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High-expression of V-domain Imuunoglobulin Suppressor of T-cell Activation (VISTA) Is Correlated with Advanced Pathological Features in Patients with Pancreatic Ductal Adenocarcinoma

Hamid Nickho[1,2], Reza Falak[1,2], Fereshteh Rezagholizadeh[2,3,4], Majid Khoshmirsafa[1,2], Mohammad Taghi Joghataei[3,4,5], Shabnam Mollazadeh Ghomi[6,7], and Elahe Safari[1,2]

[1] Immunology Research Center, Institute of Immunology and Infectious Disease, Iran University of Medical Sciences, Tehran, Iran
[2] Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[3] Cellular and Molecular Research Centre, Iran University of Medical Sciences, Tehran, Iran
[4] Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
[5] Department of Innovation in Medical Education (DIME), Faculty of Medicine, University of Ottawa, Ottawa, Canada
[6] Department of Pathology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[7] Immunology Board for Transplantation and Cell-Based Therapeutics (ImmunoTACT), Universal Scientific Education and Research Network (USERN), Tehran, Iran

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ABSTRACT

V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) seems a promising immune checkpoint target in cancer treatment; however, its prognostic significance in pancreatic ductal adenocarcinoma (PDAC) remains unknown.

Herein, 29 fresh PDAC tissue samples were used to evaluate the mRNA expression level of VISTA by real-time polymerase chain reaction (PCR). Besides, 40 formalin-fixed paraffin-embedded PDAC tissues were collected to evaluate VISTA protein expression by immunohistochemistry.

Real-time PCR indicated that high expression of VISTA was significantly correlated with advanced stages of the cancer, based on the tumor/node/metastasis (TNM) stagingand tumor cell differentiation. Immunohistochemistry results also showed significant correlation of the elevated cytoplasmic expression of VISTA with advanced TNM stages, older age of the patients and was a worsening indicator, regarding the disease-specific survival.

In conclusion, we found that the expression levels of VISTA can be a potential prognostic biomarker in PDAC patients and its elevated levels are correlated with poor prognostic outcomes.

Keywords: Immune checkpoint; Pancreatic cancer; Pancreatic ductal adenocarcinoma; V-domain immunoglobulin suppressor; T cell activation

Corresponding Author: Elahe Safari PhD;
Immunology Research Center, Institute of Immunology and Infectious Disease, Iran University of Medical Sciences, Tehran, Postal code: 1449614535, Iran. Tel: (+98 912) 5975 038, Fax: (+98 21) 8862 2625, E-mail: safari.e@iums.ac.ir

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent histopathological type of pancreatic

cancer. Despite the recent advances in its treatment and diagnosis, PDAC is still among the world's 7 most common cancers and its control requires special reconsiderations.[1] Radiation therapy, chemotherapy, and targeted therapy are the current available therapeutic options for PDAC.[2] Therefore, oncologists try to find new target molecules for its early diagnosis, prognosis and treatment. PDAC tumor microenvironment (TME) shows a high amount of tumor-infiltrating immune cells and studying the characteristics of these cancer cells and their targets can lead us toward novel immunotherapy methods.[3]

Recently, immune checkpoint inhibitory antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death 1 (PD-1) molecule, and its ligand (PD-L1) have been developed for the monotherapy of various cancers and are available as ipilimumab, pembrolizumab, atezolizumab, receptively.[4,5] However, similar to other traditional therapies, focusing merely on the blockade of each of them, due to the diversity of resistance mechanisms and possible toxic side effects may result in poor effects in the treatment of PDAC.[6]

One of the inhibitory immune checkpoint molecules that has been newly considered in tumor immunotherapy is the V-domain immunoglobulin suppressor of T-cell activation (VISTA), which is expressed in TME cells.[7] VISTA is an immunoregulatory receptor, which is also expressed on myeloid cells, dendritic cells, T cells, and natural killer (NK) cells; transmitting inhibitory signals to hinder T cell activation, proliferation, and cytokine release.[8] Based on the high expression of VISTA in pancreatic cancer, this molecule was introduced as a potential immunotherapy target in this cancer.[9] Moreover, tumor-associated macrophages are abundantly found in the TME of patients with PDAC [10] and express VISTA higher than T cells.[11]

Even though several studies have demonstrated the association of VISTA with various cancers, its association with PDAC is still not clear. Thus, the aim of this study is to better understand the landscape of immune cells in resected PDAC by investigating the presence of VISTA in cancer cells and determining their prognostic relevance in terms of overall survival (OS).

MATERIALS AND METHODS

Patients and Samples

To evaluate VISTA expression at the mRNA level, fresh tumor tissue samples were collected from 29 PDAC patients who underwent pancreas surgery during 2018 to 2020 at Firozgar Hospital, Tehran, Iran. Simultaneously, tumor-adjacent tissue samples from each of the patients were studied as controls. All samples were preserved in RNA Later (Qiagen, Phoenix, AZ, USA) solution at −70. The samples from early-stage cancers and patients who have received concurrent chemotherapy were omitted. Patient's clinical and demographic data, including age, sex, tumor size, distant metastasis, and tumor differentiation were also collected from available digital records or paper files of the patients.

To evaluate VISTA expression at the protein level, 40 formalin-fixed paraffin-embedded blocks (FFPE) from PDAC patients who were diagnosed from 2013 to 2018 were recruited from the pathology department of the hospital. The histopathological grade was determined based on tumor/node/metastasis (TNM), lymph node (LN) metastasis, distant metastasis, vascular invasion (VI), tumor size, and recurrence through hematoxylin-eosin-staining (H&E), and the patients' recorded clinical or demographic data. Human tonsil tissue was also used as a positive control in the experiments. Ethical consent was obtained from all patients participating in this study and the present research was approved by the Research Ethics Committee of Iran University of Medical Sciences (Ethics code: IR.IUMS.FMD.REC.1399.161).

The distance between the date of surgery to the date of the patient's death was considered as disease-specific survival (DSS). Moreover, the break between the first surgery and the last follow-up visit, if the patient had no evidence of metastasis, recurrence, or disease-related death was considered progression-free survival (PFS). Tumor grade and cancer stage were considered based on the classification suggested by the FIGO-cancer Report 2018 and the criteria proposed by the College of American Pathologists (CAP) 2018.

Tissue Microarray

For the preparation of PDAC tissue microarray (TMA) blocks, the H&E-stained slides were examined by a pathologist to select and mark the most representative areas of tumors on the slides. The selected regions were punched from the original tissue blocks into the recipient TMA blocks, using a precision arraying tool. Finally, three slide copies from each TMA block were obtained. To compare the expression patterns of VISTA in each TMA block, marginal tumor

tissue samples, together with tumor tissue samples were included in the blocks.

Immunohistochemistry (IHC) Staining

Tissue immunohistochemistry and gene expression analysis are essential instruments for determining gene and protein expression patterns in cancer studies. To better understand the essential mechanisms of biological processes, it is necessary to assign whether the changes observed in mRNA can also be displayed in the translated protein, and to precisely identify the cell types that show these changes. mRNA and protein levels typically display rational association, incorporating both kinds of data can disclose exciting biology and is a crucial phase in clarifying our understanding of the principles of gene expression regulation. TMA sections were deparaffinized at 60°C for 30 minutes in the oven, immersed in xylene for rehydration for 10 minutes, and followed by immersion in serial dilutions of alcohol (70%, 96%, and 100%; respectively) for 5 minutes. For curbing the endogenous peroxidase activity, TMA slides were incubated with 3% H_2O_2 for 20 minutes at 25°C. Epitope retrieval was performed by autoclaving tissue sections in sodium citrate buffer (pH 6.0) for 20 minutes. After cooling, the slides were washed 3 times for a total of 5 minutes in tris-buffered saline (TBS, pH 7.4). Then the sections were blocked with a protein blocker (Dako, CA, USA) within 20 minutes. Afterward, all sections were incubated with a specific primary antibody against VISTA (clone D1L2G, 1:200; Cell-Signaling Technology, MA, USA), overnight at 4°C. After 3 washing steps in TBS, sections were incubated with anti-mouse envision IgG–HRP (EnVision, Dako, USA) as the secondary antibody for one hour. For visualization, chromogen 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, USA) as the substrate for treating the FFPE slides was used for 5 min at 25°C. The slides were then counterstained with hematoxylin (Dako, USA) and analyzed and imaged using a light microscope. VISTA expression was distinguished in the membrane or cytoplasm of tumor cells and tumor-infiltrating immune cells.

RNA Extraction, Reverse Transcription, and Real-time polymerase chain reaction

Fresh tissue samples which were collected and kept in RNA later were finally homogenized and lysed in TRIzol (Sinaclon, Tehran, Iran). Then, the total RNA was extracted by RNX-Plus (Sinaclon, Tehran, Iran), based on the manufacturer's protocol. cDNA synthesis was carried out on 1 μg of total RNA using the miScript Reverse Transcription Kit (Takara, Cat. #RR037A, USA) according to the manufacturer's protocols. Quantitative real-time polymerase chain reaction (PCR) assay was performed on the Qiagen Rotor Gen Q system (QIAGEN, Hilden, Germany) using the SYBR green Master Mix (Biofact, Daejeon, Korea). The cycling settings were adjusted for an initial 15-minute denaturation at 95°C and 40 repeated cycles (15 seconds at 95°C, 35 seconds at 59°C, and 45 seconds at 72°C). At the end of each PCR test, a melting curve was generated to confirm that merely the target product was replicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was considered as housekeeping gene. The expression level of VISTA mRNA was identified in 29 pairs of PDAC and marginal tissue samples. The primer sequences were mentioned in suplementrary table 1. The expression levels of VISTA mRNA were normalized against GAPDH levels based on the $2^{-\Delta\Delta Ct}$ approach.

Scoring of IHC Slides

In the PDAC tissue samples, VISTA was expressed at numerous severities in the membranous and cytoplasmic areas. The intensity of the immunostaining was scored using a 4-point scale as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The percentages of positive tumor cells were scored in the range of 0 to 100. The total score was obtained by multiplying the staining intensity by the histochemical score (H-score) and the percentage of positive cells. Each score was then given a final score of 0 to 300. The approximate mean of H-scores of VISTA (H-score=100) was used as a cutoff point to classify the tumors with high and low expressions.

Statistical Analysis

SPSS version 26.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA) was used for plotting the graphs. The chi-square $\chi 2$ and Spearman's correlation test were performed to examine the association between the expression of VISTA in the TME and clinicopathological parameters. Mann–Whitney U and Kruskal-Walli's tests were used for pairwise comparisons between groups. The consequences of this study were plotted via the Kaplan-Meier method and compared using log-rank tests to compare the estimated curves between the groups with

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95% confidence intervals (CI). the Cox proportional hazards regression was used for univariate analyses. Also, $p<0.05$ was considered statistically significant.

RESULTS

Characteristics of the Patients

We studied 29 fresh tissue samples from PDAC patients (20 male and 9 female; male/female ratio=2.2). The median age of patients was 59 years (SD=12.6), ranging from 24 to 78 years; 17 patients (58/2%) were younger than 59 years and 12 patients (41.8%) were older. Nine patients (31%) were diagnosed at stage I, 12 (41.3%) at stage II, and 8 (27.5%) at stage III.

Regarding adenocarcinoma grading, pathology results indicated that 3 patients (10.3%) had low-grade, 9 patients (31%) had moderately differentiated, and 17 patients (58.6%) had poorly differentiated PDAC. Metastasis to LN, VI, and NI were found in 21 (72.4%), 13 (44.8%) and 15 (51.7%) of the subjects, respectively.

VISTA Gene Expression and Its Correlation with the Clinicopathological Findings

Real-time PCR results showed that PDAC patients had significantly higher levels of mRNA expression for VISTA than controls ($p<0.001$) (Figure 1).

Mann–Whitney U test was applied to assess the discrepancy between the median expressions of VISTA between the groups, which indicated a significant difference regarding the mean of mRNA level and tumor differentiation in the groups ($p=0.046$) (Table 1); representing a positive correlation between the high-level expression of VISTA and an increased tumor differentiation. Furthermore, the results represented a significant correlation between the expression levels of VISTA mRNA and PDAC stages, particularly stage III ($p=0.025$) (Figure 2). We found a positive association between the PDAC stages and VISTA gene expression.

Table 1. The association between VISTA mRNA expression and clinicopathological parameters of fresh tissue pancreatic ductal adenocarcinoma samples. Data are presented as fold change (min; max). *p*=<0.05

Variables		mRNA Expression of VISTA	*p*
Age (year)	≤ median age (n= 17)	1.38 (0.65; 5.85)	0.563*
	> median age (n= 12)	1.79 (1.05; 6.02)	
Gender	Male (n= 20)	2.41 (1.21; 4.31)	0.632*
	Female (n= 9)	1.93 (0.91; 4.02)	
Tumor differentiation	Well (n= 3)	1.23 (0.82; 3.05)	**0.046***
	Moderate (n= 9)	1.36 (0.91; 3.89)	
	Poor (n= 17)	3.63 (1.83; 6.43)	
Vascular invasion	No (n= 16)	2.41 (1.65; 4.45)	0.165*
	Yes (n= 13)	2.63 (1.41; 3.85)	
Lymph node invasion	No (n= 8)	1.34 (0.31; 3.71)	0.086*
	Yes (n= 21)	2.11 (1.15; 4.31)	
Neural invasion	No (n= 14)	1.73 (1.02; 3.54)	0.234*
	Yes (n= 15)	1.52 (0.91; 3.09)	
Stage	I	1.25 (0.45; 2.26)	**0.025****
	II	1.68 (0.86; 3.20)	
	III	5.74 (2.56; 7.52) [a]	

VISTA: V-domain Imuunoglobulin suppressor of T-cell activation, *Data analysis was done using the Mann-Whitney U test. **Data analysis was done using the Kruskal-Wallis test. Data are presented as fold change (min; max). [a]$p=0.001$ compared to Stage I

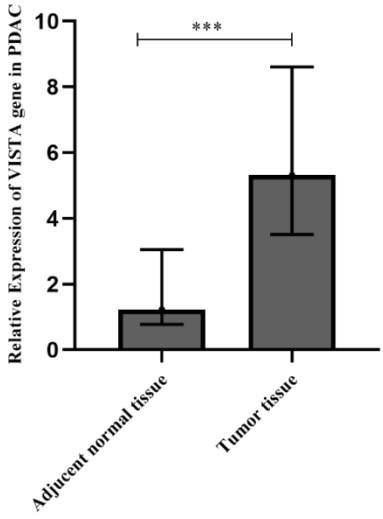

Figure 1. The mRNA expression levels of V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) in pancreatic ductal adenocarcinoma (PDAC) cancer.

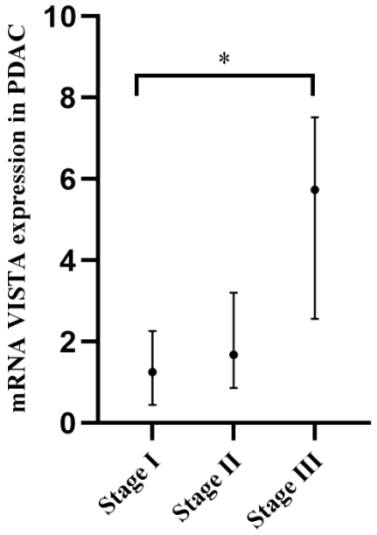

Figure 2. Gene expression level of V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) mRNA in different stages of pancreatic ductal adenocarcinoma (PDAC).

The mRNA expression levels of VISTA were studied in fresh PDAC tissues using real-time PCR. We saw that there was a difference between the expression levels in different cancer stages. Mann–Whitney U test indicated a significant difference between the median of VISTA mRNA expression between stages I and III (p=0.025). There were no statistically significant differences in the mean level of VISTA mRNA expression among other stages.

Demographic and Pathological Analysis of FFPE Samples

We studied 40 FFPE tissue samples from PDAC patients. The studied tissue blocks were from 20 male and 20 female patients (male/female ratio=1). The median age of the patients was 62 (SD=9.3), ranging from 44- to 85 years.

Tumor tissue diameters ranged from 1 to 7 cm, with a median tumor size of 3.8 cm. Accordingly, 28 cases

(72.5%) had tumors with diameter≤3.8 cm; and 12 (41.3%) suffered from a tumor>3.8 cm.

Based on FIGO staging distribution, the patients were in the following condition: 14 (35%) were classified as Stage I, 20 (50%) as Stage II, 4 (10%) as Stage III, and 4 (10%) as Stage IV.

Based on cancer grading, the patients were in the following condition: 10 patients (50.0%) were classified as grade I, 16 patients (40.0%) as grade II, and 2 patients (5.0%) as grade III. We did not find any histological grading data for 2 of the patients (5.0%).

The lymph vascular invasion (LVI) and perineural invasion (PNI) were found in 11 (27.5%) and 21 (52.5%) cases, respectively.

VISTA Protein Expression

To determine the association between the expression level of VISTA protein and clinical findings, the expression level of VISTA was evaluated on TMA sections prepared from 40 FFPE samples using IHC. We used 3 scoring systems, including H-score, intensity of staining, and percentage of positive tumors during the study (Table 2). The expression of VISTA level was assessed in tumor tissues and their marginal regions. VISTA-positive immunostaining was mainly detected in the cytoplasmic and cell membrane regions. Intensity of VISTA positivity was detected in cytoplasmic regions of 39 (95.0%) and cell membranes of 11 (27.5%) PDAC samples. The mean expression level of VISTA was 14.2 in the cytoplasm and 30 in the cell membrane regions of the cancer cells. According to the established H-score (cut off=100), 37 patients (92.5%) showed lower cytoplasmic expression, and 3 patients (7.5%) had higher expression of VISTA. Also, regarding the membranous expression of VISTA, 36 patients (90%) showed a low expression rate and 4 patients (10%) showed a high expression rate, respectively (Table 2). The expression of VISTA was observed in the membranous and cytoplasmic regions of all marginal tissues. Representative stained samples of VISTA are shown in Figure 3.

Table 2. Cell membrane (CM) and cytoplasmic V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) expression (Intensity of staining, percentage of positive tumor cells, and H-score) in pancreatic ductal adenocarcinoma tissue and its margins.

Scoring System	Cytoplasmic Expression N (%)		Membranous Expression N (%)	
	Tumor	Margins	Tumor	Margins
Intensity of staining				
Negative (0)	2 (5.0)	4 (10.0)	27 (72.5)	8 (20.0)
Weak (+1)	19 (47.5)	4 (10.0)	4 (10.0)	1 (2.5)
Moderate (+2)	15 (37.5)	1 (2.5)	3 (7.5)	0 (0.0)
Strong (+3)	5 (10.0)	0 (0.0)	4 (10.0)	0 (0.0)
Not identified	0 (0.0)	0 (0.0)	0 (0)	0 (0.0)
Percentage of positive tumor cells				
< 25%	30 (75.0)	9 (100.0)	36 (90.0)	9 (100.0)
25–50%	7 (17.5)	0 (0.0)	3 (7.5)	0 (0.0)
> 50%	3 (7.5)	0 (0.0)	0 (0.0)	0 (0.0)
Not identified	0 (0.0)	0 (0.0)	1 (2.5)	0 (0.0)
H-score cut off =100				
Low	36 (90.0)	9 (100.0)	37 (92.5)	9 (100.0)
High	4 (10.0)	0 (0.0)	3 (7.5)	0 (0.0)
Total	40 (100)	9 (100)	40 (100)	9 (100)

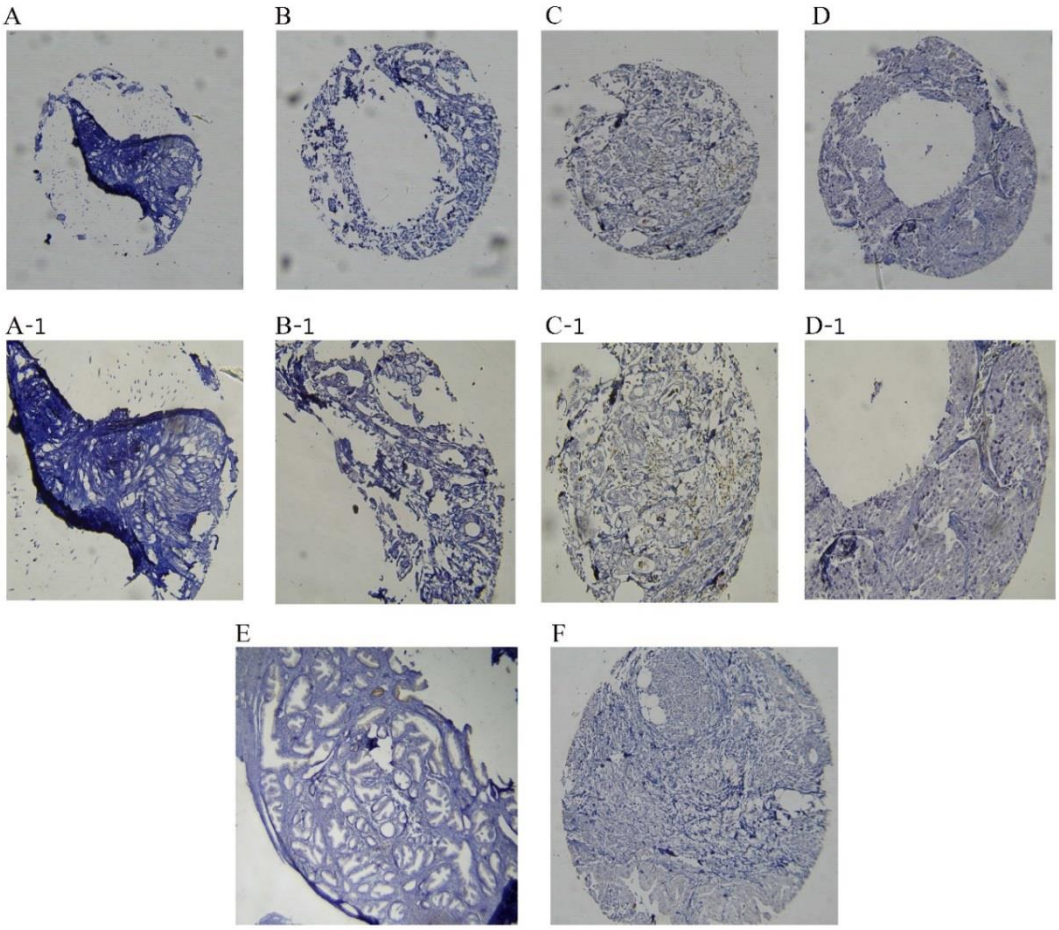

Figure 3. Immunohistochemical (IHC) staining of V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) in human pancreatic ductal adenocarcinoma (PDAC). PDAC tissue microarray (TMA) blocks were stained with anti-VISTA antibodies. expression of VISTA at various levels: Stage I (A), Stage II (B), Stage III (C) and Stage IV (D) in magnification of × 100; Stage I (A-1), Stage II (B-1), Stage III (C-1) and Stage IV (D-1) in magnification×200. IHC staining of prostate tissue was presented as positive (E) and negative (F) controls.

Associations of VISTA Expression Pattern with Clinicopathological Features

Pearson's chi-square test indicated a correlation between VISTA expression and clinicopathological features of the PDAC patients. VISTA protein expression was determined based on cytoplasmic and cell membrane expression levels (intensity of staining and H-score). VISTA CM expression in PDAC tissues had a significant association with pathological tumor differentiation (p=0.04) and the age of the patients (p=0.05) (Table 4).

The Mann–Whitney U test was used to analyze the correlations between the difference of the FIGO stages, revealing a significant difference in the mean level of VISTA protein expression between stages I and IV (p=0.038), and stages III and IV (p=0.043). We did not find any statistically significant differences between other FIGO stages of PDAC cases.

Spearman's and Pearson's $\chi 2$ test revealed a significant difference in the association of cytoplasmic expression of VISTA and pathological FIGO stages (p=0.041), as well as patients age (p=0.03) (Table 3).

No correlation was found between cytoplasmic and cell membrane expression of VISTA protein and the other clinicopathological features.

Table 3. The result of Pearson's χ2 and Spearman's test association between cytoplasmic V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) expression and clinicopathological parameters of pancreatic ductal adenocarcinoma samples (intensity of staining and H-score). H-score indicates Histological score. *p* values in bold are statistically significant.

Patients and Tumor Characteristics	Total Samples N (%)	Intensity of Staining N (%) Cytoplasmic				*p*	H-score N (%) Cytoplasmic		*p*
		0	1+	2+	3+		Low	High	
Median age, years (Range)	62 (44-85)								
≤ Median age	21 (52.5)	1(50)	10 (52.6)	6 (40)	4 (100)	**0.05**	18 (50)	3 (75)	0.72
> Median age	19 (47.5)	1(50)	9 (47.3)	9 (60)	0 (0)		18 (50)	1 (25)	
Gender									
Male	20 (50)	1(50)	8 (42.1)	9 (60)	2 (50)	0.78	18 (50)	2 (50)	0.62
Female	20 (50)	1(50)	11 (57.8)	6 (40)	2 (50)		18 (50)	2 (50)	
Mean tumor size (cm)	3.8 (1-7)								
> Median	22 (55)	2 (100)	12 (63.2)	6 (40)	2 (50)	0.66	20 (55.5)	2 (50)	0.36
≤ Median	18(45)	0(0)	7 (36.8)	9 (60)	2 (50)		16 (44.4)	2 (50)	
Tumor differentiation									
Well	20 (50)	1(50)	12 (63.1)	4 (26.6)	3 (75)	0.15	17 (85)	3 (15)	0.42
Moderate	16 (40)	1(50)	5 (26.3)	10 (66.6)	0 (60)		16 (100)	0 (0)	
Poor	2 (5)	0(60)	0 (60)	1 (6.7)	1 (25)		1 (50)	1 (50)	
Not identified	2 (5)	0(0)	2 (10.5)	0 (0)	0 (0)		1 (50)	1 (50)	
Lymph vascular invasion									
Present	11 (27.5)	0 (0)	5 (26.3)	5 (33.3)	1 (25)	0.81	9 (81.8)	2 (18.1)	0.35
Absent	18 (45)	2 (100)	8 (42.1)	6 (40)	2 (50)		17 (94.4)	1 (5.5)	
Not identified	11 (27.5)	0 (0)	6 (31.5)	4 (26.6)	1 (25)		10 (90.9)	1 (9)	
Perineural invasion									
Present	21(52.5)	0 (0)	12 (63.1)	6 (42)	3 (75)	0.24	18 (85.7)	3 (14.3)	0.55
Absent	14 (35)	2 (100)	5 (26.3)	7 (46.6)	0 (0)		14 (100)	0 (0)	
Not identified	5 (12.5)	0 (0)	2 (10.6)	2 (13.3)	1 (25)		4 (80)	1 (20)	
Stage									
I	14 (35)	1 (50)	7 (36.8)	5 (26.3)	1 (25)	0.51	13 (92.8)	1 (7.1)	**0.041**
II	18 (45)	1 (50)	7 (36.8)	9 (60)	1 (25)		17 (94.4)	1 (5.5)	
III	2 (5)	0 (0)	2 (10.5)	0 (0)	0 (0)		2 (100)	0 (0)	
IV	6 (15)	0 (0)	3 (15.7)	1 (6.6)	2 (50)		4 (66.6)	2 (33.3)	
Distant metastasis									
yes	21 (52.5)	2 (100)	9 (47.3)	8 (53.3)	2 (50)	0.56	19 (90.4)	2 (9.5)	0.19
no	19 (47.5)	0 (0)	10 (52.6)	7 (46.6)	2 (50)		17 (89.4)	2 (10.5)	

Table 4. The result of Pearson's χ2 and Spearman's test association between cell membrane (CM) of V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) expression and clinicopathological parameters of pancreatic cancer (PDAC) samples (Intensity of staining and H-score). H-score indicates Histological score. P Values in bold are statistically significant.

Patients and Tumor Characteristics	Total Samples N (%)	Intensity of Staining N (%) CM				*p*	H-score N (%) CM		*p*
		0	1+	2+	3+		low	high	
Median age, years (Range)	62 (44-85)								
≤ Median age	21 (52.5)	16 (55.1)	0 (0)	2 (66.6)	3 (75)	**0.031**	19 (51.3)	2 (66.6)	0.06
> Median age	19 (47.5)	13 (44.8)	4 (100)	1 (33.3)	1 (25)		18 (48.6)	1 (33.3)	
Gender									
Male	20 (50)	13 (44.8)	4 (100)	1 (33.3)	2 (50)	0.2	18 (48.6)	1 (33.3)	0.51
Female	20 (50)	16 (55.1)	0 (0)	2 (66.6)	2 (50)		19 (51.3)	2 (66.6)	
Mean tumor size (cm)	3.8 (1-7)								
> Median	22 (55)	17 (58.6)	2 (50)	2 (66.6)	1 (25)	0.89	21 (56.7)	2 (66.6)	0.89
≤ Median	18 (45)	12 (41.3)	2 (50)	1 (33.3)	3 (75)		16 (43.2)	1 (33.3)	
Tumor differentiation									
Well	20 (50)	15 (51.7)	2 (50)	2 (66.6)	1 (25)	0.18	20 (54.0)	0 (0)	**0.04**
Moderate	16 (40)	12 (41.3)	2 (50)	0 (0)	2 (50)		14 (37.8)	2 (66.6)	
Poor	2 (5)	2 (6.9)	0 (0)	0 (0)	0 (0)		2 (5.4)	0 (0)	
Not identified	2 (5)	0 (0)	0 (0)	1 (33.3)	1 (25)		1 (2.7)	1 (33.3)	
Lymph vascular invasion (LVI)									
Present	11 (27.5)	7 (24.1)	2 (50)	0 (0)	2 (50)	0.45	10 (27)	1 (33.3)	0.43
Absent	18 (45)	14 (48.2)	1 (25)	1 (33.3)	2 (50)		16 (43.2)	2 (66.6)	
Not identified	11 (27.5)	8 (27.5)	1 (25)	2 (66.6)	0 (0)		11 (29.7)	0(0)	
Perineural invasion (PNI)									
Present	21(52.5)	15 (51.7)	3 (75)	1 (33.3)	2 (50)	0.11	20 (54)	1 (33.3)	0.08
Absent	14(35)	11 (37.9)	1 (25)	0 (0)	2 (50)		12 (32.4)	2 (66.6)	
Not identified	5(12.5)	3 (10.3)	0 (0)	2 (66.6)	0 (0)		5 (13.5)	0(0)	
Stage									
I	14(35)	10 (34.4)	1 (25)	2 (66.6)	1 (25)	0.96	13 (35.1)	1 (33.3)	0.95
II	18(45)	13 (44.9)	2 (50)	1 (33.3)	2 (50)		17 (45.9)	1 (33.3)	
III	2(5)	2 (6.9)	0 (0)	0 (0)	1 (25)		2 (5.4)	0(0)	
IV	6 (15)	4 (13.7)	1 (25)	0 (0)	0 (0)		5 (13.5)	1 (33.3)	
Distant metastasis									
yes	21 (52.5)	16 (55.1)	1 (25)	2 (66.6)	2 (50)	0.67	19 (51.3)	2 (66.6)	0.33
no	19 (47.5)	13 (44.8)	3 (75)	1 (33.3)	2 (50)		18 (48.6)	1 (33.3)	

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Clinical Outcomes in Patients with Pancreatic Ductal Adenocarcinoma

We investigated the associations between expression of VISTA and survival in patients with PDAC. Later on, excluding patients with imperfect treatment or with a follow-up duration of less than six months, survival analysis was accomplished on 40 PDAC patients for whom follow-up information was accessible. Tumor metastasis was found in 5 patients (12.5%) and recurrence was observed in 19 patients (47.5%), also 21 (52.5%) and 35 subjects (87.5%) presented negative results. In addition, 16 patients (40.0%) were negative for both tumor metastasis and recurrence. In 28 cases (65.0%), cancer-associated death was documented at the mean survival of 22.0 months. No significant difference was observed between groups, for survival analysis of 40 patients in terms of clinicopathological characteristics and immune markers. The follow-up duration ranged from 1-39 months.

Survival Outcomes Based on Expression of VISTA in Pancreatic Ductal Adenocarcinoma

Kaplan–Meier survival analysis was performed to compare PFS or DSS based on the expression of VISTA (H-score). Survival analysis showed significant differences between the patients with cytoplasmic expression levels of VISTA and DSS (Log Rank test, p=0.038). In addition, there were no significant differences between patients with cytoplasmic expression levels of VISTA and PFS (Log Rank test, p=0.076) (Figure 4). The average DSS and PFS time for the patients whose samples expressed low amounts of VISTA were 21 (SD=3.1) and 22 (SD=4.4) months, and for high amounts of VISTA were 16 (SD=2.7) and 22 (SD=4.6) months, respectively.

Univariate analyses were also used to evaluate the clinical significance of numerous parameters of VISTA expression that can affect PFS/DSS in patients with PDAC. Among all clinicopathological parameters, only stage III (p=0.039), and tumor size (p=0.010), affected DSS in univariate analysis. Some other variables, including H-score, cytoplasmic (p=0.933) and membranous VISTA expression (p=0.712), PNI (p=0.974), LNI (p=0.918), tumor recurrence (p=0.364), distant metastasis (p=0.567) had p-values greater than 0.05; though, hazard ratio (HR) was not more than 1 (Tables 5). Remarkably, the other clinicopathological variables in the multivariate analysis did not show any significant difference for PFS or DSS.

Kaplan–Meier analysis of membranous expression of VISTA indicated that there were not any significant differences between both PFS or DSS (low expression and high expression) (Log Rank test; p=0.914, p=0.706), respectively.

Table 5. Univariate Cox regression analyses of potential prognostic factors for progression-free survival in patients with pancreatic ductal adenocarcinoma

Covariate		Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*	HR (95% CI)	*p*
H-score (cytoplasmic)		0.958 (0.352–2.608)	0.933	0.842 (0.432–1.501)	0.154
H-score ( )		0.797 (0.238–2.667)	0.712	0.531 (0.124–2.112)	0.756
Age		0.738 (0.338–1.609)	0.444	0.159 (0.118–1.762)	0.081
Stage	I	0.452 (0.132–1.543)	0.205	0.487 (0.354–2.466)	0.641
	II	0.583 (0.181–1.877)	0.366	0.695 (0.304–2.197)	0.915
	III	0.321 (0.048–2.133)	**0.039**	0.129 (0.634–3.615)	0.364
Perineural invasion		0.985 (0.399–2.433)	0.974	——	----
Lymph node invasion		0.950 (0.361–2.503)	0.918	——	----
Distant metastasis		0.798 (0.369–1.727)	0.567	——	----
Tumor recurrence		0.603 (0.203–1.796)	0.364	——	----
Tumor size		3.162 (1.242–8.051)	**0.016**	——	----

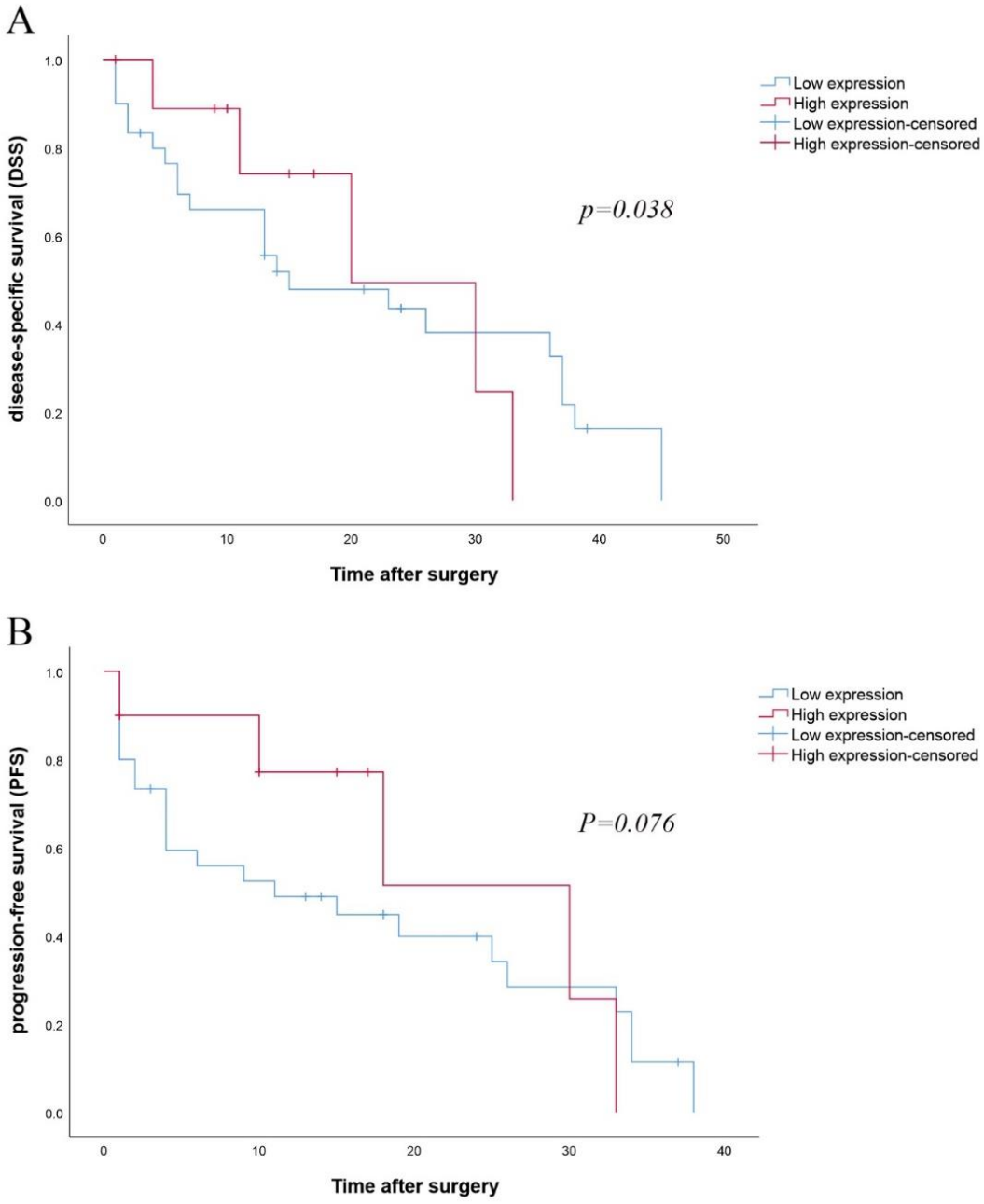

Figure 4. Survival rate curve for diseasespecific survival and progressionfree survival according to protein expression levels of VISTA in pancreatic ductal adenocarcinoma patients.

Kaplan–Meier survival curves for diseasespecific survival (DSS) and progressionfree survival (PFS) according to the expression levels of V-domain Imuunoglobulin suppressor of T-cell activation (VISTA) proteins in PDAC. (A) High-level expression of VISTA proteins was associated with shorter DSS (p=0.038). (B) Low levels of VISTA protein expression are not significantly related to PFS (P=0.076).

DISCUSSION

Immune cells play a role in the initiation, progression, and control of PDAC. However, their spreading pattern could be very variable.[12,13] Therefore, a detailed study and understanding of their infiltration in the TME of the pancreatic tumors is essential to provide an effective targeted immune therapy. Immune checkpoints are molecules that are expressed on the surface of lymphocytes and are responsible for the exhaustion of T cells and escape of the tumor cells from immunological eradication. Expression of VISTA is positively correlated with T-cell penetration rate in TME of PDAC.[14] VISTA is a novel immune checkpoint molecule, which was previously studied in murine models, various cell lines,[15] and human malignancies such as pancreatic cancer[16] and cancer immunotherapy.[17] VISTA in tumor microenvironment suppressed effector T-cell activation, proliferation, and cytotoxicity and downregulating proinflammatory cytokines such as (interleukin [IL]-12, IL-23, and IL-6), also induce tumor-specific adaptive $CD4^+Foxp3^+$ Tregs in microenvironment tumor cells.[18,19] In hypoxic conditions, HIF1α contributes to MDSC-mediated T-cell suppression by increasing VISTA expression at the level of MDSCs.[20]

Tumor-associated macrophages (TAMs) are an ample population of inflammatory cells that play an important role in remodeling tumor microenvironments and tumor progression. studies have established which VISTA may hinder T-cell activation and suppress the development of tumor-specific immunity, $VISTA^+CD68^+$ macrophages as one of the potential inhibitory cell types that can affect clinical outcomes in patients with pancreatic cancer. IL-8 secreted by $CD68^+$ macrophages leads to promoting metastasis and flexibility of PDAC cells in vivo and in vitro.[19] Liu CY et al. studies indicated TAMs-derived IL-10 boosted the metastasis of PDAC cells.[21] VISTA in TAMs suppresses activation and proliferation of T cells and hinders cytokine production (TNF-α and IFN-γ), Digomann et al.'s blocking of VISTA receptor decreases tumor growth in PDAC mouse models.[1] VISTA with CCL2/CCR2 axis induces the recruitment of inflammatory monocytes to the tumor microenvironment. which plays an important role in the induction of cancer and metastasis.[22] Nevertheless, the role of VISTA in PDAC was unclear. In this study, we examined the expression of VISTA in the TME of PDAC cases, and its association with advanced pathological features and cancer prognosis.

Many researchers have studied the expression levels of VISTA in various cancers using western blotting,[23] enzyme-linked immunosorbent assay (ELISA),[24] and in-silico prediction methods;[25] however, few studies have simultaneously reported its expression level in mRNA and protein levels in PDAC patients.

In this study we analyzed 29 fresh and 40 FFEP tissue samples from PDAC patients. Based on real-time PCR on fresh tissues, we found high expression of VISTA in tumor tissues compared to their marginal sections. We demonstrated higher mRNA expression of VISTA in correlation with increased TNM grades and tumor differentiation. Also, IHC showed a significant correlation between the cytoplasmic expression of VISTA and increasing the patient age and TNM grade. Moreover, we found a correlation with the membranous expression of VISTA with age and tumor differentiation in the clinicopathological parameters. The comparison of the low and high stages demonstrated that mRNA expression of VISTA and cytoplasmic protein expression levels were related to poor prognosis of the PDAC.

VISTA expression with a cytoplasmic or membranous pattern exacerbates immunosuppression in the TME.[11] A previous study has reported that VISTA was mostly expressed by tumor-infiltrating lymphocytes. Hou et al. showed that VISTA expression in TME of PDAC was only observed in 38.1%, 25.6%, and 26.0% of immune cells, tumors, and endothelial cells, respectively.[3] In PDAC, tumor grade has been recognized as a significant independent prognostic indicator of overall survival after resection.[26,27] This may be the reason that poorly differentiated tumors have more aggressive biology, leading to metastasis to nearby or distant regions.[28] Most pancreatic cancers that are detected at an early stage have long-term survival.[29] Our findings on VISTA protein expression are in agreement with previous evidence which showed that high expression of VISTA on tumor cells is correlated with progressive tumor stages,[30] suggesting that expression of VISTA is involved in PDAC development. Zong et al. have reported that VISTA is expressed in early-stage tumors is correlated with a better prognosis and can be a forecaster of improved survival autonomous of FIGO stage.[31] VISTA expression on tumor cells leads to

repressed T-cell functions .[23] Rabadi et al. reported that VISTA expression was correlated with the infiltration of immune cells in the TME of PDAC.[32] In conclusion, the correlation of progressive PDAC stages with high expression of VISTA can be explained by the capacity of this molecule to keep VISTA-positive tumor cells that repress metastasis and progressive tumor growth.[33] blocking VISTA via antibodies has been related to reduced VISTA interaction with their ligand (VSIG-3), leading to increased inflammatory cytokines and chemokines such as IL-2, IL-17, CCL-3, CXCL11, and CCL-5. VISTA-blocking raises the tumor proliferation, infiltration, cytokine production, and effector function of T (helper and cytotoxic) cells[34] anti-inhibitory effects of the VISTA also reduce suppression function of $Foxp3^{+}CD4^{+}$ Tregs. By upregulating the production of IL-12 and TNF-α and elevating the expression of MHC-II and CD80, leads to induce the activation of tumor-related myeloid DCs.[18] VISTA antibody indicated a raised in NF-κB phosphorylation, IFN-γ production, and $CD4^{+}$ T-cell proliferation of human T cells.[35]

In the current research, the Kaplan–Meier curve results on protein expression level demonstrated that PDAC tumors with lower expression levels of the VISTA protein lead to poor prognosis for DSS. Low cytoplasmic expression of VISTA compared with its high expression may be considered a worse prognostic factor of DSS in PDAC patients. The prognostic value of VISTA may be increased with a longer follow-up time. Loeser et al. reported that the expression of VISTA was associated with improved DSS in patients with Stage pT1/2 esophageal adenocarcinoma, which is in contrast with our observations among patients with PDAC in our current study.[36] Mulati et al. reported that VISTA was distinguished in all of their cases but was not correlated with patients' survival time.[23] It must be declared that VISTA may be a very contributory marker for poor prognosis of DSS in PDAC patients. Furthermore, the IHC application may confirm the real-time PCR results and launch VISTA status in clinical practice.

This research revealed that expression of high-level VISTA mRNA/protein is correlated with a more destructive form of tumor behavior in PDAC patients. Besides, we reported that cytoplasmic expression of VISTA has clinical significance in PDAC patients and is correlated with augmented poor prognosis hazard for DSS in univariate analysis. In general, evaluation of the expression of the cytoplasmic form of VISTA in tumor cells may be useful as a prognostic evaluation index and could be a predictor of cancer progression in PDAC patients. Our outcome approved that the expression of VISTA predicts short survival. Also, its inhibition may be beneficial for PDAC cancer patients. More study is essential to explain the basic controlling mechanisms of VISTA and its biological function in PDAC cancer.

STATEMENT OF ETHICS

This research was approved by the Research Ethics Committee of the Iran University of Medical Sciences. (reference number: IR.IUMS.FMD.REC.1399.161).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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