

VISTA and PD-L1 synergistically predict poor prognosis in patients with extranodal natural killer/T-cell lymphoma

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ABSTRACT

Although PD-1/PD-L1 blockade therapy confers salutary effects across cancer types, their efficacy in Extranodal Natural killer/T-cell lymphoma (ENKTCL) patients is limited and unpredictable. Here, we comprehensively evaluated the expression profile of a panel of immune-regulatory makers to identify novel prognostic biomarkers and/or therapeutic targets for this malignancy. Using immunohistochemistry and multiplex immunofluorescence, we found that the expression of VISTA (88.1%) was predominantly in CD68+ macrophages and much higher than PD-L1 expression (68.7%) in ENKTCL. B7-H4 and HHLA2 proteins were not detected in ENKTCL. B7-H3 was expressed in minority of ENKTCL patients (13.7%) and mainly colocalized with CD31. A close correlation was detected between VISTA and PD-L1, but they were not co-expressed in the same cells. High expressions of VISTA or PD-L1 were significantly associated with detrimental clinicopathological characteristics, dismal prognosis, and high density of CD8+ TILs, and high VISTA expression was also significantly associated with high density of Foxp3+ TILs. VISTA combined with PD-L1 was an independent prognostic factor for PFS and OS. Moreover, the patients with high VISTA showed a poor response to PD-1 blockades in ENKTCL. In conclusion, these findings provide a rationale for VISTA as an ideal immunotherapeutic target next to PD-L1 for ENKTCL.

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Introduction

Extranodal natural killer (NK)/T-cell lymphoma (ENKTCL) is a rare but aggressive subtype of non-Hodgkin lymphoma, which is derived from NK cells or $\gamma\delta$ T cells.^{1–4} ENKTCL is more prevalent in Asia and South America than that in European countries.⁵ A typical immunophenotype of ENKTCL is positivity for CD3 ϵ , CD56, and cytotoxic molecular such as granzyme B and TIA1.⁶ Of note, ENKTCL is strongly associated with Epstein-Barr virus (EBV) infection and EBV encoded RNA (EBER) positive is an important auxiliary diagnostic maker for ENKTCL.⁷ Unfortunately, the consensus on standard treatment strategy remains unavailable for ENKTCL patients, partially due to limited cases. Chemoradiotherapy or chemotherapy yield curative effects on ENKTCL patients in the early-stage.^{8,9} However, the outcomes of those treatments for patients with advanced or relapsed ENKTCL are disappointing, and the estimated 3-year overall survival is 25% in advanced patients.¹⁰ Therefore, there is an urgent medical need to explore new therapeutic strategies for advanced ENKTCL.


Over the past decade, immunotherapy is, once again, a promising approach to cancer treatment, due to the

remarkable salutary effectiveness of immune-checkpoint inhibitors (ICIs) targeting programmed death-1 (PD-1) or programmed death ligand-1 (PD-L1), in numerous solid and hematological malignancies.^{11–14} Aberrant expression of PD-1/PD-L1 is also observed and associated with poor prognosis in these malignancies.^{15–18} In terms of ENKTCL, the complete response rate of PD-1/PD-L1 blockades ranged from 24% to 71%.^{14,19,20} However, the association between PD-L1 expression and effectiveness of the blockade of PD-1/PD-L1 is discrepant and there are still partial PD-L1 high expression ENKTCL cases suffering from disease progression following the blockades of PD-1/PD-L1 axis treatment.^{14,20} Therefore, it is speculated that the PD-1/PD-L1 axis independent immunosuppressive mechanisms that restrain T cell activation in ENKTCL and it is reasonable to develop alternative immunomodulatory therapies that may be functional in the non-responder cases.

Beyond PD-L1, several novel B7 family members, including B7-H3, B7-H4, V-domain immunoglobulin suppressor of T cell activation (VISTA) and HERV-H LTR-associating 2 (HHLA2), are aberrantly expressed and hamper anti-tumor immunity in cancers. Of these, VISTA, encoded by c10orf54

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gene, is one of the most promising immunotherapeutic targets with sequence homology to PD-1 and PD-L1.^{21,22} As a negative immune checkpoint protein, VISTA displays an inhibitory role in the function of T cell via suppressing T cell proliferation and blunting cytokine production and makers activation.^{23,24} Of note, the mechanism of VISTA-induced T cell exhaustion is distinct from the PD-1/LD-L1 pathway.²⁵ VISTA is predominantly expressed within hematopoietic cells, with the highest expression level in myeloid cells.^{21,26} The high expression of VISTA is also observed in numerous cancers, including colorectal carcinoma, oral squamous cell carcinoma, melanoma and ovarian cancer.^{27–30} Moreover, VISTA expression is elevated after treatment with an anti-PD-1 antibody and/or anti-CTLA4 antibody.^{31,32} Additionally, VISTA is preferentially expressed in pancreatic cancer, which is considered as “cold” tumor due to low response to ICIs.³³ Furthermore, previous *in vivo* experiment demonstrates that blocking of PD-L1 and VISTA shows a synergistic therapeutic effect in colon cancer models.²⁵ These findings indicate that VISTA may be a potential target for tumor immunotherapy. However, the expression and clinicopathological relevance of VISTA in ENKTCL remain unknown.

In the present study, we first examined the expression of PD-L1, B7-H3, B7-H4, VISTA and HHLA2 in ENKTCL cases using immunochemistry (IHC) and immunofluorescence (IF). Then, we evaluated the correlation between VISTA and PD-L1, clinicopathological characteristics, and the density of tumor-infiltrating lymphocytes (TILs). Finally, we evaluated the synergistic effect of PD-L1 and VISTA on predicting the prognosis and immunotherapeutic efficacy in ENKTCL patients.

Methods

Patients and samples

Upon approval by the Institutional Ethical Boards of Sun Yat-sen University Cancer Center (SYSUCC). One hundred and nine consecutive ENKTCL cases diagnosed at SYSUCC between 2003 and 2019 were collected in this study. Written informed consent was obtained from all cases before enrollment. All enrolled cases met the following criteria: (1), pathologically confirmed ENKTCL; (2), received no anti-cancer treatments before biopsy; (3), Formalin-fixed, paraffin-embedded (FFPE) blocks were available; (4), no history and concurrence of other malignant tumors; (5), complete clinicopathological and follow-up data. Progression-free survival (PFS) was defined as the period span from the date of first treatment for ENKTCL to the date of cancer progression or death, and the overall survival (OS) was defined as the period span from the date of first treatment for ENKTCL to the date of death. The follow-up was censored on 31 December 2019, the date of the last follow-up for patients without progression or death event.

Immunohistochemistry and *in situ* hybridization

The immunohistochemistry (IHC) staining was performed on 4 μ m FFPE ENKTCL sections by a professional pathologist according to our previous study.³⁴ Briefly, after deparaffinization, rehydration and endogenous peroxidase inactivation, antigen

retrieval was performed using EDTA buffer (PH 9.0) in microwave, followed by blocking nonspecific binding with protein block (Novocastra, Newcastle, UK). Then, slides were incubated with primary antibody (anti-PD-L1: cell signaling technology (CST), #13684; anti-VISTA: CST, #54979; anti-B7-H3: CST, #14058; anti-B7-H4: CST, #14572; anti-HHLA2: Sigma-Aldrich, HPA055478; anti-CD8: CST, #85336; anti-Foxp3: Abcam, ab215206) at 4 °C overnight. After incubation with the corresponding second antibody, the slides were visualized with a DAKO EnVision Detection System (Dako). *In situ* hybridization (ISH) was performed to detect the EBV encoded small RNA (EBER) in FFPE tissue slides as previously described.³

Quantification of PD-L1, VISTA and CD8+ or Foxp3 + T cells

The PD-L1 and VISTA expressions were evaluated by two senior pathologists separately, based on the percentage of positive cells (e.g., number of positive cells/numbers of total cells). CD8+ or Foxp3+ TILs were calculated in the same manner as described in the previous study.¹⁶ In brief, the average of five independent high-power fields (400 \times), which represented the densest lymphocytic infiltrates, was used to reflect the extent of T cell infiltration.

Immunofluorescence

In line with our previous study,³⁵ immunofluorescence (IF) staining was performed on slides, which have been prepared in the same manner as for IHC before incubation of antibodies, by using primary and secondary antibodies as follows: PD-L1 (the same as IHC and ab210931 abcam), VISTA (the same as IHC), B7-H3 (the same as IHC), CD56 (Abcam, ab9272), CD68 (Abcam, ab955), CD31 (Abcam, ab9498), goat anti-rabbit (Alexa Fluor 488, ab150077; Alexa Fluor 594, ab150088, Abcam), goat anti-mouse (Alexa Fluor 488, ab150117; Alexa Fluor 594, ab150116, Abcam). DAPI (4', 6-diamino-2-phenylindole) fluorochrome was used for nuclei staining of all cells.

Statistical analysis

Optimal cutoff values of PD-L1 or VISTA levels for predicting survival were determined via the receiver operating characteristics (ROC) curve analysis. Associations between PD-L1 and VISTA expression, and clinicopathological characteristics were calculated using the chi-square test or Fisher's exact test, displayed by cross-table. The student's t-test was used for two-group analysis. Kaplan–Meier method was performed to depict the survival curves of PFS and OS, and the intergroup differences of survival were determined with the log-rank test. Univariate and multivariate analyses were carried out based on the Cox proportional hazard model. All data were analyzed with SPSS V.24.0 software (SPSS, Chicago, IL, USA) and Graphpad Prism 7 software (La Jolla, California, USA). Statistical significance was defined as a $p < .05$.

Results

Patient clinicopathological characteristics

In the present study, a total of 109 patients with ENKTCL were included, and the baseline clinicopathological characteristics were summarized in supplementary table S1. Of those, 74 (67.9%) patients were male. The median age at diagnosis was 41 (range: 10–70) and 96 (88.1%) patients were younger than 60 years old. The primary lesion of 85 (78.0%) patients was present at nasal cavity and adjacent tissues (Table S1). Nineteen (17.4%) patients had distal lymph node (LN) involvement and 35 (32.1%) patients had stage III or IV disease (Table S1). Elevated lactate dehydrogenase (LDH) was detected in 34 (31.2%) patients (Table S1). Sixty-four (58.7%) patients were assigned to intermediate high-risk and high-risk groups based on the nomogram-revised index (NRI), and 26 (23.8%) patients were assigned to high prognostic index of natural killer/T cell (PINK) group^{10,36} (Table S1). EBER positive was detected in 104 (95.4%) cases. Eighty-nine (81.6%) cases were positive for CD56. All patients had received chemotherapy and the chemotherapy can be categorized as anthracycline (ANT)-based ($n = 19$) and non-ANT-based regimens ($n = 90$) (Table S1). 17 out of 19 ANT-based regimens included CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone). Whereas the mainly used non-ANT-based regimens were asparaginase (ASP)-containing regimens, including ASP/ANT based (8.3%), ASP/gemcitabine (GEM) based (48.6%), ASP/methotrexate based (9.2%) and ASP/not otherwise specified based (2.8%) regimens, followed by platinum or other-containing regimens (13.8%)³⁷ (Table S1).

At the end of follow-up, 73 (67%) cases experienced disease progression and 58 (53.2%) cases had died. The median PFS time was 20 months (range: 1–111 months) and the median OS time was 60 months (range: 1–173 months). The 1-, 3-, and 5-year PFS rates were 60.6%, 43.8%, and 37.7%, respectively. The 1-, 3-, and 5-year OS rates were 89.0%, 56.8%, and 53.5%, respectively.

Expression of B7 family proteins in NK/T cell lymphoma

To explore the expression characteristics of B7 family proteins, including PD-L1, VISTA, B7-H3, B7-H4 and HHLA2, IHC, IF, and ISH were performed. Staining using serial sections showed that PD-L1 was expressed in tumor cells (marked with EBER) as well as in tumor-infiltrating immune cells (TIICs) (Figure S1A-B). The double IF for CD56 and PD-L1 further demonstrated that CD56 positive lymphoma cells partially co-expressed PD-L1 (Figure 1(a)). Notably, the double IF staining for CD68 and PD-L1 also confirmed that CD68+ tumor associated macrophages (TAMs) were partially co-expressed PD-L1 (Figure 1(b)). Through integral analysis of the PD-L1 expression in lymphoma cells and TAMs, 64 out of 109 cases (68.7%) were PD-L1 positive staining in ENKTCL.

Unlike PD-L1 expression, both IHC and IF demonstrated that VISTA expression was not detected in lymphoma cells (marked with EBER or CD56) (Figure 1(c) and S1C-D). However, double IF staining showed that VISTA expression was predominantly present on CD68+ TAMs (Figure 1(d)). VISTA positive staining was detectable to varying degrees in 96

cases (88.1%), which was more prevalent than PD-L1 expression in ENKTCL cases. Of note, double IF staining for PD-L1 and VISTA demonstrated that PD-L1 and VISTA were expressed on distinct cell subsets (Figure 1(e)). However, they mainly co-existed in the same area within tumors and displayed a significant correlation ($r = 0.4103$, $p < .001$) (Figure 1(e-g)).

Conversely, the IHC illustrated that the expression of B7-H3, B7-H4 and HHLA2 were observed neither in tumor cells nor TIICs (Figure S2A-C). While, B7-H3 expression was detected on partial membranes of vascular endothelial cells in 15 (13.7%) cases (Figure S2D-E). Taken together, these data demonstrated that, among B7 family members, only PD-L1 and VISTA were frequently expressed in ENKTCL.

Correlations between VISTA, PD-L1 expression, and clinicopathological characteristics

To explore the clinical relevance of PD-L1 and VISTA, we first divided cases into two subgroups based on the optimal cutoff values of percentage of PD-L1 (32.5%) and VISTA (27.5%) expression, which determined by ROC analysis, respectively. As shown in Table 1, high PD-L1 expression ($\geq 32.5\%$) was significantly associated with distal LN metastasis ($p = .033$), advanced Ann Arbor stage ($p = .006$) and high NRI ($p = .009$) and PINK ($p = .004$) (Table 1).

Similarly, high VISTA expression ($\geq 27.5\%$) was also significantly correlated with the distal LN metastasis ($p = .004$), advanced Ann Arbor stage ($p = .002$), and high NRI ($p = .001$) and PINK ($p = .003$) (Table 1). Additionally, primary tumor in nasal had much higher VISTA expression, compared with primary tumor in others ($p = .039$) (Table 1). Moreover, a significant correlation was identified between PD-L1 and VISTA expression ($p < .001$) (Table 1). These results indicated that both high expressions of PD-L1 or VISTA were associated with detrimental clinicopathological characteristics.

VISTA and PD-L1 synergistically predict a poor prognosis in ENKTCL

To further evaluate the prognostic significance of PD-L1 and VISTA, a univariate analysis was performed. In addition to lesion location (HR = 1.93, 95%CI: 1.14 to 3.19, $p = .013$), distal LN metastasis (HR = 2.17, 95%CI: 1.27 to 3.76, $p = .006$), Ann Arbor stage (HR = 2.34, 95%CI: 1.46 to 3.77, $p < .001$), NRI score (HR = 1.72, 95%CI: 1.06 to 2.78, $p = .027$), PINK (HR = 1.82, 95%CI: 1.09 to 3.02, $p = .023$), and chemotherapy regimens (HR = 0.38, 95%CI: 0.22 to 0.65, $p < .001$), patients with high PD-L1 or VISTA expression were associated with increased risks of disease progression, compared with patients with low PD-L1 or VISTA expression, respectively (PD-L1: HR = 1.93, 95% CI: 1.22 to 3.07, $p = .005$; VISTA: HR = 2.05, 95% CI: 1.29 to 3.25, $p = .001$) (Table 2). Kaplan-Meier analysis showed that the high PD-L1 or VISTA expression was significantly associated with shorter PFS (PD-L1: $p = .004$, VISTA: $p = .002$) (Figure 2(a-b)). In terms of OS, univariate analysis demonstrated that patients with high PD-L1 or VISTA expression experienced significantly increased overall mortality than those with low PD-L1 or VISTA expression, respectively (PD-L1: HR = 2.44, 95% CI: 1.45 to 4.11,

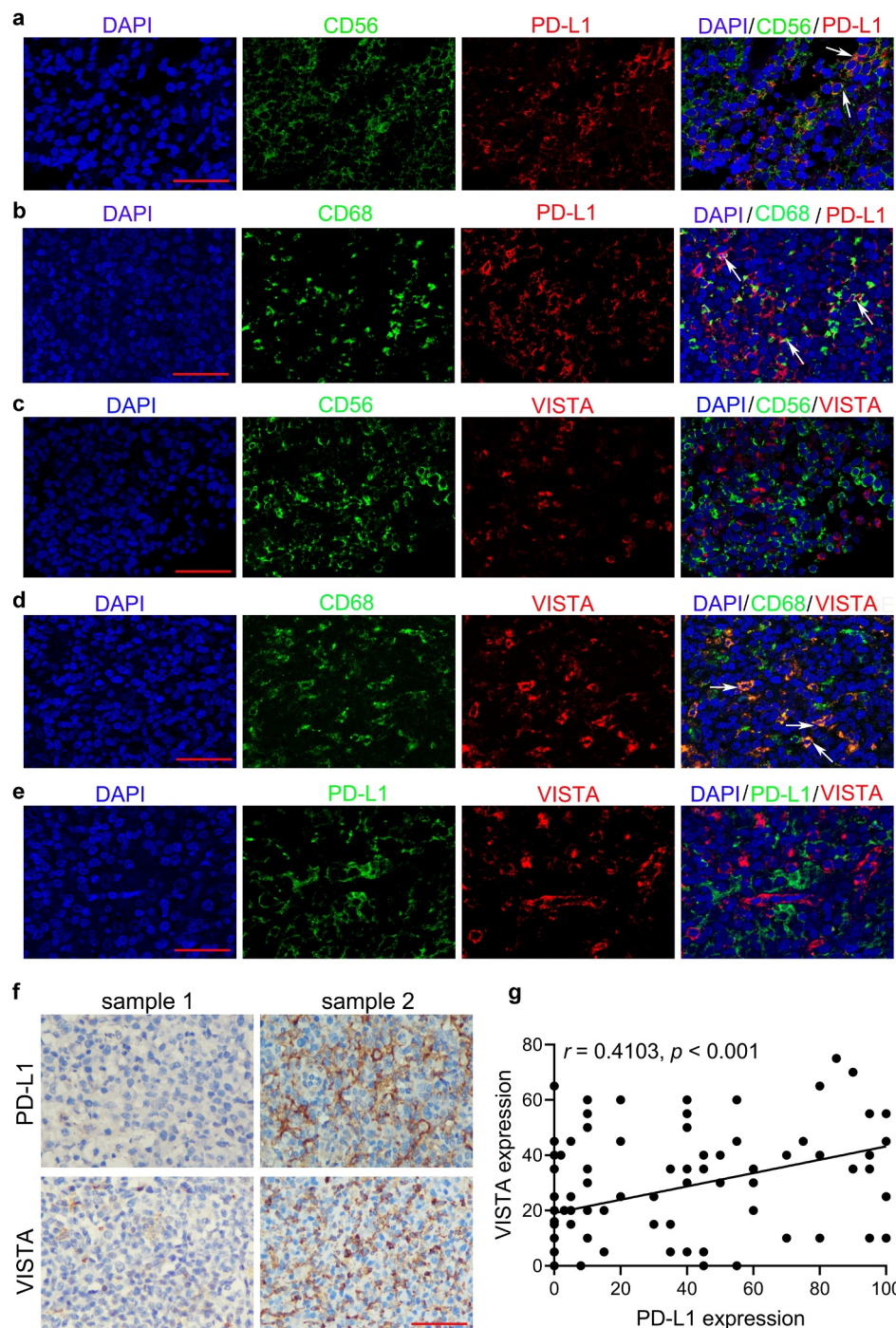


Figure 1. Expression of PD-L1 and VISTA in tumor tissues from ENKTCL patients. **A-E.** Representative double immunofluorescence images of CD56 (green) and PD-L1 (red) (a), CD68 (green) and PD-L1 (red) (b), CD56 (green) and VISTA (red) (c), CD68 (green) and VISTA (red) (d), and PD-L1 (green) and VISTA (red) (e). The nucleus is labeled with DAPI (blue). Arrows indicate the colocation of indicated proteins. **F.** The representative IHC images of PD-L1 and VISTA from serial sections. **G.** The correlation between PD-L1 expression and VISTA expression depicted with scatter plot. Scale bar is 50 μ m.

$p = .001$; VISTA: HR = 2.27, 95% CI: 1.34 to 3.83, $p = .002$), in line with the elevated LDH (HR = 1.74, 95%CI: 1.01 to 3.00, $p = .048$), distal LN metastasis (HR = 1.87, 95%CI: 1.01 to 3.48, $p = .048$), Ann Arbor stage (HR = 2.59, 95%CI: 1.53 to 4.38, $p < .001$), NRI score (HR = 2.24, 95%CI: 1.28 to 3.93, $p = .005$) and chemotherapy regimens (HR = 0.39, 95%CI: 0.22 to 0.69, $p = .003$) (Table 2). Kaplan-Meier analysis also confirmed that high PD-L1 or VISTA expression was significantly associated with worse OS (PD-L1: $p < .002$, VISTA: $p = .001$) (Figure 2c-d).

Furthermore, multivariate analysis demonstrated that Ann Arbor stage (HR = 3.70, 95%CI: 1.57 to 8.73, $p = .003$), PINK (HR = 0.19, 95%CI: 0.05 to 0.73, $p = .016$) and chemotherapy regimens (HR = 0.33, 95%CI: 0.18 to 0.59, $p = .001$) remained significant independent factors for PFS and chemotherapy regimens (HR = 0.27, 95%CI: 0.14 to 0.52, $p = .001$) was the only significant factor for OS. However, neither PD-L1 nor VISTA was an independent prognostic factor for both PFS and OS (Table 3).

Table 1. The correlation between PD-L1 and VISTA expression and clinicopathological characteristics.

Variables	PatientsNO.	PD-L1 expression			VISTA expression		
		Low	High	<i>p</i>	Low	High	<i>p</i>
Age(y)				0.412			0.666
<60	96	55	41		53	43	
≥60	13	9	4		8	5	
Gender				0.307			0.158
Male	74	41	33		38	36	
Female	35	23	12		23	12	
Location				0.325			0.039
Nasal	85	52	33		52	33	
Others	24	12	12		9	15	
B symptoms				0.098			0.317
Yes	49	33	16		30	19	
No	60	31	29		31	29	
ECOG score				0.500			1.000
0-1	105	61	44		59	46	
≥2	4	3	1		2	2	
Elevated LDH				0.096			0.093
Yes	75	48	27		46	29	
No	34	16	18		15	19	
Distal LN involvement				0.033			0.004
Yes	90	57	33		56	34	
No	19	7	12		5	14	
Ann Arbor stage				0.006			0.002
I-II	74	50	24		49	25	
III-IV	35	14	21		12	23	
NRI score				0.009			0.001
0-1	45	33	12		34	11	
≥2	64	31	33		27	37	
PINK				0.004			0.003
0-1	83	55	28		53	30	
≥2	26	9	17		8	18	
VISTA				<0.001			-
Low	70	48	13		-	-	
High	39	16	32		-	-	

Abbreviation: ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; LN, lymph node; NRI, nomogram-revised risk index; PINK, prognostic index of natural killer lymphoma. *p*-value <0.05 marked in bold font shows statistically significant.

Table 2. Univariate analysis of prognostic factors correlated with PFS and OS in patients with ENKTCL.

Variables	PFS		OS	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Age (y) (≥60/<60)	1.10 (0.54–2.20)	0.800	1.57 (0.77–3.19)	0.217
Gender (male/female)	0.72 (0.43–1.19)	0.200	0.77 (0.44–1.36)	0.369
Location (nasal/others)	1.93 (1.14–3.19)	0.013	1.31 (0.72–2.40)	0.378
B symptoms (yes/no)	1.30 (0.82–2.06)	0.272	1.26 (0.75–2.12)	0.388
ECOG score (≥2/0-1)	1.23 (0.39–3.92)	0.725	1.76 (0.55–5.62)	0.343
Elevated LDH (yes/no)	1.43 (0.87–2.35)	0.158	1.74 (1.01–3.00)	0.048
Distal LN involvement (yes/no)	2.17 (1.27–3.76)	0.006	1.87 (1.01–3.48)	0.048
Ann Arbor stage III–IV/I–II	2.34 (1.46–3.77)	<0.001	2.59 (1.53–4.38)	<0.001
NRI score (≥2/0-1)	1.72 (1.06–2.78)	0.027	2.24 (1.28–3.93)	0.005
PINK (≥2/0-1)	1.82 (1.09–3.02)	0.023	1.59 (0.89–2.83)	0.117
Chemotherapy (non-ANT/ANT based)	0.38 (0.22–0.65)	<0.001	0.39 (0.22–0.69)	0.003
PD-L1 expression (high/low)	1.93 (1.22–3.07)	0.005	2.44 (1.45–4.11)	0.001
VISTA expression (high/Low)	2.05 (1.29–3.25)	0.002	2.27 (1.34–3.83)	0.002

Abbreviation: ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; LN, lymph node; NRI, nomogram-revised risk index; PINK, prognostic index of natural killer lymphoma; ANT, anthracycline; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval. *p*-value <0.05 marked in bold font shows statistically significant.

It is well known that both PD-L1 and VISTA are capable of suppressing antitumor T-cell responses. However, the combinatorial analysis of PD-L1 and VISTA expressions in prognosis of ENKTCL remained unknown. The patients were initially categorized as PD-L1^{low}/VISTA^{low}, PD-L1^{low}/VISTA^{high}, PD-L1^{high}/VISTA^{low} and PD-L1^{high}/VISTA^{high}. Interestingly, Kaplan–Meier analysis demonstrated that patients with PD-L1^{high}/VISTA^{high} had the shortest PFS and OS among the ENKTCL patients. Patients with PD-L1^{low}/VISTA^{high} or PD-

L1^{high}/VISTA^{low} were associated significantly with worse PFS and OS than patients with PD-L1^{low}/VISTA^{low} (Figure S3A-B). However, no differences in PFS and OS were noted between patients with PD-L1^{low}/VISTA^{high} or PD-L1^{high}/VISTA^{low} (Figure S3A-B). Therefore, we further classified patients into three immune types: Type I, PD-L1^{low}/VISTA^{low}; Type II, PD-L1^{low}/VISTA^{high} or PD-L1^{high}/VISTA^{low}; Type III, PD-L1^{high}/VISTA^{high}. As shown in Table S2, type III was significantly associated with distant lymph node metastasis (*p* = .009),

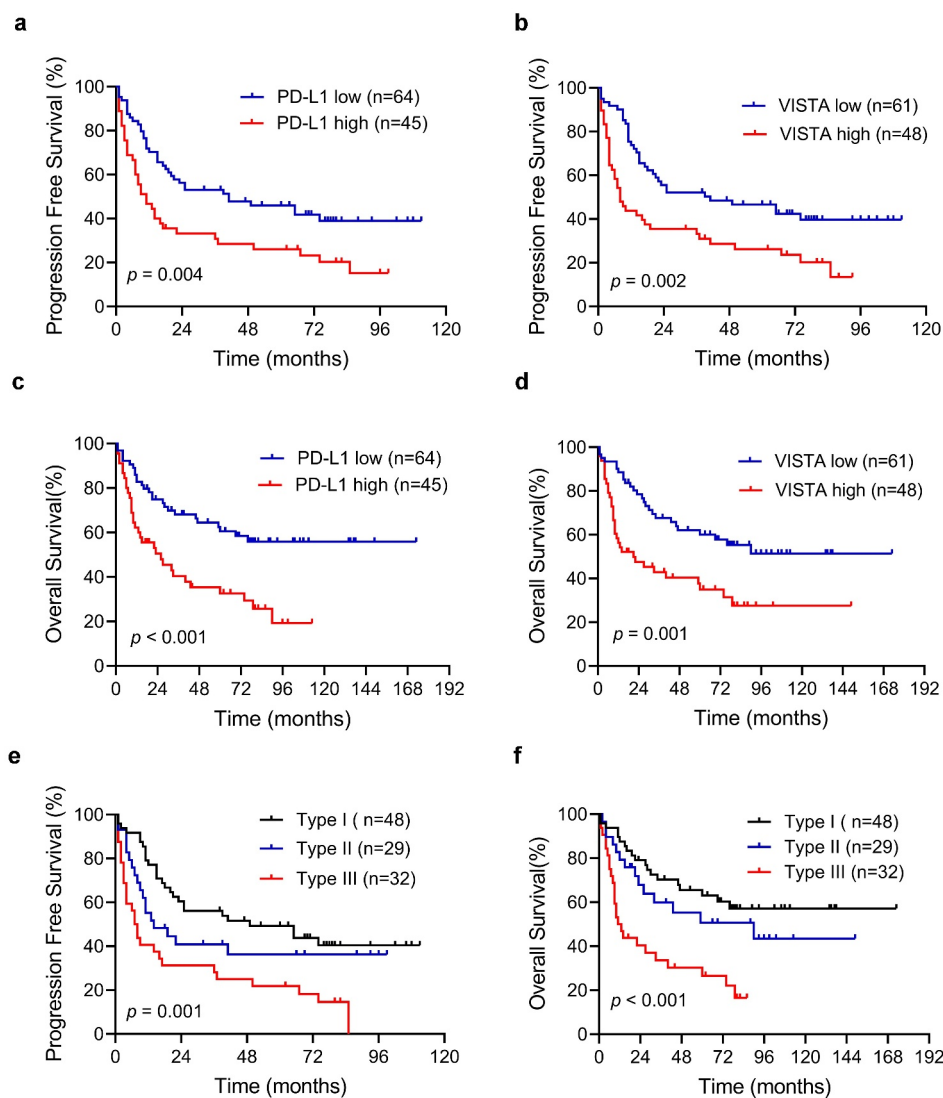


Figure 2. Kaplan-Meier survival curves for progression-free survival (PFS) and overall survival (OS) of patients with ENKTCL according to PD-L1 and VISTA expression. **A-B.** PFS for ENKTCL patients based on PD-L1 (a) and VISTA (b) expression. **C-D.** OS for ENKTCL patients based on PD-L1 (c) and VISTA (d) expression. **E-F.** PFS (e) and OS (f) for ENKTCL patients based on combination of PD-L1 and VISTA expression. Type I, PD-L1^{low}/VISTA^{low}; Type II, PD-L1^{low}/VISTA^{high} or PD-L1^{high}/VISTA^{low}; Type III, PD-L1^{high}/VISTA^{high}.

advanced Ann Arbor stage ($p = .002$) and high NRI ($p = .001$) and PINK ($p = .003$). In terms of survival end-points, both PFS and OS in type III were significantly shorter than the other two types ($p < .001$, Figure 2(e-f)). Interestingly, multivariate analysis demonstrated that type III had a significant influence on PFS and OS, with high hazard ratios (PFS: HR = 1.47, 95% CI: 1.10–1.98, $p = .010$; OS: HR = 1.53, 95% CI: 1.10–2.12, $p = .012$) after adjusting for possible confounding variables (Table 4). In conclusion, PD-L1 and VISTA showed synergistic functions in predicting the poor prognosis in ENKTCL.

High expression of PD-L1 or VISTA were associated with infiltration of TILs

Given that both PD-L1 and VISTA display suppressive effects on TILs, we evaluated the correlation between PD-L1 and VISTA, and TILs, including CD8⁺ TILs and Foxp3⁺ TILs by

IHC in serial slides (Figure 3(a)). As shown in Figure 3(b-c), the density of CD8⁺ TILs was significantly higher in tumors with PD-L1 or VISTA high expression than that in corresponding PD-L1 or VISTA low expression tumor (PD-L1, $p < .001$; VISTA, $p < .001$). Moreover, the counts of CD8⁺ TILs were much higher in both PD-L1 and VISTA high expression tumors than that in single marker high expression tumors (Figure 3(d)). No significant difference of Foxp3⁺ TIL infiltration was detected between PD-L1 high expression tumors and PD-L1 low expression tumors (Figure 3(e)). However, the count of Foxp3⁺ TILs was significantly increased in VISTA high expression tumors, compared with that in VISTA low expression tumors ($p = .03$) (Figure 3(f)). Additionally, the count of Foxp3⁺ TILs was significantly higher in both marker high expression tumors compared with the other two types ($p < .05$ Figure 3(g)). These data showed high expression of PD-L1 or VISTA was associated with infiltration of TILs.

Table 3. Multivariate analysis of prognostic factors correlated with PFS and OS in patients with ENKTCL.

Variables	PFS		OS	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Location (nasal/others)	1.65 (0.92–2.98)	0.094	-	-
PINK (2–3/0–1)	0.19 (0.05–0.73)	0.016	-	-
Elevated LDH (yes/no)	-	-	1.14 (0.62–2.13)	0.671
Distal LN involvement (yes/no)	2.34 (0.77–7.08)	0.134	0.71 (0.32–1.57)	0.392
Ann Arbor stage III–IV/I–II	3.70 (1.57–8.73)	0.003	2.01 (0.91–4.44)	0.083
NRI score ($\geq 2/0-1$)	1.28 (0.68–2.42)	0.443	1.78 (0.85–3.74)	0.127
Chemotherapy (non-ANT/ANT based)	0.33 (0.18–0.59)	0.001	0.27 (0.14–0.52)	0.001
PD-L1 expression (high/low)	1.46 (0.82–2.62)	0.200	1.75 (0.93–3.28)	0.081
VISTA expression (high/Low)	1.48 (0.82–2.68)	0.198	1.32 (0.68–2.55)	0.412

Abbreviation: LDH, lactate dehydrogenase; LN, lymph node; NRI, nomogram-revised risk index; PINK, prognostic index of natural killer lymphoma; ANT, anthracycline; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval. *p*-value <0.05 marked in bold font shows statistically significant.

Table 4. Multivariate analysis of prognostic factors correlated with PFS and OS in patients with ENKTCL.

Variables	PFS		OS	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Location (nasal/others)	1.65 (0.92–2.97)	0.094	-	-
PINK (2–3/0–1)	0.19 (0.05–0.73)	0.016	-	-
Elevated LDH (yes/no)	-	-	1.14 (0.62–2.13)	0.670
Distal LN involvement (yes/no)	2.34 (0.77–7.08)	0.134	0.69 (0.31–1.53)	0.361
Ann Arbor stage III–IV/I–II	3.70 (1.57–8.73)	0.003	2.02 (0.91–4.47)	0.082
NRI score ($\geq 2/0-1$)	1.28 (0.68–2.42)	0.443	1.77 (0.84–3.70)	0.131
Chemotherapy Non-ANT/ANT based	0.33 (0.18–0.59)	<0.001	0.28 (0.15–0.53)	<0.001
PD-L1/VISTA expression Type III/II/I	1.47 (1.10–1.98)	0.010	1.53 (1.10–2.12)	0.012

Abbreviation: LDH, lactate dehydrogenase; LN, lymph node; NRI, nomogram-revised risk index; PINK, prognostic index of natural killer lymphoma, ANT, anthracycline, PFS, progression-free survival, OS, overall survival; HR, hazard ratio; CI, confidence interval; Type I, PD-L1^{low}/VISTA^{low}; Type II, PD-L1^{low}/VISTA^{high} or PD-L1^{high}/VISTA^{low}; Type III, PD-L1^{high}/VISTA^{high}. *p*-value <0.05 marked in bold font shows statistically significant.

The PD-L1 and VISTA expression predicted the response to blockade of PD-1

To further evaluate the clinical relevance of PD-L1 and VISTA for predicting the immunotherapeutic effect, we retrospectively investigated the PD-L1 and VISTA expression in tumors from 12 refractory and recurrent ENKTCL patients who were treated with PD-1 blockade in a validation cohort (Table S3). 2-deoxy-2-fluorine-18-fluoro-D-glucose positron emission tomography integrated with computed tomography (PET-CT) was used to assess the response to PD-1 blockade. As shown in Figure 4(a–b), all three patients with PD-L1^{low}/VISTA^{low} showed no response to PD-1 blockade. Similarly, one patient with PD-L1^{low}/VISTA^{high} did not respond to PD-1 blockade. Conversely, three of the four patients with PD-L1^{high}/VISTA^{low} displayed complete remission (CR) to PD-1 blockade and maintained this status for more than 12 months. However, another patient with PD-L1^{high}/VISTA^{low} showed partial response (PR) after the treatment of PD-1 blockade. Intriguingly, among the four patients with PD-L1^{high}

/VISTA^{high}, two patients achieved CR, but the maintain time of CR was less than that in two patients with PD-L1^{high}/VISTA^{low}; however, the other two patients unfortunately showed transient PR or no response to PD-1 blockade (Figure 4(b)). The data indicated that treatment with PD-1 blockade alone might not achieve a satisfying effect in patients with VISTA high expression. Thus, combination treatment with PD-L1 and VISTA blockade might be a potential option for ENKTCL patients with PD-L1^{high}/VISTA^{high}.

Discussion

With the development of research for immune checkpoints, it is increasingly clear that immune checkpoints can facilitate tumor escape from immune surveillance via inhibiting the T cell response. Thus, the ICIs, particularly PD-L1 and CTLA-4, have been the focus of investigation in basic and clinical research. Nevertheless, the response rate following ICIs, targeting CTLA-4, PD-1 or PD-L1, is generally less than 30%. Similarly, the efficacy of PD-1/PD-L1 blockades is limited in relapsed or refractory ENKTCL cases, indicating that alternative immune checkpoint pathways hamper antitumor immunity.²⁰ To our knowledge, this is the first study to survey the expression of a panel of immune checkpoint markers in ENKTCL. We found that PD-L1 and VISTA were variably expressed, while B7-H3 was sporadically expressed in endothelial cells in partial ENKTCL. However, no expression of B7-H4 and HHLA2 was detected in ENKTCL. These data indicate that, among the B7 family proteins, perhaps only PD-L1 and VISTA could perform the immune-modulatory function in ENKTCL.

Previous studies consistently demonstrated that PD-L1 was expressed variably by tumor cells and CD68+ TAMs in ENKTCL, which has been further confirmed in the present study.^{38,39} However, the clinical relevance and prognostic significance of PD-L1 in ENKTCL remain controversial. Two studies from South Korea showed that high PD-L1 expression was significantly associated with better clinical outcomes and a prolonged OS in ENKTCL.^{40,41} In this study, however, based on a more larger cohort, we confirmed that high PD-L1 expression in all cells (including tumor cells and immune cells) was correlated with worse clinical outcomes and shorter PFS and OS. Similarly, two studies from our center also determined that high PD-L1 expression in tumor cells or TIICs were associated with poor prognosis.^{17,18} Moreover, we previously found that patients with the high level of soluble PD-L1 in serum showed significantly worse prognosis, which was confirmed by another study.^{17,39} Given this contradiction, it is possible that the expression of other immune checkpoints may affect the prognostic significance of PD-L1 in ENKTCL.

As a novel immune checkpoint of B7 family, VISTA has received an increasing amount of attention. The VISTA protein was detected in tumor cells and immune cells in melanoma, colorectal cancer, ovarian cancer, and craniopharyngioma.^{27,28,42} However, this study found that the VISTA was predominantly expressed in CD68+ TAMs but not in tumor cells, and VISTA expression was more prevalent, compared with PD-L1 expression, in ENKTCL. Taking this further, VISTA overexpression was common in PD-L1 low

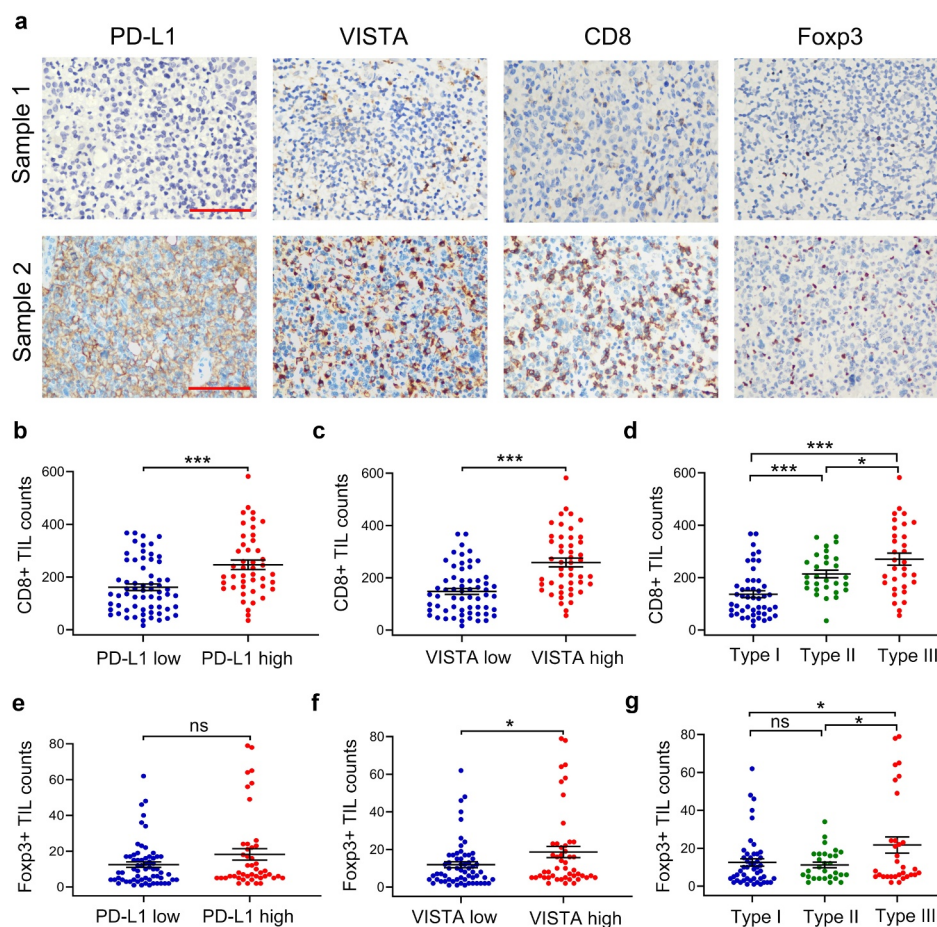


Figure 3. The correlation between the expression of VISTA and PD-L1, and the density of tumor-infiltrating lymphocytes (TILs). **A.** The representative images of PD-L1, VISTA, CD8 and Foxp3 from serial sections from two samples, sample 1 (up) and sample 2 (down). **B-D.** The correlations between CD8+ TILs and PD-L1 expression (b), VISTA expression (c) or novel immune classification (d) depicted with scatter plot. **E-G.** The correlations between Foxp3+ TILs and PD-L1 expression (e), VISTA expression (f) or novel immune classification (g) depicted with scatter plot. All data display mean \pm SEM. Scale bar is 50 μ m. * $p < .05$, *** $p < .001$. ns: no significance. Type I, PD-L1^{low}/VISTA^{low}; Type II, PD-L1^{low}/VISTA^{high} or PD-L1^{high}/VISTA^{low}; Type III, PD-L1^{high}/VISTA^{high}.

expression ENKTCL, and positively associated with detrimental clinical features. As for survival, previous studies demonstrated that VISTA expressed in TCs and ICs displayed discrepant prognostic significance in cancers. VISTA expression in TCs was identified as a favorable prognostic factor in patients with hepatoma, non-small cell lung cancer (NSCLC), or advanced ovarian cancer.^{27,43,44} In contrast, VISTA expression in ICs was associated with poor prognosis in patients with melanoma.²⁸ Consistent with the latter, this study also confirmed VISTA mainly on ICs as an extremely poor prognostic factor in ENKTCL. Thus, VISTA expressed in TCs or ICs may exert distinct immunomodulatory functions in malignancies and a great deal of effort is necessary to define the exact mechanism of VISTA for distinct cell types.

A positive correlation between PD-L1 expression and VISTA expression was detected in this study, which corresponds to the findings in NSCLC and craniopharyngioma.^{42,44} Furthermore, Wang et al. reported that VISTA and PD-L1 co-expression was detected in the same TIICs but not in tumor cells.⁴² In contrast, another study showed a differential expression of PD-L1 and VISTA

on distinct CD68+ subsets.³³ In this study, we also demonstrated that PD-L1 and VISTA were expressed in distinct immune cells. Despite this, the regional co-localization of PD-L1 and VISTA proteins was frequently observed within ENKTCL. Additionally, PD-L1 and VISTA proteins displayed a synergistic effect on predicting PFS and OS in ENKTCL. These data indicate that VISTA upregulation may be mediated by locally secreted cytokines, which could also induce PD-L1 overexpression, but VISTA and PD-L1 are capable of facilitating immune escape via separate inhibitory pathways. Of note, Bharaj et al. showed that VISTA in monocytes was prominently upregulated by IL-10 and IFN- γ .²⁶ CD8 + T cells are the main IFN- γ -producing immune cells in tumors. In this study, we found that CD8 + T cells were mainly aggregated in the region of high PD-L1 and/or VISTA expression and the mean counts of CD8 + T cells was highest in patients with both PD-L1 and VISTA high expression. Besides, a higher mean count of Foxp3+ Tregs was detected in patients with high VISTA expression. Considering both PD-L1 and VISTA function as brakes for T-cell activation, we speculate that there is a negative feedback immunomodulatory mechanism in

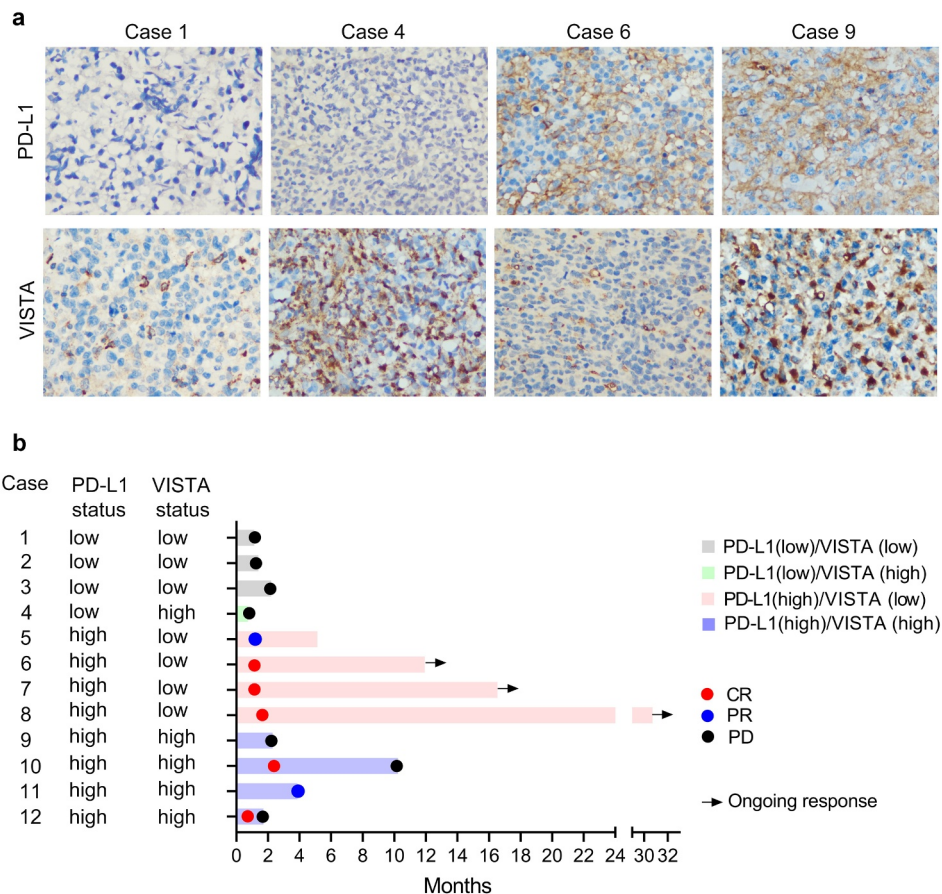


Figure 4. The PD-L1 and VISTA expression were associated with the response to blockade of PD-1. (a). The representative images of the PD-L1 and VISTA expression in indicated groups. (b). Swimmer's plot showing the response to PD-1 blockade in extranodal NK/T-cell lymphoma patients, who were classified into four groups as indicated. CR, complete response; PR, partial response; PD, progressive disease.

ENKTCL as follows: activated CD8 + T cells induce the upregulation of PD-L1 and VISTA expression via secreting IFN- γ ; then, overexpressed PD-L1 and VISTA, in turn, suppress the proliferation and activation of CD8 + T cells through nonredundant inhibitory pathways, and finally undermine antitumor immune response in ENKTCL.

Actually, Liu et al. reported that a combination of PD-L1 and VISTA blockade showed a synergistic therapeutic effect in a colon cancer mouse model.²⁵ In this study, we retrospectively analyzed PD-L1 and VISTA expression in ENKTCL patients who received monoclonal therapy against PD-1. We found that ENKTCL patients with low PD-L1 expression, regardless of VISTA expression, could not benefit from the immunotherapy of PD-1 blockade. Three out of four patients with PD-L1 high expression alone achieved CR, but one patient with PD-L1 high expression alone did not respond to PD-1 blockade. These data indicated that other non-B7 family proteins, such as IDO1, TIM3, might also display an immunosuppressive role within ENKTCL. More importantly, despite the certain effect of PD-1 blockade in patients with both high expression of PD-L1 and VISTA, it was not as effective as that in patients with PD-L1 high expression alone, indicating that VISTA may be involved in resistance to PD-1 blockade. Therefore, simultaneous blockade of both PD-L1 and VISTA pathways may be a potential therapeutic regimen for ENKTCL patients bearing the high expression of PD-L1 and VISTA.

Despite a series of novel findings, our study also had some limitations as follows: First, given the tumor heterogeneity, the biopsy sample may not be able to represent the immune microenvironment of the entire tumor. Second, although we conducted the largest existing cohort study to analyze PD-L1 expression in ENKTCL, this is a retrospective, single-center study and the selection bias was inevitable. Third, this study only analyzed the expression of B7 family proteins but not other immunosuppressive factors, such as IDO1 and adenosine, in ENKTCL. Finally, because only 12 ENKTCL cases were enrolled in the validation cohort, it is hard to accurately evaluate the clinical reliability of predicting immunotherapeutic effectiveness based on the expression of PD-L1 and VISTA.

In conclusion, we comprehensively explore the expression profile of numerous B7 family proteins and elucidated that VISTA expression was more prevalent than PD-L1 in ENKTCL. Both high expressions of PD-L1 and VISTA were significantly associated with worse clinical outcomes and more infiltrating CTLs. VISTA synergized with PD-L1 as an independent predictor of poor prognosis, and patients with both high expressions of PD-L1 and VISTA suffered from the worst prognosis. More importantly, VISTA was involved in the resistance to PD-1/PD-L1 blockades in ENKTCL. These findings indicate VISTA might be an ideal target for therapeutic modulation alone.

or in combination with PD-L1 blockade for ENKTCL immunotherapy.

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Authors' contributions

Conception and Design: Gao Yan, Xu Chen and Hui-qiang Huang; Performing experiments: Hai-xia He, Qiang-hua Zhou and Jian-chang Fu; Statistical analyses: Hai-xia He, Qi-xiang Rong, Cheng Huang and Yan-xia He; Drafting of the article: Hai-xia He and Qiang-hua Zhou; Critical revision of manuscript: all authors; All authors read and approved the final manuscript.

Availability of data and materials

All datasets used and/or analyzed during the current study are available in the Research Data Deposit public platform (www.researchdata.org.cn, number RDDDB2021001093) from the corresponding author on request.

Disclosure of potential conflicts of interest

None potential conflicts of interest were disclosed

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Ethics approval

This study was approved by the Clinical Research Ethics Committee of Sun Yat-sen University Cancer center (B202100301).

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