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Original Article

Potential value of V-domain Ig suppressor of T-cell activation for assessing progn osis in cervical cancer and as a target for therapy

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Abstract: V-domain Ig suppressor of T-cell activation (VISTA), which belongs to the B7 family and is expressed predominantly on hematopoetic cells, myeloid, granulocytic and T cells, can suppresses T-cell activation in vivo and vitro. The blockade of VISTA has displayed brilliant results in certain murine tumor models. But to date, little is known about the expression and impact of VISTA in human cervical cancer (CC). To fill this gap of information, we systemically investigated the expression of VISTA on tumor cells, intratumoral immune cells (ICs) and vascular endothelial cells (VECs) in a group of patients with CC by performing immunohistochemical analysis. The associations between VISTA expression and different clinicopathologic features were evaluated using Fisher's exact test, and the analysis of overall survival in different groups was performed by the construction of Kaplan-Meier models. The results indicated that high expression of VISTA on ICs or VECs was significantly related to advanced tumor stage and the lymph node metastasis (LNM) of CC. Furthermore, we performed multivariate Cox regression analysis, which showed that there was no association between VISTA expression and the 5-year overall survival rate, and LNM was the only independent predicting factor of poor prognosis for CC.

Keywords: V-domain immunoglobulin suppressor of T cell activation, cervical cancer, immunohistochemical staining, immune checkpoint, immunotherapy, overall survival analysis

Introduction

Cervical cancer (CC) is one of the most common tumors in women globally. 530,000 new cases are diagnosed annually, and as a major health problem for women especially in developing countries, it causes approximately 275,000 deaths annually [1]. There are several treating strategies that are currently available for CC including radiotherapy, chemotherapy and surgery. For early-stage patients in those who have lost fertility, surgery is an effective way to cure their disease [2]. But for advanced-stage patients and young women who want to preserve their fertility, CC is usually treated effectively with a combination of radiotherapy and platinum-based chemotherapy [3]. However, normal cells may be exposed to damage done by these latter drugs. Still, the prognosis for advanced stage disease remains poor. So, there is an urgent need to identify damages done by these latter drugs. Still, the prognosis is uncertain.

Immunotherapy for cancers involves utilizing the immune system of the patients to attack tumor cells by targeting tumor-specific antigens. These strategies have yielded breakthroughs in the treatment of certain types of cancer, including therapeutic vaccines, immunomodulators, immune checkpoint inhibitors and adoptive T cell transfer [4]. Although gynecologic cancers have not been historically classified as immunogenic tumors, growing evidence has shown that they are able to elicit endogenous antitumor immune responses, suggesting that patients with these cancers may benefit from immunotherapy. Modest clinical success has been accomplished in early trials using immunotherapeutic modalities for major gynecologic cancers including ovarian, cervical, and endometrial cancer [5].

Recently, a novel negative checkpoint regulator (NCR) has surfaced called V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation (VISTA). With a similar structure to the B7 Ig superfamily that includes PD-L1, this NCR is predominantly expressed on hematopoietic cells [6]. In several murine cancer models, VISTA expression is found at particularly high levels on myeloid cells and granulocytic cells, and weaker expression on cluster of differentiation CD4+ and CD8+ T cells [7]. In vitro and in vivo, VISTA exerts immunosuppressive activities on T cells and could be an important mediator in controlling the development of autoimmunity and the immune responses to cancer. In murine fibrosarcoma models, VISTA overexpression on tumor cells was demonstrated to elicit immune protection against the growth of control tumor cells [6]. Additionally, it was suggested that the use of an anti-VISTA monoclonal antibody in murine cancer models may impair the growth of tumor, with remarkable results when used in combination with a tumor vaccine [6]. In oral squamous cell carcinoma patients, VISTA expression associates with poor overall survival in patients with low CD8+ TILS [8]. These observations indicate that VISTA is a promising target in cancer therapy. Preclinical studies with VISTA blockade have shown great improvement in antitumor T-cell responses, leading to hampered tumor growth and improved survival. CTLA-4 and VISTA combination treatment will compensate for the disadvantage and promote antitumor effificacy [9]. These conclusions indicate that VISTA may be a favorable target for immunotherapy in cancers.

Nevertheless, to our knowledge, no direct evidence of the VISTA expression in CC and association between VISTA and CC has been provided so far. The expression of VISTA in CC and the exact role in predicting the prognosis of CC are far from understanding. In the present study, VISTA expression in tumor tissues was examined and the prognostic value of VISTA in different types of CC was evaluated, to show its clinical significance.

Materials and methods

Patients and tissue samples

In this study, VISTA immunohistochemical analysis was performed on tissues attained from CC. Tissue sample collection from 127 patients were collected in the Pathology Department of West China Hospital, Sichuan University between Jan, 2013 and Dec, 2013. Patients were included in our research following the criteria below: i) Accessible clinical data and at least 5 years of routine follow-ups; ii) no chemotherapy or radiation therapy before surgery; and iii) CC confirmed by clinical and histopathological diagnosis. Among the 127 cases, 23 cases were excluded because of the loss of follow-up. The clinicopathologic features of patients, including age (age range, 31-68 years, median 42 years), and stage of CC, are summarized in **Table 1**.

All patients were followed until death or the end of the follow-up period (Sept, 2019). Overall survival (OS) was calculated from the date of surgery to the date of mortality or the last follow-up. Patients were censored at the date of the last visit or at the time of death not related to CC-associated causes. The study was approved by the ethics committee of West China Second Hospital of Sichuan University and written informed consent was obtained from all patients undergoing surgery.

Immunohistochemistry (IHC)

For immunohistochemical analysis of VISTA expression in CC tissues, the CC tissues were fixed in 10% (v/v) formalin at room temperature for 48 hours once being excised from the patients during surgery, and then embedded in paraffin until use. 4 μm sections cut from the paraffin-embedded (FFPE) tissue blocks were deparaffinized and rehydrated using xylene and a graded series of ethanol (absolute, 95%, 80%, 50%). Sections were then rinsed twice with PBS containing 0.1% Tween-20 (PBST). 10 mmol/l boiling (~95°C) sodium citrate buffer was used to performed high-temperature antigen retrieval at pH 6.0 for 15 min. To block the endogenous peroxidase activity, samples were immersed in 3% hydrogen peroxide for 30 min at room temperature, and then incubated in 5% bovine serum albumin (BSA) (cat. no. 9048-46-8; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min to reduce non-specific binding. Sections were then incubated overnight with a primary monoclonal rabbit anti-human VISTA antibody (cat. no. 64953, Cell Signaling Technology, Inc., Danvers, MA, USA; 1:50 dilution in 5% BSA) at 4°C. After rinsing in

Table 1. Coorelation between VISTA expression and clinicopatholoical features in patients with cervical cancer (n=104)

Characteristics	Total (%)	VISTA-postive tumor cells			VISTA-positive ICs/200 ICs			Vascular endothelial cells		
		Low, n (%)	High, n (%)	*P*-value	Low, n (%)	High, n (%)	*P*-value	Negative, n (%)	Positive, n (%)	*P*-value
	104	93 (89.42)	11 (10.58)		59 (56.73)	45 (43.27)		82 (78.85)	22 (21.15)	
Age, years				0.865			0.555			0.159
<55	88	78	10		51	37		72	16	
≥55	16	15	1		8	8		10	6	
Therapy				1.000			0.690			0.123
Operation	19	17	2		10	9		12	7	
Operation + others	85	76	9		49	36		70	15	
Stromal invasion				0.456			0.235			0.411
Negative	44	41	3		22	22		33	11	
Positive	60	52	8		37	23		49	11	
Vaginal wall extension				1.000			0.183			0.167
Negative	84	76	8		45	39		69	15	
Positive	20	17	3		14	6		13	7	
Parametrial extension				1.000			0.448			0.724
Negative	58	52	6		31	27		45	13	
Positive	46	41	5		28	18		37	9	
Intravascular space involvement				0.254			0.404			0.114
Negative	58	46	12		35	23		49	9	
Positive	46	32	14		24	22		33	13	
Lymph node metastasis				0.545			0.005			0.008
Negative	87	79	8		55	32		75	12	
Positive	17	14	3		4	13		10	7	
Stage				0.467		0.030				0.025
Early (in situ, I)	80	73	7		50	30		67	13	
Advanced (II, III, IV)	24	20	4		9	15		15	9	
Differentiation				0.320			0.552			0.112
Poor	75	66	9		40	35		61	14	
Moderate	24	23	1		16	8		19	5	
Well	5	4	1		3	2		2	3	
Tumor size				0.790			0.914			0.199
<4 cm	37	34	3		22	15		26	11	
≥4 cm	22	19	3		12	10		17	5	
Unknown	45	40	5		25	20		39	6	
Histology type				1.000			0.022			0.466
SCC	93	82	11		57	36		77	16	

ADC	6	6	0		1	5		4	2	
ASC	5	5	0		1	4		4	1	
tumor-specific survival, months										
Total/event/censored	104/14/90	93/12/81	11/2/9	0.558	59/4/55	45/10/35	0.018	82/9/73	22/5/17	0.160
Median survival	73.792±1.924	74.360±1.943	62.545±6.724		78.717±1.277	67.311±3.944		75.115±1.994	68.136±5.019	
95% CI	70.022-77.563	70.552-78.168	49.355-75.725		76.312-81.121	59.582-75.040		71.207-79.023	58.299-77.973	

VISTA: V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation; ICs: immune cells; SCC: squamous cell carcinoma; ADC: adenocarcinoma; ASC: adenosquamous carcinoma; event: cancer-related mortality; censored: patients lived to the date of the last visit or at the time of mortality due to non-CC-related causes; 95% IC: 95% confidence interval.

PBST, they were incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. A0208; Beyotime Institute of Biotechnology, Haimen, China) for 1 hour at room temperature in accordance with the manufacturer's recommendations. Sections were then rinsed thoroughly in PBST and later incubated with streptavidin peroxidase for 30 min at room temperature. Sections were then incubated with 1% (w/v) 3,3'-diaminobenzidine solution to develop color for 10 min at room temperature after attentive rinsing with PBST three times. Lastly, before being examined underaLeicaDM1000lightmicroscopeatamagnification of ×400 (Leica Microsystems GmbH, Wetzlar, Germany), sections were counterstained with 0.5% (w/v) hematoxylin at room temperature for 5 min and then mounted with neutral balsam. Cell cytoplasm was stained and considered to manifest VISTA-positive expression, and samples from healthy cervical tissues were used as negative controls. Females included in our study as healthy controls (age range, 30 to 61; median age, 45) were from West China Second University Hospital, Sichuan University with benign gynecological diseases including uterine myoma or cervical myoma. Patients were totally aware of the procedure of our study and the applications of their tissues, and written informed consent was signed prior to surgery, during which small pieces of normal cervical tissues were obtained from them.

Evaluation of VISTA protein expression

As described previously [10-12], evaluation of the immunohistochemical data was a reproducible semiquantitative method that took the staining intensity and proportion of positive tumor cells or immune cells into consideration. The process of evaluation was performed by three independent pathologists who were blinded to patient information. Based on the following criteria, the staining intensity was scored as 0 (no staining), 1 (weak, light yellow), 2 (moderate, yellow-brown), or 3 (strong, brown) (**Figure 1**). The percentage of VISTA-positive cells was scored as follows: 0, no stained cells; 1, 1-30% positive cells; 2, 31-60% positive cells; 3, 61-90% positive cells; 4, 91-100% positive cells. Scoring was performed in three distinct fields per slide, and the three scores were averaged and rounded off to the nearest whole number. Adding the score of intensity and percentage of VISTA-positive cells, the final immunoreactivity score (IS) was obtained of each sample. Overall scores were dichotomized into two groups: Low expression (IS<5); and high expression (IS≥5) in CC samples. Additionally, VISTA-positive immune cells (ICs) were also counted regarding the proportion of VISTA-positive immune cells per 200 ICs in intratumoral hotspot regions, where the highest density of VISTA-positive immune cells accumulated. With ≤35 VISTA-positive ICs, samples of CC were identified as exhibiting low VISTA expression in terms of the proportion of VISTA-positive ICs/200 ICs, >35 VISTA-positive ICs/200 ICs as high VISTA expression. Immunostaining of vascular endothelial cells was defined as present or absent.

Statistical analysis

The correlations of demographic and clinical variables with VISTA expression were assessed using Pearson's χ^2 test or Fisher's exact test for categorical variables. The Kaplan-Meier method and the log-rank test were adopted to assess the cumulative survival rate. Univariate and multivariate Cox proportional hazard models were used to evaluate the relationship between VISTA expression and clinicopathologic characteristics with overall survival. For all the tests, a two-tailed $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed by SPSS v22 software (IBM Corp, Armonk, NY, USA).

Results

Patient characteristics

A total of 104 patients with CC (aged 31-68 years, median 42 years) were included in our study. At the end of the follow-up period, 90 cases of survival were censored, while the other 14 events were CC-related mortalities. The average survival time of this group was 73.792±1.924 months (95% CI, 70.022-77.56 months) and the 5-year OS rate was 88.5% (**Figure 2**). The characteristics of the patients included in our study are summarized in **Table 1**.

VISTA expression in normal cervix and cervical cancer tissues detected by IHC

It is showed in **Figure 2** that positive immunostaining for VISTA was observed in the tumor

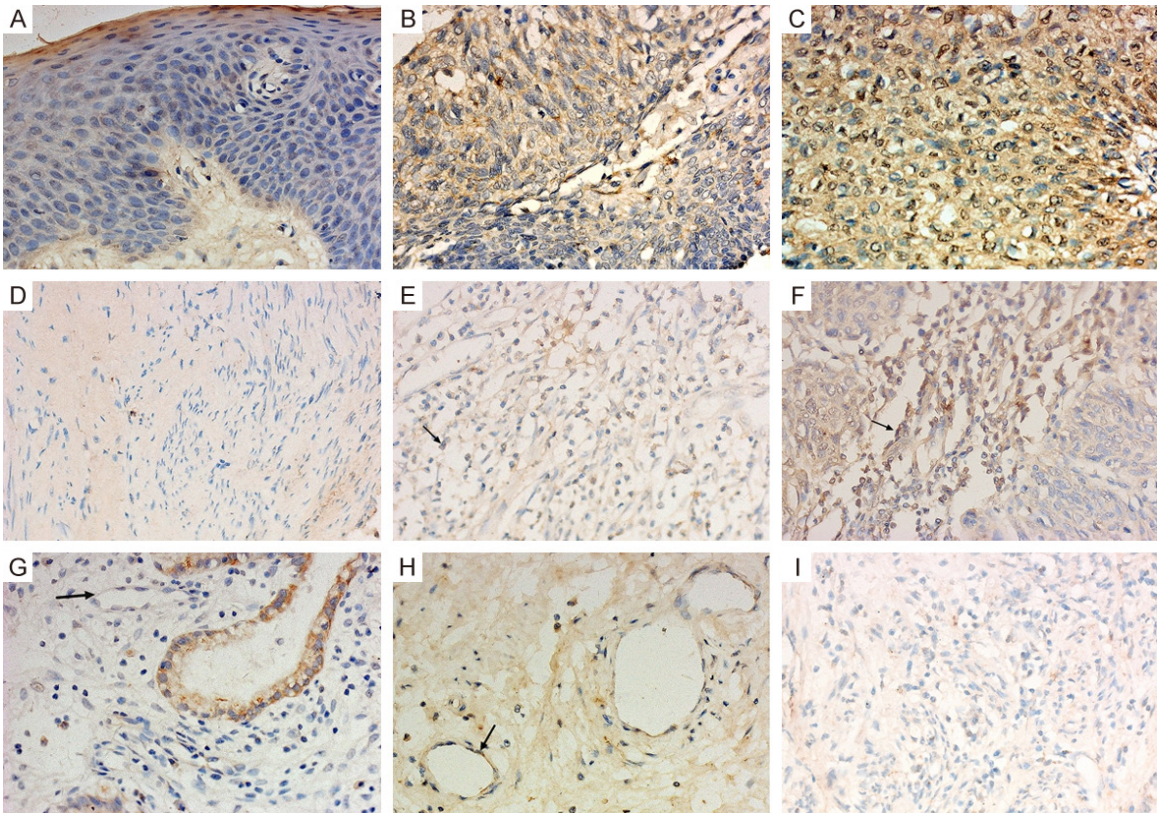

Figure 1. VISTA expression in cervical cancer. VISTA-positive tumor cells were observed in 32/104 cases (30.76%), and staining was graded as (A) weak, (B) moderate or (C) strong. VISTA-positive immune cells were detected in 85/104 cases (81.73%), and were classified as (D) absent, (E) low and (F) high. The expression of VISTA on vascular endothelial cells was categorized as (G) absent and (H) present, with 22/104 cases (21.15%) identified as present and the majority as absent. (I) Normal cervical samples were obtained to act as negative control. VISTA expression is pointed by the black arrow. Scale bar, 20 μm. VISTA, V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation.

cells, ICs and VECs in the CC tissues. Based on a combination of the staining intensity which ranges from negative (0) to strong (3) (median, 0) and the percentage of VISTA-positive cells which ranges from 0 to 95% (median, 1), the final IS was calculated respectively in the tumor cell, ICs and VECs. A total of 32 out of 104 cases (30.76%) showed a VISTA expression in tumor cells which were located predominantly in the adenoid structure of the tumor lesions. Only 11/104 cases (10.57%) were defined as high VISTA expression (IS≥5). In short, the percentage of positive tumor cells and the staining intensity were low in the cervical cancer cases in our study. In a great percentage of cases, the tumor-infiltrating ICs accumulated in the interstitial regions which were defined as intratumoral hotspot regions. VISTA-positive cells were detected in 85 cases (81.73%), and the proportion of VISTA-positive ICs/200 ICs ranged from 8 to 79 (median, 31). A total of 45/104 cases (43.27%) were classified as presenting high expression of VISTA-positive immune cells (>35 ICs/200 ICs). VISTA-positive VECs (yellow-brown circles under light microscopy) were identified in 22 cases (21.15%). Additionally, it was detected that several VISTA-positive ICs were sporadically distributed in the normal cervical tissue.

Clinical significance of VISTA expression in CC

As shown in **Table 1**, though none of the 11 clinicopathologic parameters reaches any statistical significance in VISTA expression on tumor cells, there was a trend that more cases with stromal invasion or with vaginal wall extension or with intravascular space involvement or tumor size larger than 4 cm or with advanced stages or with lymph node metastasis had an

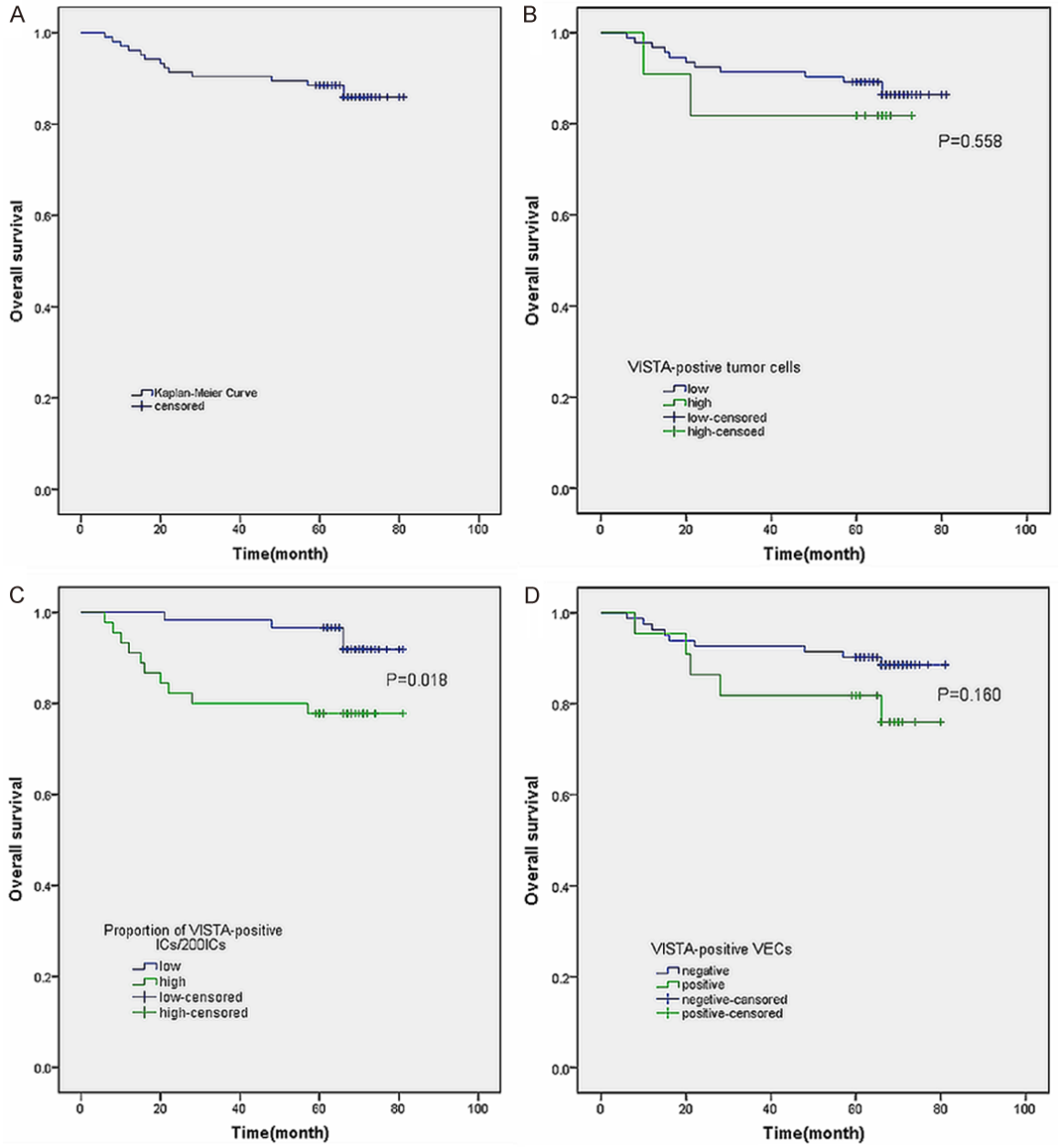

Figure 2. Kaplan-Meier survival analysis of patients with cervical cancer, subdivided according to the status of VISTA expression on tumor cells, ICs and VECs. (A) The median survival time of the 104 patients with CC was 73.792+1.924 months, and the 5-years OS rate is 88.5%. Notably, significant association was observed between VISTA expression on ICs and tumor-specific overall survival. High VISTA expression of (C) ICs/200 ICs negatively indicated shorter overall survival (median survival, 78.717+1.277 vs. 67.311+3.944 months; P=0.018), suggesting that VISTA expression on ICs is a factor of predicating poor prognosis in patients with cervical cancer. No significant association between tumor-specific overall survival and VISTA expression on (B) tumor cells (median survival, 74.360+1.943 vs. 62.545+6.724 months; P=0.558) or (D) VECs (median survival, 75.115+1.994 vs. 68.136+5.019 months; P=0.160) was detected. VISTA, V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation.

elevated ratio of presenting high expression of VISTA-positive tumor cells. The relatively low number of patients with high VISTA expression of tumor cells may have biased the results. Furthermore, the proportion of high VISTA-expression on ICs was higher in cases with lymph node metastasis compared with those without (P=0.005). High expression of VISTA on ICs was also associated with the tumor stage and histologic type, with significantly higher fre-

quencies of advanced stage (II+III+IV) (P=0.03) and non-SCC (ADC+ASC) (P=0.022) of cervical cancer. Similarly, VISTA expression in VECs was associated with LNM status and tumor stage. Higher frequency was shown in cases with an advanced stage (P=0.008) and lymph node metastasis (P=0.025). However, it was not statistically significant in associations between patient age, therapy, stromal invasion, vaginal wall extension, parametrial extension, intravascular space involvement, tumor differentiation, or tumor size and VISTA expression in ICs and VECs.

Survival analysis and prognostic significance of VISTA expression in CC

By constructing Kaplan-Meier curves, we performed OS analysis of cervical cancer patients to explore the potential association between VISTA expression and the prognosis of CC. Comparing patients with high VISTA expression in tumor cells, ICs and VECs with patients with lower VISTA expression, the median survival time was clearly decreased (74.360±1.943 vs. 62.545±6.724 months in VISTA-positive tumor cells; 78.717±1.1.277 vs. 67.311±3.944 months in VISTA-positive ICs/200 ICs; 75.115± 1.994 vs. 68.136±5.019 months in VISTA-positive VECs). As indicated in **Table 1**, median survival time was 67.311±3.944 months and the 5-year OS was 77.8% for the 45 patients with high VISTA expression in ICs/200 ICs, significantly lower than the 59 patients with low VISTA expression (MST=78.717±1.1.277 months, 5-year OS=96.6%; P=0.018). However, there was no statistically significant difference in the 5-year OS rate of patients with high VISTA expression (n=11) in tumor cells compared with those with low VISTA expression (n=93; 81.8% vs. 89.2%; P=0.558). Similarly, there was no significant difference in the 5-year OS rate of patients with positive-VISTA expression in VECs compared with those with negative-VISTA expression (81.8% vs. 90.2%; P=0.160). All the results above showed that expression of VISTA in ICs is negatively associated with OS of CC. The correlation of 11 clinicopathologic parameters and OS in patients with CC was estimated through a univariate Cox regression model. In the univariate Cox regression analysis, it was noted that LNM (HR=9.346, P< 0.001), stage (HR=3.831, P=0.012), histologic type (HR=4.386, P=0.013) and VISTA-positive ICs/200 ICs (HR=3.704, P=0.027) were correlated with poorer OS of patients with CC (**Table 2**). Using the Cox regression model in multivariate analysis for the three factors, it was revealed that LNM may play a role in independently predicting the poor prognosis of CC (HR=8.333; P=0.012).

Discussion

Cervical cancer is the fourth most common malignancy in women worldwide [13]. Though preventable through early diagnosis and intervention of precancerous lesions, once it progresses to the metastatic or recurrent status, the prognosis is devastating. Since the utilizing of antiangiogenic therapy, treatment for relapsed/advanced disease has improved over the last 5 years. But the median overall survival for advanced cervical cancer is 16.8 months and the 5-year overall survival for all stages is 68% in spite of modest advances achieved [4]. It is imperative to search new therapeutic approaches and novel biomarkers that can indicate prognosis and serve as therapy targets, especially for late-stage cervical cancer.

VISTA, a novel negative checkpoint regulator, is predominantly expressed on hematopoietic cells, with the greatest densities on myeloid and granulocytic cells, and weaker expression on T cells. It is a new member of the Ig superfamily that contains an Ig-V domain with distant sequence similar to PD-L1 [14]. The expression of VISTA on myeloid APCs is inhibitory for T cell responses in vitro, and overexpression on tumor cells impairs protective antitumor immunization in vaccinated mice [6]. The role of VISTA has been elucidated previously in a group of patients with human gastric carcinoma, oral squamous cell carcinoma and ovarian cancer [8, 10, 15]. However, no data concerned about the role of VISTA in human CC are currently available. Our study is the first one to investigate the expression of VISTA in tumor cells, ICs, and VECs in patients with CC. To improve our understanding of the role of VISTA in human CC, we perform an analysis of association between VISTA-expression and clinicopathologic features, and relevance of VISTA-expression and OS.

Immunotherapy has been proven effective in several cancers, so diverse immune checkpoint inhibitors are currently authorized to treat melanoma, kidney cancer, and lung cancer [4]. There are also many attempts to investigate

Table 2. Univariate and multivariate Cox analyses for cancer-specific overall survival in patients with cervical cancer (n=104)

Characteristics	N	Univariate analysis		Mutivariate analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age, years		0.596 (0166-2.142)	0.428		
<50	88				
≥50	16				
Therapy		0.351 (0.117-1.049)	0.061		
Operation	19				
Operation + others	85				
Stromal invasion		1.441 (0.503-4.124)	0.496		
Negetive	44				
Positive	60				
Vaginal wall extension		2.545 (0.850-7.576)	0.095		
Negetive	84				
Positive	20				
Parametrial extension		1.287 (0.451-3.676)	0.638		
Negetive	58				
Positive	46				
Intravascular space involvement		1.496 (0.500-4.473)	0.471		
Negetive	58				
Positive	46				
Lymph node metastasis		9.346 (3.195-27.778)	<0.001	8.333 (1.603-43.478)	0.012
Negetive	87				
Positive	17				
Stage		3.831 (1.337-10.989)	0.012	0,690 (0.139-3.413)	0.649
Situ +I	80				
II+III+IV	24				
Differentiation		1.440 (0.402-5.163)	0.576		
Poor	75				
Otherwise*	29				
Histologic type		4.386 (1.366-14.085)	0.013	2.469 (0.697-8.772)	0.161
SCC	93				
ADC+ASC	11				
VISTA-positive tumor cells		1.558 (0.348-6.993)	0.562		
Low	93				
High	11				
VISTA-positive ICs/200 ICs		3.704 (1.160-11.765)	0.027	2.653 (0.800-8.772)	0.111
Low	59				
High	45				
VISTA-positive VECs		2.151 (0.720-6.410)	0.170		
Negative	82				
Positive	22				

*Otherwise: moderately-differential and well-differential carcinoma; VISTA: V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation; HR: hazard ratio; 95% IC: 95% confidence interval; SCC, squamous cell carcinoma; ADC+ASC, adenocarcinoma and, adenosquamous carcinoma; ICs, immune cells; VECs, Vascular endothelial cells.

this approach in cervical cancer and the possibility that immunotherapy may take part in the therapy for CC. Usually, the complexity of tumor-infiltrating immune cells (TICs) widely affects

the immune status and biological behavior of the host and plays a pivotal role in the response to immunotherapy [16]. The tumor microenvironment (TME) of cervical cancer is closely related to its etiology. Due to long-term viral infection, cervical cancer masses are infiltrated by various inflammatory immune cells [17, 18]. A study about TME of cervical cancer showed that most of the enriched infiltrates were CD8+ T cells and macrophages. CD8+ T cells are key cytotoxic lymphocytes that target cancer [4]. A previous report indicated that the mean levels of CD8+ T cells, which may perform immune surveillance against cancer, were higher in cervical cancer tissue than in peripheral blood in mice [19].

VISTA, homologous to the B7 family ligand PD-L1, with greatest mRNA detected in either hematopoietic tissues such as spleen, lymph nodes, and peripheral blood or those tissues with significant infiltration by leukocytes, exerts its immunosuppressive activities on resting and activated human CD4+ and CD8+ T cells in vitro and in vivo [6, 14]. Interestingly, in human gastric carcinoma and ovarian cancer, VISTA expression on tumor cells has also been detected [10, 15]. In our study, tumor-infiltrating VISTA-positive ICs were easy to detect in CC tissues, with almost one-half (43.27%) defined as exhibiting high VISTA expression. Additionally, VISTA expression on tumor cells cytoplasmically was observed in CC tissues, with only a small group (10.58%) exhibiting high VISTA expression, and no apparent association between expression level of VISTA and any of the clinicopathological features. It is worth mentioning that high VISTA expression on ICs and VECs was closely related to advanced-stage CC and the presence of LNM. As is well known, either the stage of CC, or LNM is prognostic factor widely used in clinic. The information above suggests that VISTA is probably involved in CC progression. Similarly, Wu et al [8] also demonstrated that the expression of VISTA was associated with lymph node status in human oral squamous cell carcinoma. By preventing promiscuous resting T-cell responses to self-antigens [7], activated VISTA plays a role in tumor cells evasion from the immune surveillance. It was suggested that VISTA expression was associated with the expression of the PD-L1 in gastric cancer, indicating that VISTA cooperates with PD-L1 in the mechanism of immune evasion [10]. Therefore, the association of advanced disease stage with high VISTA expression in CC may be explained by the protective role of this molecule to free VISTA-positive cells from the immune regulations that impair tumor growth and metastasis [22]. Other studies showed that the increased expression of cleaved caspase-3 [23], the PD-L1 [13], the expression of TIM-3 and the increased CTLA-4 expression and reduced CD28 expression [21] in CC have also been identified as predictive factors for CC prognosis. The Kaplan-Meier analysis of our study suggested that the high expression VISTA on ICs was negatively correlated with median survival in CC patients. Nevertheless, in the univariate and multivariate Cox regression analyses, apart from LNM that independently predicted poor OS, there was no significant association between VISTA expression and OS of patients with CC. May the small cohort (n=104) of patients included in these analyses biased the results. Enough samples are required to draw the conclusion whether VISTA expression participates in the progression of CC. However, there are other studies that indicated that the expression of VISTA alone was not related to OS, but collaboratively predicating OS with CD8+ T cells in human oral squamous cell carcinoma [8]. Although there was no association between VISTA expression and OS in our study, positive VISTA expression elevated with late stage and LNM, indicating VISTA as a potential predictor in CC progression. Hence, VISTA may be considered as a candidate biomarker of advanced stage and lymph node status in CC. Furthermore, due to long-term viral infection, cervical cancer are infiltrated by a wide range of immune cells, of which some participate in immune evasion. Therefore, VISTA can be included as a potential immunotherapeutic target for CC. The underlying mechanisms of immunotherapy targeting immune checkpoints, including PD-L1, CTLA-4 and VISTA remain to be confirmed, pre-clinical and clinical trials have offered great excitement [13, 20].

However, it is challenging to apply immunotherapy in CC due to the difficulty in classification of the patients who will suit the immune checkpoint therapy. So before starting immunotherapy, the measurement of VISTA-expression level in the tumor tissue may be a favorabe potential biomarker used to assess patients for

inclusion in VISTA-associated therapy and contribute to the formulation of individualized intensive treatment strategies.

In nutshell, VISTA-positive tumor cells, ICs and VECs were detected in CC tissues. Additionally, VISTA expression on ICs and VECs was negatively associated with advanced CC stage and LNM, indicating that this molecule may be engaged in the progression of CC. In our study, LNM was identified as an independent indicator of poor survival in CC. Though not suitable as a prognostic predictor of CC, VISTA can still serve as a potential biomarker for inclusion of patients for VISTA-associated immunotherapy in the future either as a single target or in combination with other immunotherapeutic strategies. Further investigations and comprehensive explorations should be performed to deepen our understanding of the predictive value of VISTA.

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Disclosure of conflict of interest

None.

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