. 1). Positive PD-L1 (i.e., a CPS ≥ 1) was observed in 57.9% of the samples, and B7-H4 was observed in the TCs of 44.8%. VISTA was detected in the ICs of 92.6% of samples, while only 4.8% expressed VISTA in TCs. B7-H3 was detected in TCs of 62.8% of the samples, while its expression in stromal compartments was observed in 94.0% (Table 1).

Table 1. Expression of B7 family proteins in primary and recurrent cervical cancer.

B7 family proteins	Cervical cancer		*P*-value
	Primary *N* (%)	Recurrent *N* (%)	
PD-L1			0.022
Negative	249 (42.1)	36 (57.1)	
Positive	342 (57.9)	27 (42.9)	
B7-H3 in TCs			0.012
Negative	225 (37.2)	14 (21.5)	
Positive	380 (62.8)	51 (78.5)	
B7-H4 in TCs			0.010
Negative	334 (55.2)	48 (71.6)	
Positive	271 (44.8)	19 (28.4)	
VISTA in ICs			<0.001
Negative	45 (7.4)	18 (26.9)	
Positive	561 (92.6)	49 (73.1)	
VISTA in TCs			0.145
Negative	577 (95.2)	61 (91.0)	
Positive	29 (4.8)	6 (9.0)	

B7-H3 B7-homolog 3, *B7-H4* B7-homolog 4, *ICs* immune cells, *PD-L1* programmed cell death-ligand 1, *TCs* tumor cells, *VISTA* V-domain Ig suppressor of T-cell activation.

The median age of the 67 patients with recurrent cervical cancer was 45 years; most received initial treatment at other hospitals and were admitted to Peking Union Medical College Hospital to receive salvage treatment upon experiencing recurrence. Thus, the samples from these 67 recurrent patients were not matched with the abovementioned primary cervical cancers; their clinicopathological features are summarized in Supplementary Table S2. PD-L1, B7-H4, and VISTA positivity in ICs was more frequent in primary tumors than in recurrent counterparts, while tumoral B7-H3 expression was observed more frequently in recurrent cervical cancer (Table 1).

Association between B7 family proteins and clinicopathological features

The associations between the expression of B7 family checkpoint proteins and patients' clinicopathological features are presented in Supplementary Table S3. PD-L1, VISTA in ICs and B7-H3, B7-H4, and VISTA in TCs were observed significantly more frequently in SCCs than in adenocarcinomas (Fig. 2A). PD-L1, B7-H3 (in stromal cells and TCs), B7-H4, and VISTA (in ICs) were more frequently positive in moderately and poorly differentiated carcinomas (Fig. 2B). B7-H3 expression in stromal cells was associated with unfavorable pathological features (i.e., the presence of LVSI and lymph node metastasis). B7-H4 positivity correlated with small tumor size (<4 cm) and the presence of LVSI. None of the 4 investigated B7 family proteins were associated with FIGO stage or stromal invasion. Additionally, there was no difference in B7 family protein expression between patients who received neoadjuvant chemotherapy and those who did not.

Association between PD-L1, B7-H3, B7-H4, and VISTA

As shown in Table 2, VISTA expression in ICs correlated positively with PD-L1, B7-H4, and B7-H3 in TCs; VISTA in TCs was associated with PD-L1 but not with B7-H3 or B7-H4; both PD-L1 and B7-H4 positively correlated with B7-H3 in TCs; and no association was observed between PD-L1 and B7-H4. When evaluated as a continuous variable, the extent of VISTA positivity in ICs was significantly and directly correlated with the CPS of PD-L1 (Spearman's $\rho = 0.382$, $P < 0.001$). Co-expression of PD-L1 with B7-H3 (in TCs), B7-H4, and VISTA (in ICs) was observed in 38.8%, 25.4%, and 57.9% of the samples, respectively. Notably, 83.1% of the PD-L1-negative samples expressed VISTA in ICs, 58.1% expressed B7-H3 in TCs, 46.6% expressed B7-H4, and 92.7% expressed at least 1 of these checkpoint proteins (B7-H3, B7-H4,

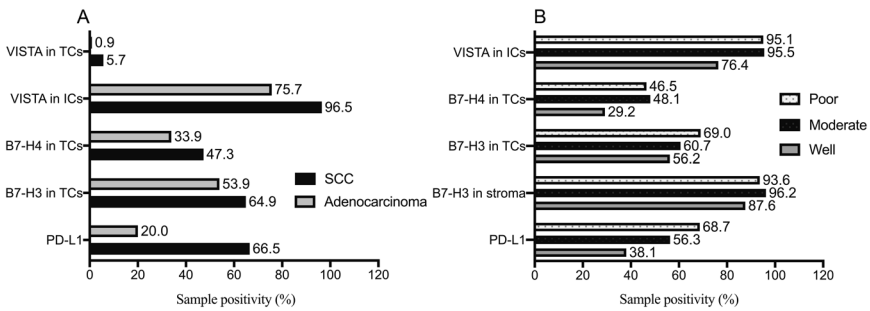

Fig. 2 Distribution of B7 family proteins in cervical cancer. PD-L1, B7-H3 in tumor cells (TCs) and in stroma, B7-H4 in TCs, VISTA in TCs, and VISTA in ICs across **A** histological types: squamous cell carcinoma (SCC) and adenocarcinoma and **B** tumors with well, moderate, and poor differentiation. *PD-L1* programmed cell death-ligand 1, *B7-H3* B7 homolog 3, *B7-H4* B7 homolog 4, *VISTA* V-domain Ig suppressor of T-cell activation, *TCs* tumor cells, *ICs* immune cells.

Table 2. Association among PD-L1, B7-H3, B7-H4, and VISTA in cervical cancer.

Variables	B7-H3 in TCs		B7-H4 in TCs		PD-L1	
	Negative	Positive	Negative	Positive	Negative	Positive
B7-H4 in TCs	$P = 0.002$					
Negative	142	191				
Positive	83	188				
PD-L1	$P = 0.027$		$P = 0.531$			
Negative	104	144	133	116		
Positive	113	229	191	150		
VISTA in ICs	$P = 0.045$		$P = 0.002$		$P < 0.001$	
Negative	23	22	35	10	42	0
Positive	202	358	299	261	207	342
VISTA in TCs	$P = 0.273$		$P = 0.997$		$P = 0.044$	
Negative	217	359	318	258	242	320
Positive	8	21	16	13	7	22

B7-H3 B7-homolog 3, *B7-H4* B7-homolog 4, *ICs* immune cells, *PD-L1* programmed cell death-ligand 1, *TCs* tumor cells, *VISTA* V-domain Ig suppressor of T-cell activation.

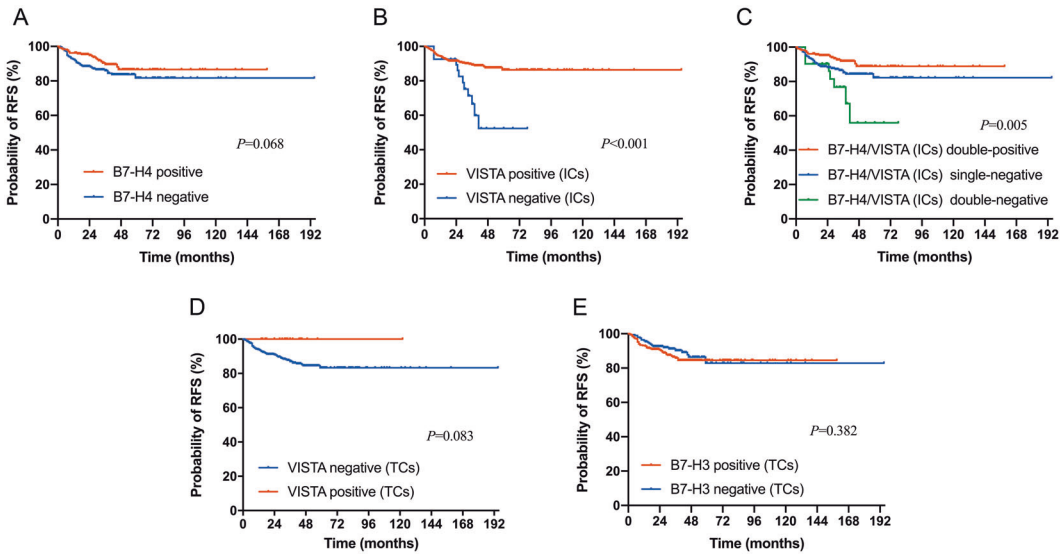

Fig. 3 Kaplan-Meier curves showing the RFS in the entire cohort of 531 patients with cervical cancer according to B7 family proteins. **A** B7-H4, **B** VISTA in ICs, **C** B7-H4 and VISTA in ICs, **D** VISTA in TCs, **E** B7-H3 in TCs. *RFS* recurrence-free survival, *B7-H4* B7 homolog 4, *B7-H3* B7 homolog 3, *VISTA* V-domain Ig suppressor of T-cell activation, *ICs* immune cells, *TCs* tumor cells.

and/or VISTA). Of the 605 patient samples for which data regarding both B7-H4 and VISTA in ICs were available, 261 (43.1%) were double positive for B7-H4 and VISTA in ICs, 35 (5.8%) were double negative, and 309 (51.1%) had only one or the other (single positive).

Association between B7 family protein expression and prognosis in patients with cervical cancer

After excluding patients who only had conization specimens available and those whose follow-up times were under 3 months, 531 patients with primary cervical cancer who underwent radical surgeries with complete adjuvant systemic therapy (when necessary) were subjected to survival analysis. There were no significant differences between the entire cohort of 606 patients and this survival analysis-only subgroup in terms of clinicopathological parameters (data not shown). After a median follow-up of 36 months (range, 9–195 months), 64 patients (12.1%) had relapsed and 49 (9.2%) had died owing to cervical cancer as of July 2020.

Univariate analysis showed that FIGO stage, lymph node metastasis, and VISTA in ICs were significantly associated with survival across the entire cohort (Fig. 3, Supplementary Fig. S1, and Table S4). Positive B7-H4, negative B7-H3 in TCs, and small tumor size (<4 cm) tended to be associated with improved DSS, although the relationship was not significant. Although VISTA in TCs was not associated with RFS ($P = 0.083$), none of the 24 patients with VISTA-positive TCs relapsed (Fig. 3). Moreover, 230 patients were double positive for B7-H4 and VISTA in ICs, 31 were double negative, and 270 were single positive. Kaplan-Meier analysis showed that patients with B7-H4 and VISTA double-positive tumors had excellent outcomes, those with double-negative tumors had poor RFS, and those with single-positive status had intermediate outcomes (Fig. 3 and Supplementary Table S4). On multivariate analysis adjusted for FIGO stage and lymph node metastasis, B7-H4 combined with VISTA in ICs remained significantly associated with RFS and DSS (Table 3). Additionally, B7-H3 positivity in TCs was a predictor of poor DSS independent of B7-H4 and VISTA in ICs or of FIGO stage.

Table 3. Multivariate analysis of factors potentially predictive of survival in patients with cervical cancer after surgery ($N = 531$).

Variables	Relapse-free survival			Disease-specific survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Lymph node metastasis			0.039			
Yes vs. no	1.71	1.03–2.83				
VISTA in ICs and B7-H4			0.005			0.026
Double negative *vs.* double positive	4.01	1.73–9.31	0.001	3.02	0.97–9.44	0.057
Single positive *vs.* double positive	1.93	1.09–3.43	0.024	2.23	1.15–4.34	0.018
2009 FIGO stage						0.036
IB2–II *vs.* IA1–IB1				1.84	1.04–3.25	
B7-H3 in TCs						0.031
Positive vs. negative				2.07	1.07–3.99	

B7-H3 B7-homolog 3, *B7-H4* B7-homolog 4, *CI* confidence interval, *FIGO* International Federation of Gynecological Oncology, *HR* hazard ratio, *IC* immune cell, *TC* tumor cell, *VISTA* V-domain Ig suppressor of T-cell activation.

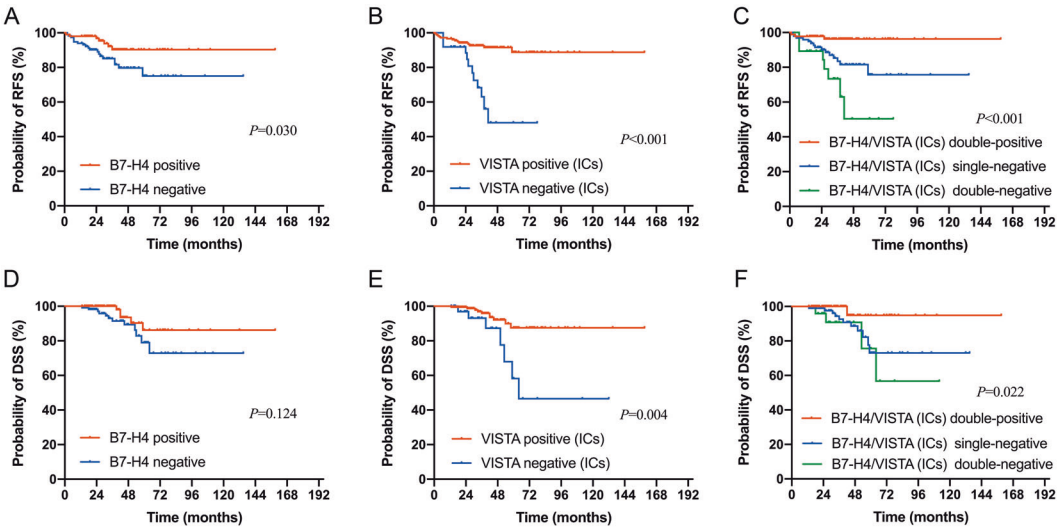

Fig. 4 Kaplan-Meier curves of 216 patients with programmed cell death-ligand 1-negative cervical cancer according to other B7 family proteins. A–C RFS and **D–F** DSS according to **A, D** B7-H4, **B, E** VISTA in ICs, and **C, F** B7-H4 combined with VISTA in ICs. *RFS* relapse-free survival, *DSS* disease-specific survival, *B7-H4* B7 homolog 4, *VISTA* V-domain Ig suppressor of T-cell activation, *IC* immune cell.

Subgroup survival analyses were performed according to PD-L1 status. VISTA expression in ICs was significantly associated with improved RFS and DSS in 216 patients with PD-L1-negative cervical cancer, while B7-H4 positivity was associated with improved RFS (Fig. 4). Consistent with results observed in the entire cohort, patients with B7-H4 and VISTA double-positive tumors had favorable outcomes, while those with double-negative tumors had poor outcomes (Fig. 4). On univariate and multivariate analyses, double-negative B7-H4 and VISTA in ICs was the only predictor of poor RFS and DSS in patients with PD-L1-negative tumors (Table 4). B7-H4 expression or B7-H4 combined with VISTA in ICs was not associated with survival in patients with PD-L1-positive cervical cancer (data not shown). The association between VISTA in ICs and survival was not analyzed in patients with PD-L1-positive tumors considering all had VISTA-positive ICs.

DISCUSSION

To our knowledge, this is the first study of the expression profiles of the inhibitory B7 family checkpoint proteins PD-L1, B7-H3, B7-H4, and VISTA in a large cohort of patients with cervical cancer. Despite their varying expression patterns and distributions, these proteins positively correlated with each other (the only lack of association was between PD-L1 and B7-H4). These proteins were expressed more frequently in SCC than in adenocarcinoma and were also more prevalent in moderately and poorly differentiated carcinomas. VISTA in ICs and B7-H4 were observed more frequently in primary tumors than in recurrent counterparts and were correlated with improved RFS and DSS; in contrast, B7-H3 positivity in TCs was less frequent in primary tumors than in recurrent lesions and was correlated with poor DSS. Double positivity for B7-H4 and VISTA (in ICs) predicted excellent outcomes overall as well as in the subgroup of patients with PD-L1-negative cervical cancer. These data suggest that, although B7 family proteins are closely related, their expression status and clinical significance thereof have distinct implications in patients with cervical cancer.

PD-1 inhibitors have been approved for treating PD-L1-positive cervical cancer, and many studies have investigated the expression of PD-L1 in this disease[10–12,26–30]. However, the sample sizes in most of these studies were small, and the data they produced were conflicting. In 3 previous studies, PD-L1 expression was

Table 4. Univariate analysis of survival in patients with PD-L1-negative cervical cancer after radical surgery ($N = 216$).

Variables	Relapse-free survival			Disease-specific survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
VISTA in ICs and B7-H4			0.002			0.05
Double positive	1					
Double negative	10.8	2.9–41.0	<0.001	7.8	1.4–43.1	0.017
Single positive	5.0	1.4–17.21	0.11	5.2	1.2–23.7	0.031

B7-H4 B7 homolog 4, *CI* confidence interval, *HR* hazard ratio, *IC* immune cell, *PD-L1* programmed cell death-ligand 1, *VISTA* V-domain Ig suppressor of T-cell activation.

associated with unfavorable prognosis;[10,12,28] in contrast, Kawachi et al.'s study of 127 patients with invasive adenocarcinoma found that those with PD-L1-positive tumors tended to experience longer survival[26]. In our present large-sample study, PD-L1 was not associated with patient survival, which is consistent with data from 3 older studies[11,27,29]. PD-L1 positivity was observed more frequently in SCC than in adenocarcinoma, which is consistent with the observations of Heeren et al. and Zhang et al.[12,16]. Additionally, we found that PD-L1 expression was more frequent in moderately and poorly differentiated carcinomas than in well-differentiated counterparts, which is consistent with data reported by Saglam et al.[27] and Zhang et al.[16]. These findings indicate that PD-L1 is not necessarily a predictor of survival, and that only half of the patients with SCC and with moderately and poorly differentiated tumors are candidates for anti-PD-1 immunotherapy.

The B7-H3 and B7-H4 checkpoints inhibit the activation and function of T cells[9]. Their expression profiles have been investigated in various cancers, including cervical cancer, and they were found to be associated with poor survival[9,13–16,31–34]. B7-H3 positivity was observed more frequently in SCC and in tumors with unfavorable pathological features (such as LVSI and lymph node metastasis) in both Zhang et al.'s study[16] and ours. We found that B7-H3 in TCs correlated with unfavorable prognosis and was an independent predictor of short DSS, which is consistent with previously published data[14–16,31]. Previous investigations suggested that B7-H4 expression impairs antitumor immune responses and correlates with poor survival outcomes in cervical cancer[13,31,32,34]. In contrast, our analysis showed that B7-H4 expression correlated with improved survival in the entire cohort and in the subgroup of patients with PD-L1-negative tumors. B7-H4 has been identified as an immune coinhibitory molecule; however, recent evidence suggests that it limits tumor growth in animal models[35]. Rahbar et al. found that high B7-H4 expression correlated with improved RFS in 2681 patients with breast cancer[35], which is consistent with our findings in endometrial cancer (data not published) and cervical cancer (the present study). These data suggest that B7-H3 expression is a predictor of poor outcomes and a potential immunotherapeutic target; in contrast, anti-B7-H4 therapy may be detrimental considering this protein's correlation with favorable prognosis, especially in patients with PD-L1-negative tumors.

Discovered in 2011, VISTA is a coinhibitory molecule that suppresses T-cell activation, proliferation, and cytokine release[36,37]. VISTA expression was found to be a poor prognostic factor in patients with oral squamous cell carcinoma[38] and in those with melanoma[39]. However, increasing evidence suggests that VISTA correlates with improved survival in patients with many other cancer types[40]. Consistent with our findings in endometrial cancer[25], breast and colorectal cancers[18,21], VISTA expression in ICs was associated with favorable survival outcomes in patients with cervical cancer. Interestingly, patients with B7-H4 and VISTA double-positive tumors had the most favorable outcomes, those with double-negative tumors had short survival, and those with single-positive tumors had intermediate survival durations. Importantly, co-expression of B7-H4 and VISTA in ICs was a prognostic indicator independent of FIGO stage or lymph node metastasis status. To our knowledge, our study is the first to investigate the co-expression of both B7-H4 and VISTA in terms of identifying patients with a distinct risk of relapse and cancer-specific death. T-cell-based immunoscoring has been validated and is recommended as a new component of a TNM immune classification for colon cancer[41]. As such, an immunoscore based on B7-H4 and VISTA could be considered for cervical cancer risk classification once these markers' prognostic values are validated. Additionally, the association between VISTA/B7-H4 expression and favorable prognosis should be considered in future clinical trials of immunotherapy for cervical cancer.

The association between PD-L1 and each of B7-H3, B7-H4, and VISTA has been investigated previously in various cancers[16,42–52]. Zhang et al. found that B7-H3 positively correlated with PD-L1 and that they were co-expressed in 21.0% of 552 cervical cancer samples[16]. Consistent with their study, our results showed that B7-H3 was associated with PD-L1, with 38.8% of our samples positive for both. These data support the combined blockade of B7-H3 and PD-1/PD-L1 as a therapeutic strategy. B7-H4 is positively correlated with PD-L1 in oral squamous cell carcinoma[53] but is inversely correlated with PD-L1 in breast and lung cancers[43,47]. In our present study, no association between B7-H4 and PD-L1 was observed, which corresponds to previous findings in ovarian cancer[54]. Interestingly, B7-H4 positivity was observed in 46.6% of PD-L1-negative samples and was associated with a favorable prognosis; such individuals had low response rates to PD-1 inhibitors and therefore lacked effective immunotherapeutic options. VISTA in ICs was positively associated with PD-L1; this is consistent with previous findings in lung cancer[51,52], lymphoma[44], and craniopharyngioma[50] as well as those of our studies of patients with breast cancer[18], colorectal cancer[21], and endometrial cancer[25]. Additionally, VISTA in ICs was associated with B7-H3 and B7-H4 in TCs of cervical cancer. In a study of 132 patients with craniopharyngioma, Wang et al. found that VISTA correlated with PD-L1 but not with B7-H3 or B7-H4[50]. The expression of one or more B7 family checkpoint proteins suggest that they play synergic and/or complementary roles in the immune evasion of cervical cancer. Although the regulatory mechanism of B7 family proteins in cervical cancer remains unknown, the expression profiles of B7 family proteins as elucidated in our present study ought to contribute to the better understanding of the tumor immune environment and the mechanisms by which cervical cancers evade immune surveillance. Future studies are warranted to validate the prognostic values of B7 family members in cervical cancer and identify their regulatory mechanisms as this may assist in the development of new immunotherapeutic agents.

The strengths of our study were its exclusivity to SCC and usual-type adenocarcinoma, its comprehensive analysis of B7 family proteins, and its large sample size derived from a single tertiary hospital. This study also had some limitations. First, it was a retrospective investigation that produced inherent unavoidable

biases. Second, the use of TMAs may not have accurately represented the entirety of each sample considering the intratumoral heterogeneity and variable sampling of the tumor-stroma interface across patients. PD-L1 was observed in 57.9% of the primary tumors in our study as assessed using TMAs; this proportion was approximately 80% in both the KEYNOTE-028 and KEYNOTE-158 clinical trials, which assessed PD-L1 in whole sections[6,7]. As such, the relatively low proportion of PD-L1-positive tumors may be attributed to our use of TMAs and/or to the relatively high proportion of adenocarcinomas in our study, as these tumor types express PD-L1 less frequently than do SCCs. Finally, our study was limited by its single-center nature and the lack of an independent validation cohort. Further studies from independent cohorts are needed to validate our findings.

In conclusion, we found that the inhibitory B7 family members PD-L1, B7-H3, B7-H4, and VISTA are expressed frequently in cervical cancer, particularly in SCC and moderately to poorly differentiated carcinomas. Although these 4 proteins are closely related, their expression patterns, distributions, and prognostic implications differ. B7-H3 positivity in TCs correlates with poor prognosis, while double positivity for B7-H4 and VISTA in ICs is an independent predictor of favorable outcomes. The co-expression of PD-L1 and other checkpoint proteins suggest that PD-1/PD-L1 blockade combined with the modulation (including the upregulation) of other immune checkpoints may represent a novel therapeutic approach for patients with cervical cancer. Future studies are needed to explore the underlying regulatory mechanisms of B7 family proteins and their biological roles in cervical cancer, especially in PD-L1-negative tumors.

DATA AVAILABILITY

The raw data used in this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Y.X. and J.C. made substantial contributions to the conception and design, acquisition of data, and critical revision of the manuscript. Y.Z., Y.K. and Y.G. made substantial contributions to patient selection and clinical data. L.Z., S.M. and S.Y. made substantial contributions to reviewing pathological parameters, assessing the results of immunohistochemistry, interpreting the data, and drafting the manuscript. All authors read and approved the final manuscript.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Review Board of Peking Union Medical College Hospital (approval number: S-K995).

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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