Histol Histopathol (2018) 33: 307-315

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Expression of TIM-3 and LAG-3 in extranodal NK/T cell lymphoma, nasal type

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Summary. Objective. To investigate the expression of TIM-3 and LAG-3 in extranodal NK/T cell lymphoma, nasal type (ENKTL), and evaluate the clinical and prognostic significance of TIM-3 and LAG-3 in ENKTL.

Methods. A total of 61 human paraffin-embedded specimens including 41 ENKTL and 20 rhinitis were involved. We performed immunohistochemistry to detect the expression of TIM-3 and LAG-3. We analyzed correlation between expression of TIM-3 and LAG-3 and clinicopathological features of ENKTL.

Results. TIM-3 was positively expressed in 95 (39/41) ENKTL compared with 55% (11/20) in rhinitis, LAG-3 was positively expressed in 95 (39/41) ENKTL compared with 45% (9/20) in rhinitis. A positive correlation was found between TIM-3 and LAG-3. TIM-3 and LAG-3 had no significant correlation with ENKTL patients' age, gender, international prognostic index (IPI) score, B symptoms, LDH level, Ann Arbor stage and treatment regimens. High expression of TIM-3, high IPI score, elevated LDH level and late Ann Arbor stage were shown to be correlated with worse progression free survival (PFS). A multivariate COX regression model show that TIM-3 high expression rate was an independent prognostic factor for ENKTL patients' PFS.

Conclusions. Our findings indicate that TIM-3 might be a promising predictive indicator for the ENKTL patients.

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DOI. 10.14670/HH-11-931

Key words: Extranodal NK/T cell lymphoma, Nasal type, TIM-3, LAG-3, Immunohistochemistry

Introduction

Extranodal NK/T cell lymphoma, nasal type (ENKTL) is a subtype of non-Hodgkin lymphoma with aggressive clinical behaviors (Swerdlow et al., 2008). It occurs rarely in North America and Europe, but in Asia, especially China and Korea, it has been commonly seen, and it comprises 20-46% T cell lymphoma (William and Armitage, 2013). Its commonly seen in males with a median age of 50 years old. ENKTL has a high relapse and mortality rate though clinical stage is usually early when diagnosed (Vose et al., 2008). The cause of ENKTL has not been illuminated, but Epstein-Barr virus infection is thought to be closely related with the clinical behavior of ENKTL, and may act as a prognostic factor for ENKTL (Ito and Heo, 2012; Wang et al., 2012). Chemotherapy-radiotherapy-chemotherapy sandwiched treatment model is recommended to ENKTL with early Ann Arbor stage, while advanced ENKTL is treated mainly with chemotherapy (Tse and Kwong, 2013, 2014). Several prognostic factors, such as International Prognostic Index (IPI), whole blood Epstein-Barr virus DNA quantification and pretreatment albumin to globulin ratio are used to predict the survival of ENKTL patients (Suzuki et al., 2011; Ito et al., 2012). However, these factors' prognostic roles for ENKTL are still controversial, and identifying more prognostic factors is an urgent need.

T cell immunoglobulin and mucin-domain-

containing molecule-3 (TIM-3) was found in 2002 when searching the distinct molecule between Th1 and Th2 cells, and it belongs to the TIMs family (Monney et al., 2002). TIM-3 expresses not only on the cell surface of adaptive immune cells such as IFN-γ producing $CD4^+$ Th1 and $CD8^+$ T cytotoxic 1 (Tc1) cells, but also on the cell surface of innate immune cells such as natural killer (NK) cells, dendritic cells, macrophage/mononuclear cells (Khademi et al., 2004; Anderson et al., 2007). As an immune checkpoint molecule, TIM-3 acts as a negative regulator in immune response-it can downregulate Th1/Tc1 cells immune response (Fourcade et al., 2010), induce T cell exhaustion and peripheral immune resistance. Recently, TIM-3 has been found in several types of malignant carcinomas such as melanoma, cervical cancer, colorectal cancer, gastric cancer, prostate cancer, and TIM-3 expression has correlation with malignant carcinoma patient's prognosis (Wiener et al., 2007; Cao et al., 2013; Piao et al., 2014; Cheng et al., 2015; Xu et al., 2015). For now, little is known about TIM-3 in ENKTL.

Lymphocyte activation gene-3 (LAG-3) also belongs to immune checkpoint molecules, it was found in 1990 (Triebel et al., 1990), it is a membranous protein of Ig superfamily (IgSF) which is thought to be closely related with CD4 (Gagliani et al., 2013). LAG-3 mainly expresses on the cell surface of activated $CD4^+$, $CD8^+$ T cells, activated Treg, Tr1 cells, even parts of NK cells and B cells (Huang et al., 2004; Kisielow et al., 2005). LAG-3 binds to MHC class II with high affinity, it is involved in lymphocyte activation and regulating immune response (Demeure et al., 2001). LAG-3 negatively regulates the proliferation, function and balance of T cells (Maçon-Lemaître and Triebel, 2005). High expression of LAG-3 has been detected in tumor-infiltrating lymphocytes of a variety of malignant carcinomas including ovarian cancer, clear cell renal cell carcinoma, melanoma, Hodgkin lymphoma, and it can inhibit tumor-infiltrating lymphocytes' anti-tumor ability (Demeure et al., 2001; Gandhi et al., 2006; Matsuzaki et al., 2010; Matsushita et al., 2016). Expression and prognostic value of LAG-3 in ENKTL remain unknown.

In this study, we aimed to investigate the expression of TIM-3 and LAG-3 in ENKLT patients, analyze the correlation between expression of TIM-3 and LAG-3 and clinicopathological features of ENKTL, and find out whether these two molecules could be prognostic factors for ENKTL.

Materials and methods

Sample collection

A cohort of 41 patients diagnosed with ENKTL at Xiangya Hospital of Central South University from April 2011 to December 2014 was retrospectively analyzed. The inclusion criterion was that patients are pathologically confirmed ENKTL. The exclusion criteria were that patients who received chemotherapy, radiotherapy or other anti-tumor therapies before diagnosis, and patients who had uncontrolled active infections or other illnesses. Clinical information including gender, age, IPI score, B symptoms, LDH level, Ann Arbor stage and treatment regimens was collected. All patients had complete clinical and follow-up data. We also collected 20 patients diagnosed with rhinitis from Xiangya Hospital of Central South University as control group. All paraffin-embedded specimens were collected in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, revised in 2008.

Follow-up study of the patients

All the 41 ENKTL patients were followed-up from the day diagnosed to March 2016, no patients lost to follow-up. We calculated the progression free survival (PFS) of these patients.

Immunohistochemistry

We used immunohistochemistry streptavidin-peroxidase (SP) method to detect expression, and staining was performed by following the manufacturer's instructions for the staining kit. According to the antibodies' manuals, we used mouse lung tissue as positive control for TIM-3 expression, human tonsil tissue as positive control for LAG-3 expression, and phosphate buffer instead of primary antibodies as negative control. The tissue sections were dewaxed and hydrated, and antigen retrieval was performed by heat mediation in citrate buffer (pH 6.0), and then 3% hydrogen peroxide was used for 20 minutes at 37°C, blocked with serum for 30 minutes at 37°C. Tissues were incubated at 4°C overnight with primary antibodies--anti-TIM-3 rabbit polyclonal antibody (Abcam, ab185703, at a working dilution of 1:150) and anti-LAG-3 rabbit monoclonal antibody (Abcam, ab180187, at a working dilution of 1:100). Then biotin-conjugated secondary antibody was used for 20 minutes at 37°C, horseradish peroxidase-linked avidin was then added to incubate for 30 minutes at 37°C. DAB was used to color the tissue slides, counterstained with hematoxylin, and the slides were dehydrated and mounted.

We randomly selected five high-magnification (400×) areas of high-quality staining for each slide, and these slides were evaluated double blind by two experienced pathologists. The scoring results of the two pathologists were in excellent agreement (more than 95% agreement rate), for the few disagreements, consensus scores were determined by a third experienced pathologist. For semi-quantitative evaluation, the staining intensities were classified as: negative, weak, moderate, and strong intensity. Negative

and weak intensities were considered as low expression, moderate and strong intensities were considered as high expression.

Statistical analysis

We used SPSS statistical software for data analysis (version 17.0; SPSS Inc., Chicago, IL, USA). We performed rank sum test to compare the differential expression of TIM-3, LAG-3 in ENKTL and rhinitis groups. The correlation between TIM-3 and LAG-3 was assessed by Spearman rank correlation analysis. Pearson's χ^2 test was used to compare TIM-3 and LAG-3 expression in ENKTL with clinicopathological features of ENKTL. Survival curves were calculated according to Kaplan-Meier method. Multivariate analysis was based on COX regression model. A two-side P value <0.05 was considered statistically significant.

Results

Patient characteristics

The main clinicopathological features of 41 ENKTL patients are summarized in Table 1. None of the 41 patients received any anti-tumor therapy before diagnosis, and therapy after diagnosis was known for all the patients, 4 received radiotherapy alone, 17 received chemotherapy alone, 20 received radiochemotherapy.

Table 1. Clinicopathological features of ENKTL patients.

Characteristics	Presence	Presence%
Age		
<60 years	35	85%
≥60 years	6	15%
Gender		
Male	33	81%
Female	8	19%
IPI score		
Low risk group	22	54%
Low to medium risk group	12	29%
High to medium risk group	6	15%
High risk group	1	2%
B symptoms		
Present	10	24%
Absent	31	76%
LDH		
Elevated	15	37%
Normal	26	63%
Ann Arbor stage		
I+II	31	76%
III+IV	10	24%
Treatment		
Radiotherapy	4	10%
Chemotherapy	17	41%
Radiochemotherapy	20	49%

TIM-3 and LAG-3 expression in ENKTL and rhinitis

Both TIM-3 and LAG-3 had membranous staining patterns. Immunohistochemical staining for different intensity is shown in Fig. 1. The TIM-3 and LAG-3 immunohistochemical staining is shown in Fig. 2. The positive expression rates of TIM-3 and LAG-3 in ENKTL patients were both 95% (39/41), but among rhinitis patients, only 55% (11/20) positively expressed TIM-3 and 45% (9/20) positively expressed LAG-3. Of the 41 ENKTL patients, 9 patients presented with TIM-3 low expression, 32 with TIM-3 high expression, and 8 patients presented with LAG-3 low expression, 33 with LAG-3 high expression. The positive expression rate of TIM-3 between ENKTL and rhinitis patients had statistical significance($P=1.96\times10^{-8}$), and the positive expression rate of LAG-3 between ENKTL and rhinitis patients also had statistical significance ($P=3.79\times10^{-7}$).

Table 2. Correlation between TIM-3 and LAG-3 expression.

		TIM-3		r	P value
		Low	High		
LAG-3	Low	5	3	0.482	0.001
	High	4	29		

Table 3. Association between TIM-3/LAG-3 expression and clinicopathological features.

Characteristics	No.	TIM-3		P value	LAG-3		P value
		Low	High		Low	High	
Age				0.466			0.355
<60 years	35	7	28		6	29	
≥60 years	6	2	4		2	4	
Gender				0.816			0.662
Male	33	7	26		6	27	
Female	8	2	6		2	6	
IPI score				0.494			0.115
Low risk group	22	6	16		3	19	
Low to medium risk group	12	3	9		5	7	
High to medium risk group	6	0	6		0	6	
High risk group	1	0	1		0	1	
B symptoms				0.864			0.336
Present	10	2	8		3	7	
Absent	31	7	24		5	26	
LDH				0.311			0.952
Elevated	15	2	13		3	12	
Normal	26	7	19		5	21	
Ann Arbor stage				0.054			0.073
I+II	31	9	22		8	23	
III+IV	10	0	10		0	10	
Treatment				0.727			0.986
Radiotherapy	4	1	3		2	2	
Chemotherapy	17	3	14		2	15	
Radiochemotherapy	20	5	15		5	15	

Correlation between TIM-3 and LAG-3 expression

In ENKTL patients, the expression of TIM-3 showed a positive correlation with the expression of LAG-3 (r=0.482, P=0.001, Table 2).

Association between TIM-3/LAG-3 expression and clinicopathological features

TIM-3 and LAG-3 expression had no significant correlation with ENKTL patients' age, gender, IPI score,

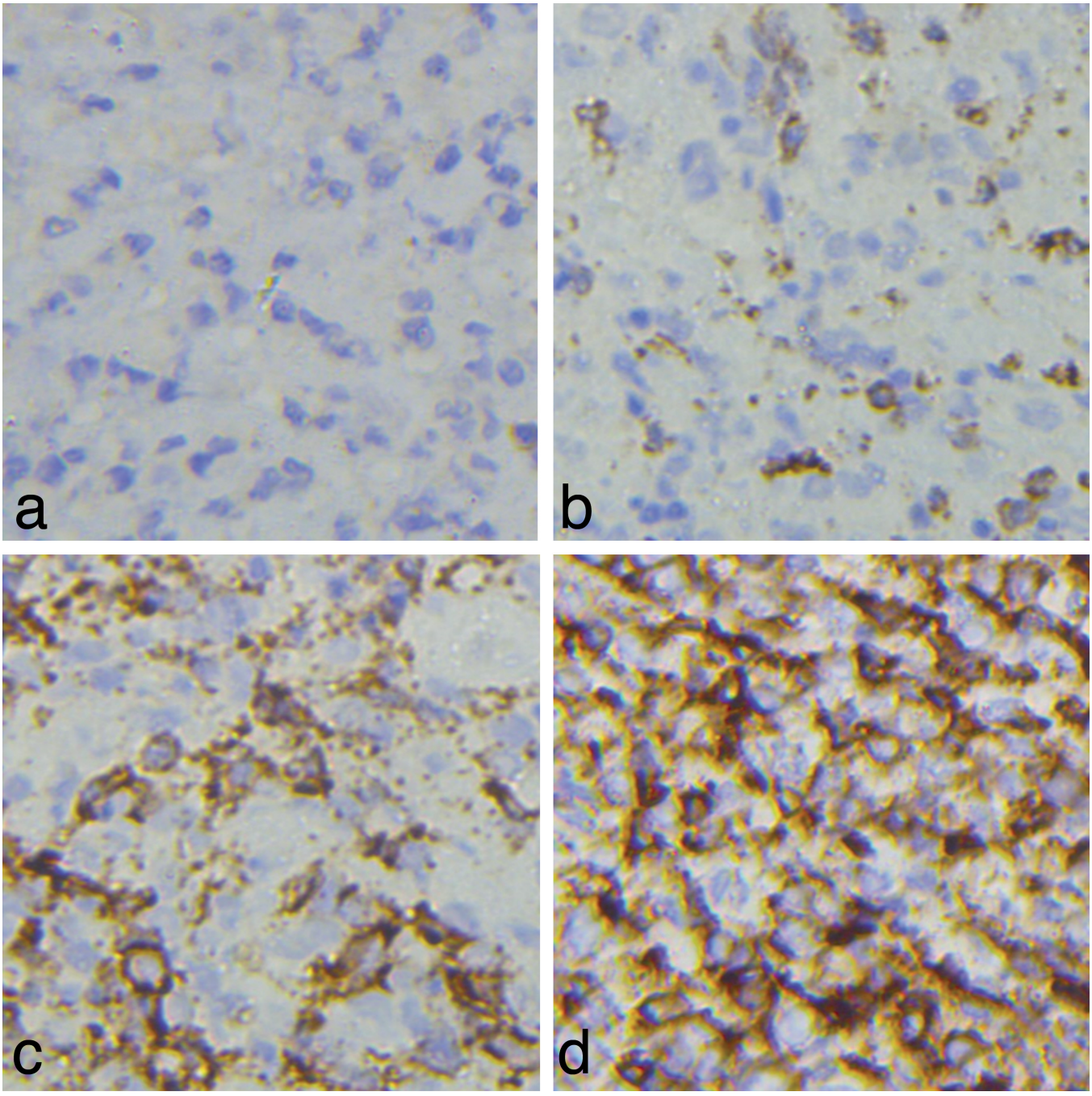

Fig. 1. Semi-quantitative scale for evaluation of immunohistochemical staining intensity. **a.** Negative intensity. **b.** Weak intensity. **c.** Moderate intensity. **d.** Strong intensity. x 400.

B symptoms, LDH level, Ann Arbor stage and treatment (P>0.05, Table 3).

Survival and prognostic factors

Among 41 ENKTL patients, 21 patients presented with disease progression, the median progression free survival (PFS) time for all the patients was 18 months. The clinicopathological features that were statistically significant predictors for PFS are shown in Table 4. IPI score, LDH level and Ann Arbor stage were statistically significant predictors for PFS (P values are listed in

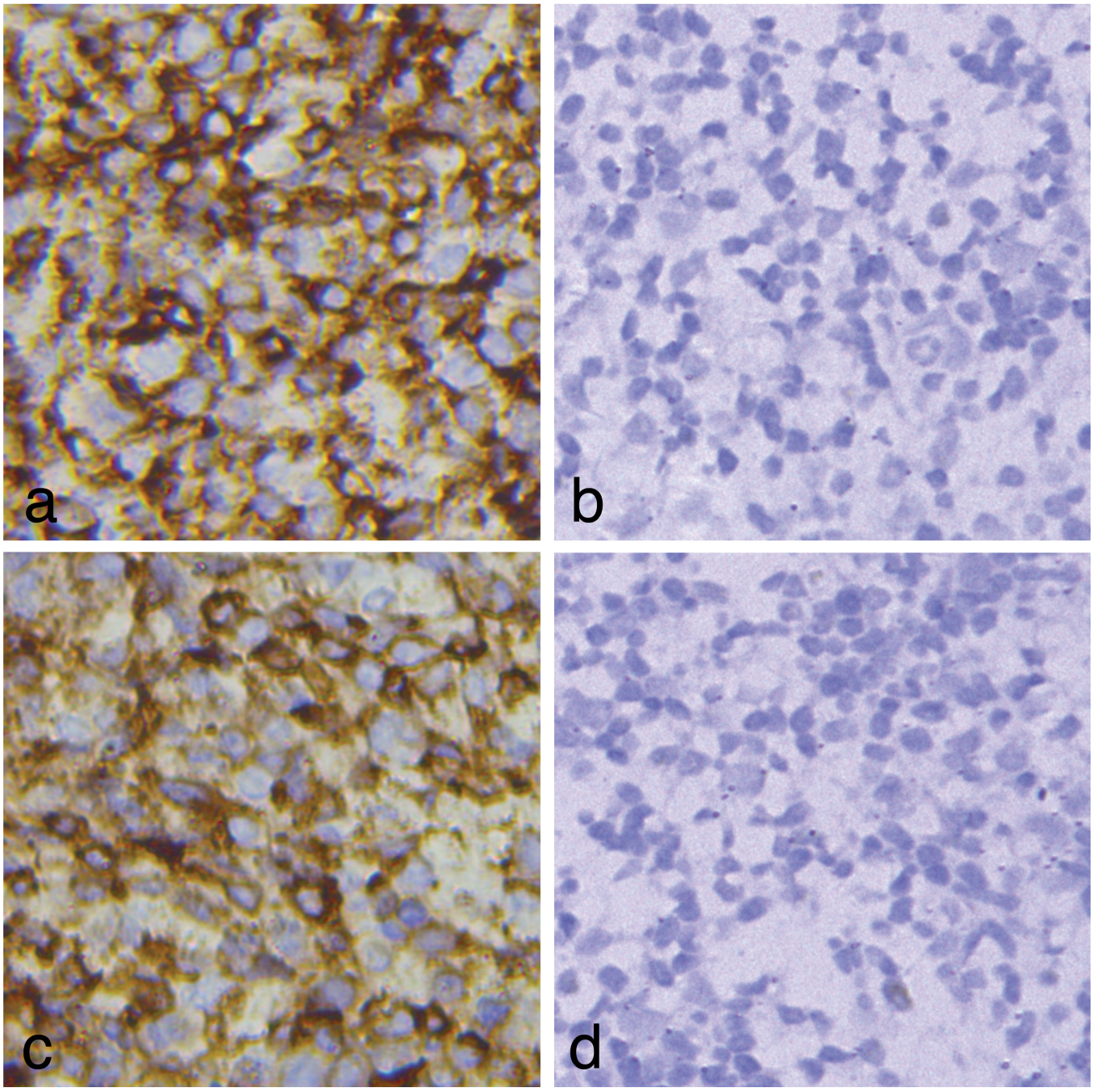

Fig. 2. Immunohistochemical staining for TIM-3 and LAG-3 in ENKTL and rhinitis. **a.** TIM-3 positively expressed in ENKTL. **b.** TIM-3 negatively expressed in rhinitis. **c.** LAG-3 positively expressed in ENKTL. **d.** LAG-3 negatively expressed in rhinitis. x 400.

Table 4), whereas age, gender, B symptoms and treatment were not statistically significant (P>0.05).

The Kaplan-Meier survival curves for PFS with respect to TIM-3 and LAG-3 expression status are shown in Fig. 3. High expression of TIM-3 contributed to statistically significant unfavorable prognostic factor for ENKTL patients (P=0.003). A multivariate Cox regression model including IPI score, LDH level, Ann Arbor stage and TIM-3 expression status showed that the expression status of TIM-3 was an independent predictor of PFS with HR (hazard ratio) at 0.073 (Table 5).

Table 4. Association of PFS and clinicopathological features.

Characteristics	Median PFS (months)	P value
Age		0.758
<60 years	40	
≥60 years	18	
Gender		0.684
Male	38	
Female	40	
IPI score		6.5×10^{-4}
Low risk group	Not reached	
Low to medium risk group	15	
High to medium risk group	9	
High risk group	1	
B symptoms		0.191
Present	16	
Absent	38	
LDH		0.008
Elevated	12	
Normal	40	
Ann Arbor stage		3.2×10^{-4}
I+II	40	
III+IV	10	
Treatment		0.232
Radiotherapy	Not reached	
Chemotherapy	14	
Radiochemotherapy	38	

Discussion

ENKTL is an aggressive disease though always at early stage when diagnosed. Due to the clinical heterogeneity of ENKTL, optimal prognostic factors are difficult to determine. However, refractory and relapse disease exists in ENKTL. Therefore, it is urgent to find new prognostic factors and therapeutic targets for ENKTL. In this study, we analyzed TIM-3 and LAG-3 expression in 41 ENKTL patients by immunohistochemistry staining, and analyzed their relationship with clinicopathological features and prognosis.

TIM-3 is a member of the TIMs family, it consists of 301 amino acids. TIM-3 expresses both on the innate immune cells and acquired immune cells (Khademi et

Table 5. Multivariate analysis of prognostic factors affecting PFS of ENKTL patients.

Parameters	HR	95% CI	P value
IPI score (0-1/2/3/4-5)		0.006-3.57	>0.05
LDH (elevated/normal)	0.425	0.129-1.403	0.16
Ann Arbor stage (I+II/III+IV)	0.39	0.082-1.847	0.235
TIM-3 status (low/high)	0.073	0.008-0.665	0.02

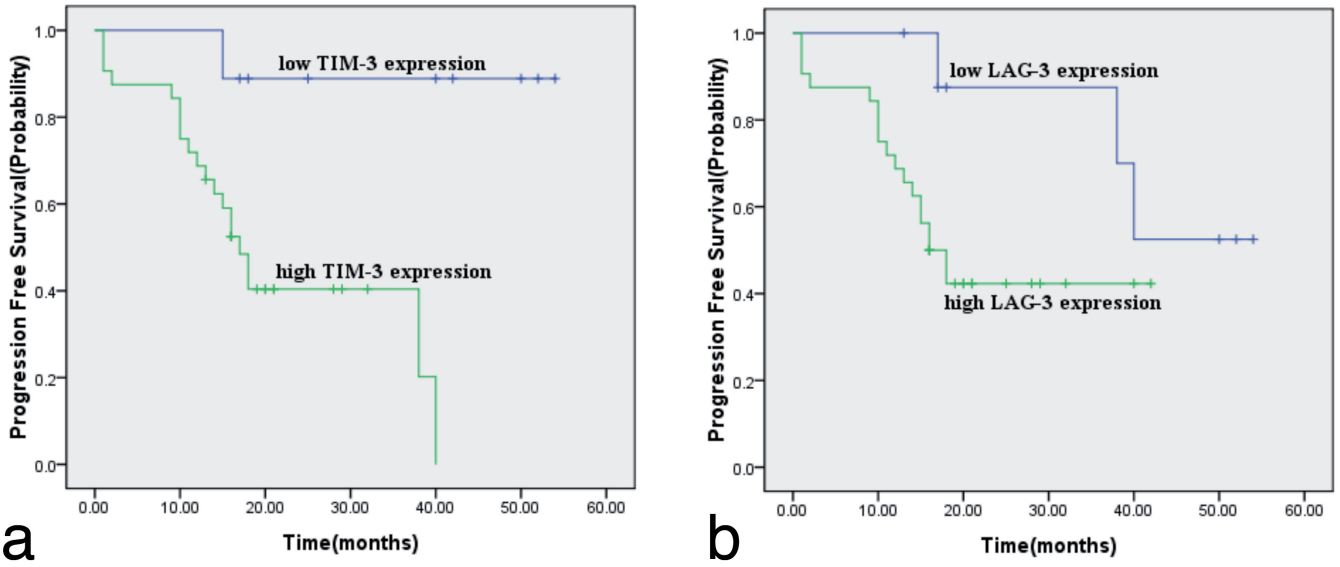

Fig. 3. Kaplan-Meier survival curves of ENKTL patients. **a.** PFS for TIM-3 low and high expression status (P=0.003). **b.** PFS for LAG-3 low and high expression status (P=0.075).

al., 2004; Anderson et al., 2007), as an immune checkpoint molecule, it plays a negative role in immune system. In malignant carcinoma patients, TIM-3 works through direct or indirect action. Direct action refers to T cell exhaustion, which is a state of T cell dysfunction, characterized by a poor effector function and sustained expression of inhibitory receptors (Wherry, 2011). Indirect action includes the effect that increases the cytokine secretion of myeloid derived suppressor cells (Anderson, 2012). It was reported that TIM-3 highly expressed on CD8+ T cells in both solid tumor and hematological malignant tumors along with PD-1, in this way, inhibiting the immune function of patients (Zhou et al., 2011). TIM-3 has been found to be highly expressed in several types of carcinomas and related to prognosis, but little is known about its role in ENKTL, so our study focused on TIM-3 expression and prognostic value in ENKTL.

Our results showed that TIM-3 positively expressed in 95% (39/41) ENKTL patients, which was higher than in rhinitis (55%). TIM-3 had no significant correlation with ENKTL patients' age, gender, IPI score, B symptoms, LDH level, Ann Arbor stage and treatment regimens. We found that patients with TIM-3 high expression had a poorer PFS compared with TIM-3 low expression. Moreover, multivariate analysis showed that TIM-3 high expression was an independent prognostic factor for ENKTL patients' PFS. (Huang et al., 2010) reported that TIM-3 high expression related to poorer prognosis of lymphoma patients. (Zhou et al., 2015) demonstrated that in patients with colorectal cancer, TIM-3 high expression indicated a shorter survival compared with low expression. Our results were consistent with the above finding, indicating that TIM-3 may act as a prognostic factor of ENKTL patients.

LAG-3, also known as CD223, is a member of the Ig superfamily. LAG-3 expresses on activated $CD4^+$, $CD8^+$ T cells, activated Treg, Tr1 cells, and parts of NK cells, B cells and plasma dendritic cells (Huang et al., 2004; Kisielow et al., 2005). Like TIM-3, LAG-3 also belongs to immune checkpoint molecules, and plays an important role in tumor immune system. In tumor microenvironment, LAG-3 works through binding to MHC class II, it can induce T cell exhaustion, and it has been found to be highly expressed on tumor-infiltrating lymphocytes, all these lead to immune tolerance (Blackburn et al., 2009; Grosso et al., 2007). It has been reported that LAG-3 highly expresses on several types of malignant carcinomas, but little is known about its role in ENKTL.

Our study showed that LAG-3 positively expressed in 95% (39/41) ENKTL patients, which was higher than in rhinitis (45%). Along with TIM-3, it had no significant correlation with ENKTL patients' age, gender, IPI score, B symptoms, LDH level, Ann Arbor stage and treatment regimens. PFS of patients with LAG-3 high expression had no statistical difference compared with LAG-3 low expression. (Kotaskova et al., 2010) reported that in lymphoma/leukemia patients, patients with LAG-3 high expression had a shorter disease-free survival compared with LAG-3 low expression. Our conflicting results may be due to small number of patients, short period of follow-up, and this study is retrospective. The role of LAG-3 in ENKTL needs further study.

The prognostic predictors of ENKTL have not been identified, and are still to be explored. (Wang et al., 2017) reported that overexpression of MYC and BCL-2 can help predict prognosis for ENKTL patients, both molecules were closely related to ENKTL patients' PFS and overall survival (OS). (Kim et al., 2009) proposed a new staging system based on treatment outcomes, which is closely related to prognosis: limited disease (stage I/II without local tumor invasiveness) and extensive disease (stage I/II with local invasiveness or stage III/IV disease). (Kim et al., 2016) demonstrated that age ≥60 years, Ann Arbor stage III and IV, distal lymph node involvement and elevated plasma EBV-DNA were poor prognostic factors which related to overall survival. Our study found that patient's IPI score, LDH level and Ann Arbor stage were statistically significant predictors in ENKTL, but patient's age, gender, B symptoms and treatment regimens had no statistical correlation with PFS.

To sum up, we detected the expression of TIM-3 and LAG-3 in ENKTL and rhinitis, and subsequently reported a statistically significant association between TIM-3 expression level and PFS of ENKTL patients, that has clinical significance. However, our study has limitations, such as limited number of patients, incomplete clinical information which leads to selection bias. The follow-up periods are short, and it is a retrospective study. The role of LAG-3 in ENKTL needs further study, and the significance of TIM-3 in ENKTL needs further confirmation.

Acknowledgements. We thank for technical support from Department of Oncology, Xiangya Hospital of Central South University.

Conflict of interest statement. The authors declare no conflicts of interest.

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Accepted September 13, 2017