Expression of microRNA-10a, microRNA-342-3p and their predicted target gene TIAM1 in extranodal NK/T-cell lymphoma, nasal type

HAOBO HUANG[1], LIPING FAN[1], RONG ZHAN[2], SHUNQUAN WU[2] and WENYAN NIU[2]

[1]Department of Blood Transfusion, Fujian Medical University Union Hospital; [2]Department of Hematology, Fujian Medical University Union Hospital and Fujian Institute of Hematology, Fuzhou, Fujian 350001, P.R. China

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Abstract. MicroRNAs (miRNAs) may act as oncogenes or tumor suppressor genes in different types of human cancer. T-lymphoma invasion and metastasis inducing factor 1 (TIAM1) participates in the development of several types of human cancer. However, the expression of miRNAs and TIAM1 in extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKTCL) remains poorly understood. In the present study, the association between the expression levels of miR-10a and miR-342-3p and the protein expression levels of TIAM1 was examined in ENKTCL tissues. The expression levels of miR-10a, miR-22, miR-340, miR-342-3p and miR-590-5p in 15 primary ENKTCL tissues were analyzed using quantitative polymerase chain reaction, and the protein expression levels of TIAM1 in 21 primary ENKTCL tissues were analyzed using immunohistochemistry. The expression levels of miR-10a and miR-342-3p were lower in ENKTCL tissues than in normal NK cells, but no significant differences were observed in the expression levels of miR-22, miR-340 and miR-590-5p in ENKTCL tissues, compared with normal NK cells. The low expression levels of miR-10a detected in the tissues of patients with ENKTCL were inversely correlated with the age of the patients, whereas the low expression levels of miR-342-3p measured in these samples were not correlated with any demographic or clinical features of the patients. The protein expression levels of TIAM1 were higher in ENKTCL tissues than in normal and reactive lymph node hyperplasia tissues, and positively correlated with the Ann Arbor stage and international prognostic index score of the tumors. Furthermore, the expression levels of miRNA-10a and miRNA-342-3p were inversely correlated with the protein expression levels of TIAM1 in ENKTCL tissues. These data suggest that TIAM1 may contribute to the pathogenesis of ENKTCL, and miRNA-10a and miRNA-342-3p may be involved in the development of ENKTCL via the TIAM1 pathway.

Correspondence to: Professor Rong Zhan, Department of Hematology, Fujian Medical University Union Hospital and Fujian Institute of Hematology, 29 Xinquan Road, Gulou, Fuzhou, Fujian 350001, P.R. China
E-mail: huanghaobo1981@163.com

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Introduction

Extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKTCL) is an aggressive type of lymphoma that occurs frequently in the Asian population (1,2). The pathogenesis of this tumor is poorly understood, and conventional chemotherapy regimens, currently employed for the treatment of other aggressive types of lymphoma, usually provide poor outcomes in patients with ENKTCL (1,2). Therefore, studies on the pathogenetic abnormalities that occur during the development of ENKTCL may contribute to improving the clinical outcomes of these patients.

MicroRNAs (miRNAs) are small non-coding RNA molecules that inhibit the transcription or translation of mRNA. Previous studies have demonstrated that dysregulation of miRNA occurs in numerous types of human cancer, indicating that miRNAs may act as oncogenes or tumor suppressor genes (3). Previous miRNA expression profiling studies conducted on a series of ENKTCL formalin-fixed paraffin-embedded (FFPE) tissues revealed that several miRNAs, including miR-10a, miR-22, miR-340, miR-342-3p and miR-590-5p, are dysregulated in ENKTCL tissues, compared with normal NK cells (1). Bioinformatic analysis of these miRNAs indicated that all of them target the T-lymphoma invasion and metastasis-inducing factor 1 (TIAM1) gene (1).

Tiam1 is a specific guanine nucleotide exchange factor for Rho-like guanosine triphosphate (GTP)ases, which exhibits its pathophysiological role via the activation of the rat sarcoma-related C3 botulinum toxin substrate signaling pathway (4). Overexpression of TIAM1 has been reported in various solid tumors (5-12). In addition, previous studies have demonstrated that TIAM1 modulates a number of cellular processes considered to be associated with tumor progression, including cell apoptosis, invasion and migration (13-18). These findings suggest that TIAM1 may be a target for cancer

Table I. Primer sequences of miRNAs for quantitative polymerase chain reaction analysis.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
miRNA-10a	TACCCTGTAGATCCGAATTTGTG	ATTCCCCTAGATACGAATTTGTGA
miRNA-22	AGCAACATGCCCTGCTC	TCTGTCACCTTCCAGATGATG
miRNA-340	ATAAAGCAATGAGACTGATTGTC	GGCTATAAAGTAACTGAGACGGA
miRNA-342-3p	GTGCTATCTGTGATTGAGGGA	CGGGTGCGATTTCTGTG
miRNA-590-5p	TTAGAGCCAACCAGCAGC	GCATTGACAGCACATCCC
U6	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	CTCAACCTACAATCAAAAACAACACAAACA

miRNA, microRNA.

therapy. However, there is limited evidence regarding the role of the TIAM1 gene in the pathogenesis of ENKTCL.

To gain insight into the potential role of miR-10a, miR-22, miR-340, miR-342-3p, miR-590-5p and TIAM1 in the pathogenesis of ENKTCL, the present study examined the expression levels of these miRNAs and their target gene TIAM1 in ENKTCL tissues, in order to assess whether the expression levels of these molecules correlated with the clinical features of patients with ENKTCL.

Materials and methods

Patients and controls. Patients who were diagnosed with ENKTCL from 2007 to 2011 were selected from the archives of the Department of Pathology of the Fujian Medical University Union Hospital (Fuzhou, China), and classified according to the 2008 World Health Organization classification of lymphomas (19). A total of 21 patients were selected for the study. The study was approved by the ethics committee of Fujian Medical University Union Hospital. The samples were collected with the patients' consent. Patients with no additional tissue available for immunohistochemical testing were excluded from the study. The FFPE tissues of 15 patients were subjected to quantitative polymerase chain reaction (qPCR) analysis. In addition, ten samples of normal and reactive lymph node hyperplasia FFPE tissue were included as controls.

Isolation of normal NK cells from peripheral blood. Human normal NK cells were isolated from whole blood samples obtained from healthy donors, which were collected with EDTA using NK Cell Isolation Kit (TBD Science, Tianjin, China).

RNA extraction. Prior to RNA extraction, five pieces of the FFPE tissue sections were treated with 1 ml xylene (Kemiou Chemical Reagent Co., Tianjin, China) for 10 sec at room temperature,then incubated at 56 degree for 3 min to remove the paraffin, and subsequently digested with 10 μl proteinase K (Qiagen, Inc., Valencia, CA, USA), followed by incubation at 56˚C for 15 min, then at 80˚C for 15 min. Total RNA was then isolated using miRNeasy FFPE Kit (Qiagen, Inc.), according to the manufacturer's protocol. Total RNA from NK cells was extracted with TRIzol (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Reverse transcription (RT) and qPCR of miRNAs. Complementary DNA was synthesized from total RNA using PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Bio, Inc., Otsu, Japan), following the manufacturer's protocol. The RT reactions contained 500 ng total RNA extracted from the samples, 2 μl 5X PrimeScript™ Buffer (Takara Bio, Inc.), 0.5 μl 1X PrimeScript™ RT Enzyme Mix I (Takara Bio, Inc.) and 0.5 μl oligo(dT) primer (Takara Bio, Inc.). The 10-μl reactions were incubated for 42 min at 37˚C, followed by 30-sec incubation at 85˚C, and then exposed to 4˚C.

To quantify the expression levels of the aforementioned miRNAs, qPCR was conducted using SYBR® Premix Ex Taq™ II (Tli RNase H Plus) Kit (Takara, Bio, Inc.) with an Applied Biosystems 7300 Real Time PCR System (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The 20-μl PCR reactions included 0.19 μl RT product, 10.0 μl 2X SYBR® Premix Ex Taq™ II (Takara Bio, Inc.), 2.0 μl primers mix (Biosune, Inc., Shanghai, China), 0.4 μl 50X ROX Reference Dye II (Takara Bio, Inc.) and 7.41 μl RNase-free dH_2O (Takara Bio, Inc.). The reaction mixtures were incubated in a 96-well plate at 95˚C for 1 min, followed by 40 cycles of amplification at 95˚C for 5 sec and 60˚C for 30 sec. The sequences of the primers used are listed in Table I. The quantification cycle (C_q) was determined using default threshold settings. All the experiments were performed in triplicate. U6 small nuclear RNA was used as control to normalize the miRNA input in the qPCR assay. The qPCR data were analyzed using the $2^{-\Delta Cq}$ method.

Immunohistochemistry. Tissue sections were subjected to antigen retrieval by incubation in 10 mmol/l sodium citrate buffer (pH 6.0) for 10 min in a microwave oven (WD900SL23-2, Galanz Enterprises Co., Ltd., Foshan, China) at the maximum power setting. Any potential endogenous peroxidase activity present in the tissues was blocked by incubation with 3% H_2O_2 for 15 min. Subsequently, the tissue sections were incubated at room temperature for 60 min with a rabbit polyclonal antibody specific for human TIAM1 (dilution,7 1:100; cat no. sc-872, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted in phosphate-buffered

Table II. Clinical features of 21 patients with ENKTCL.

Patient no.	Age (years)	Gender	Primary cancer site	Stage[a]	LDH levels	B symptoms	IPI	TIAM1 in ENKTCL (IHS)[b]
1	27	Male	Nasal cavity	I	Normal	A	0	0
2	52	Female	Nasal cavity	II	Normal	B	0	0
3	71	Male	Nasal cavity	I	Normal	A	1	0
4	68	Male	Gingiva	IV	Normal	A	4	0
5	24	Male	Adrenal gland	III	Normal	A	2	2
6	54	Male	Nasal cavity	IV	High	B	4	0
7	55	Male	Nasal cavity	I	Normal	A	0	2
8	45	Female	Bone marrow	IV	Normal	B	3	2
9	39	Male	Nasal cavity	IV	High	B	3	1
10	41	Male	Nasal cavity	I	High	A	1	2
11	26	Male	Gastric	IV	High	A	4	0
12	64	Male	Nasal cavity	I	High	A	3	1
13	13	Female	Bone marrow	IV	High	B	4	2
14	55	Male	Bone marrow	IV	High	B	4	1
15	43	Male	Bone marrow	IV	High	B	4	0
16	43	Female	Nasal cavity	II	High	B	2	0
17	71	Male	Nasal cavity	I	Normal	A	2	0
18	40	Male	Nasal cavity	IV	Normal	B	3	1
19	39	Male	Nasal cavity	III	Normal	B	3	2
20	24	Male	Nasal cavity	IV	High	B	3	2
21	32	Male	Nasal cavity	II	Normal	B	1	0

[a]Ann Arbor staging; [b]protein expression levels of TIAM1 in ENKTCL tissues. ENKTCL, extranodal natural killer/T-cell lymphoma, nasal type; LDH, lactate dehydrogenase; IPI, international prognostic index; TIAM1, T-lymphoma invasion and metastasis inducing factor 1; IHS, immunohistochemistry score.

saline (PBS). Immunoreactive proteins were visualized with MaxVision™ HRP-Polymer anti-Rabbit IHC Kit (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China), following the manufacturer's protocol, and counterstained with hematoxylin (Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China). Tissue sections corresponding to the negative control were treated with PBS under the same experimental conditions than the samples.

Quantitative evaluation of the protein expression levels of TIAM1 was performed by counting the percentage of immunoreactive cells positive for TIAM1 that were present in a number of high-power microscopic fields (magnification, x200; BX41 microscope, Olympus Corporation, Tokyo, Japan). The ENKTCL tissues were scored based on the percentage of positive tumor cells expressing cytoplasmic TIAM1, as follows: i) <15%, score 0; ii) 15-24%, score 1; iii) 25-49%, score 2; iv) 50-74%, score 3; and v) ≥75%, score 4.

Statistical analysis. Since the data corresponding to the expression levels of miR-10a, miR-22, miR-340, miR-342-3p and miR-590-5p followed a normal distribution, the expression levels of these miRNAs in ENKTCL tissues and normal NK cells were compared using Student's two-tailed t test. The association between the clinical features of patients with ENKTCL and the expression levels of miR-10a, miR-342-3p and TIAM1 protein detected in these patients was analyzed via Student's two-tailed t test and χ^2 test, respectively. Spearman's rank correlation was used to evaluate the association between the expression levels of miR-10a and miR-342-3p and the protein expression levels of TIAM1. $P<0.05$ was considered to indicate a statistical significant difference. SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Clinical features of patients with ENKTCL. The main demographic and clinical features of patients with ENKTCL are listed in Table II. The median age at the time of diagnosis was 44.1 years (range, 13-71 years), and the most frequent cancer site was the nasal cavity. According to the Ann Arbor staging of lymphoma, 6 patients were in stage I, 3 in stage II, 2 in stage III and 10 in stage IV (20). High expression levels of lactate dehydrogenase (LDH) were observed in 10 patients, and 12 patients exhibited B symptoms.

qPCR results of miR-10a, miR-22, miR-340, miR-342-3p and miR-590-5p. The expression levels of miR-10a were markedly lower in ENKTCL tissues than in normal NK cells (Fig. 1A). In addition, the expression levels of miR-342-3p in ENKTCL tissues were significantly lower than in normal NK cells (Fig. 1B). In contrast, the expression

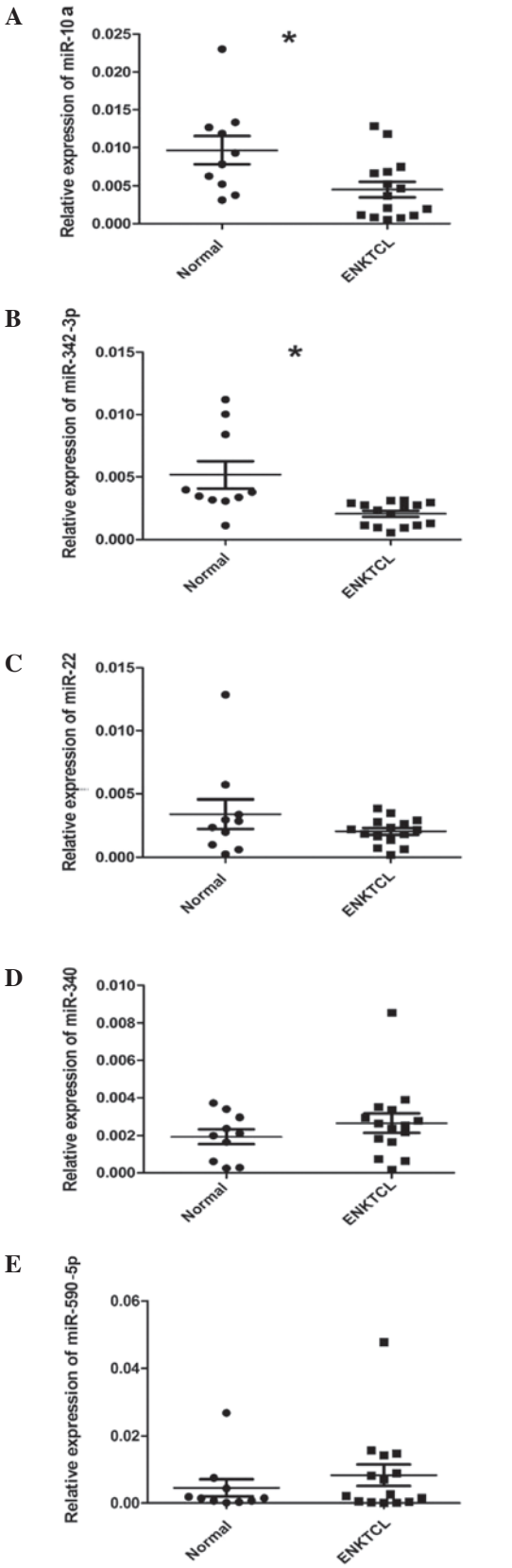

Figure 1. Expression levels of miRNAs in ENKTCL FFPE tissues and normal NK cells. The expression levels (measured relative to U6) of (A) miR-10a and (B) miR-342-3p were lower in ENKTCL tissues than in normal NK cells ($4.51\pm1.03\times10^{-3}$ vs. $9.63\pm1.88\times10^{-3}$ and $2.08\pm0.24\times10^{-3}$ vs. $5.18\pm1.08\times10^{-3}$, respectively; $^{*}P<0.05$ vs. normal NK cells). The expression levels of (C) miR-22, (D) miR-340 and (E) miR-590-5p did not differ significantly between ENKTCL tissues and normal NK cells. miRNA, microRNA; ENKTCL, extranodal NK/T-cell lymphoma, nasal type; FFPE, formalin-fixed paraffin-embedded; NK, natural killer.

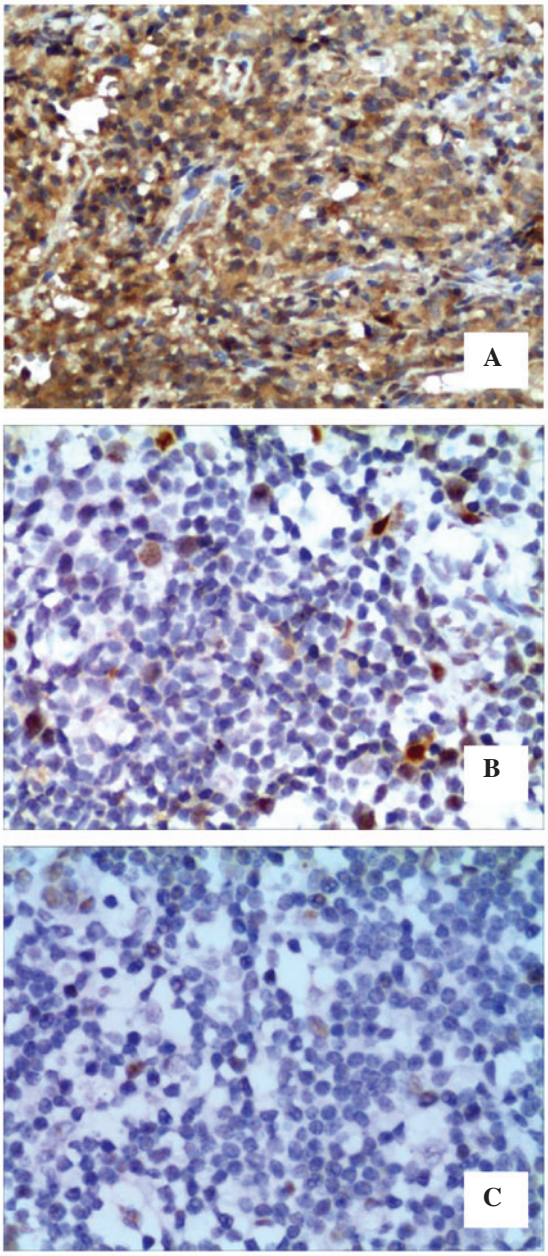

Figure 2. Immunohistochemical staining of TIAM1 in clinical FFPE tissues (magnification, x200). The rate of positive cells and the intensity of the staining for TIAM1 protein was higher in (A) extranodal natural killer/T-cell lymphoma, nasal type FFPE tissues, compared with (B) reactive lymph node hyperplasia and (C) normal lymph node tissues. TIAM1, T-lymphoma invasion and metastasis inducing factor 1; FFPE, formalin-fixed paraffin-embedded.

levels of miR-22, miR-340 and miR-590-5p did not differ significantly between ENKTCL tissues and normal NK cells (Fig. 1C-E). These results suggest that miR-10a and miR-342-3p may be involved in the pathogenesis of ENKTCL.

Correlations between the expression levels of miR-10a and miR-342-3p and the demographic and clinical characteristics of patients with ENKTCL. The expression levels of miR-10a in ENKTCL FFPE tissues were inversely correlated with the patients' age (P=0.02), but not with other demographic or clinical features, including gender, Ann Arbor stage, levels of LDH, B symptoms and international prognostic index

Table III. Association between the expression levels of miR-10a, miR-342-3p and TIAM1 protein in tissues of patients with ENKTCL and the demographic and clinical features of the patients.

		miR-10a		miR-342-3p		TIAM1		
Clinical feature	Cases (no.)	Expression ($x10^{-3}$)[a]	P-value[b]	Expression ($x10^{-3}$)[a]	P-value[b]	Cases (no.)	TIAM1^{+} cases (no.)	P-value[c]
Age (years)			0.02		0.97			0.34
<60	13	4.97±1.13		2.08±0.27		17	10	
≥60	2	1.46±0.64		2.05±0.73		4	1	
Gender			0.58		0.70			0.92
Male	12	4.03±1.02		2.02±0.28		17	9	
Female	3	6.41±3.43		2.29±0.56		4	2	
Ann Arbor stage			0.98		0.22			0.02
I-II	6	4.46±1.98		1.68±0.41		9	2	
III-IV	9	4.54±1.20		2.34±0.28		12	9	
LDH levels			0.93		0.90			0.51
High	6	4.39±1.72		2.12±0.44		10	6	
Normal	9	4.59±1.36		2.05±0.3		11	5	
B symptoms			0.06		0.88			0.53
Positive	8	6.28±1.53		2.11±0.32		12	7	
Negative	7	2.48±0.93		2.04±0.39		9	4	
IPI (score)			0.61		0.21			0.02
0-2	7	3.90±1.77		1.74±0.35		9	2	
3-5	8	5.04±1.24		2.37±0.32		12	9	

[a]Expression measured relative to U6; [b]Student's t-test; [c] χ^2 test. miR, microRNA; TIAM1, T-lymphoma invasion and metastasis inducing factor 1; ENKTCL, extranodal natural killer/T-cell lymphoma, nasal type; LDH, lactate dehydrogenase; IPI, international prognostic index.

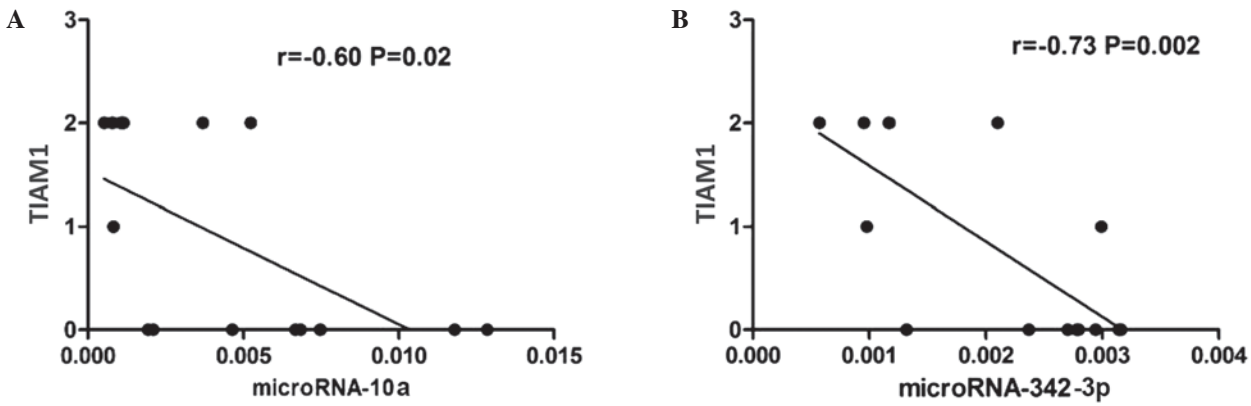

Figure 3. Correlation between the expression levels of (A) microRNA-10a and (B) microRNA-342-3p and the protein expression levels of TIAM1 in formalin-fixed paraffin-embedded tissues of patients with extranodal natural killer/T-cell lymphoma, nasal type. TIAM1, T-lymphoma invasion and metastasis inducing factor 1.

(IPI) score (Table III). The expression levels of miR-342-3p in ENKTCL FFPE tissues was not correlated with any demographic or clinical features of the patients.

Immunohistochemistry and correlation between the expression levels of TIAM1 protein and the demographic and clinical features of patients with ENKTCL. Tiam1 protein was expressed in 11 ENKTCL samples (52.4%) and in 1 of 10 paired samples of normal and reactive lymph node hyperplasia (10%), where its expression levels were low (Fig. 2). The intensity of TIAM1 protein expression detected in the ENKTCL tissues is listed in Table II. The protein expression levels of TIAM1 in ENKTCL FFPE tissues were positively correlated with Ann Arbor stage and IPI score (P=0.02; Table III), but no significant association was observed with any other demographic or clinical features of the patients. These results suggest that TIAM1 protein may be involved in the pathogenesis of ENKTCL.

Correlations between the expression levels of miR-10a, miR-342-3p and TIAM1 protein in ENKTCL FFPE tissues. Expression of TIAM1 protein was detected in the FFPE tissues of 7 of the 15 patients with ENKTCL analyzed (47%). In these patients, the expression levels of miR-10a and miR-342-3p appeared to be inversely correlated with the protein expression levels of TIAM1 (Spearman's r=-0.60 and -0.73 for miR-10a and miR-342-3p, respectively; P=0.02; Fig. 3).

Discussion

Previous studies have demonstrated that the expression levels of several miRNAs are downregulated in ENKTCL FFPE tissues, which is considered to contribute to the pathogenesis of the tumor by the loss of the suppressive effects that these miRNAs normally exert on their target genes (1,21-25). In a study by Ng *et al* (1), the expression levels of miR-10a, miR-22, miR-340, miR-342-3p and miR-590-5p appeared to be downregulated in ENKTCL FFPE tissues, compared with normal NK cells, according to the results of human miRNA microarray analysis. However, these findings were not further validated by qPCR.

The qPCR data of the present study are consistent with the previous miRNA microarray results reported by Ng *et al* (1), confirming that miR-10a and miR-342-3p are downregulated in ENKTCL FFPE tissues, compared with normal NK cells. However, in the present study, the expression levels of miR-22, miR-340 and miR-590-5p did not differ significantly between ENKTCL tissues and normal NK cells. Furthermore, the expression levels of miR-10a in the ENKTCL FFPE tissues correlated with the patients' age, but the expression levels of miR-342-3p did not correlate with any demographic or clinical feature of the patients. Therefore, further studies are required to validate the potential participation of miR-10a and miR-342-3p in the pathogenesis of ENKTCL.

Previous studies have identified several target genes of miR-10a, including high-mobility group A2 (26), cell adhesion molecule L1-like (27), homeobox (HOX)A1 (28) and HOXD4 (29), which are involved in cellular differentiation, growth, migration and invasion in various pathophysiological processes (26-29). Other studies have demonstrated that the expression of miR-342-3p is downregulated in the blood and tumor tissues of patients with cancer, including colorectal cancer (30), clinical glioblastoma multiforme (31), breast tumor (32), acute lymphoblastic leukemia (33) and Sézary syndrome (34). In addition, miR-342-3p has been previously demonstrated to be involved in cell differentiation (35), growth (36,37), invasion (37) and response to chemotherapy (38,39) in cancer cells.

Previous studies have suggested that multiple miRNAs may suppress the expression of the same target gene by directly targeting its 3' untranslated region (40). Furthermore, previous bioinformatic analysis indicated that miR-10a and miRNA-342-3p target the TIAM1 gene (1).

Tiam1 has been identified as a guanine nucleotide exchange factor that exchanges guanosine diphosphate for GTP in Rho-like GTPases, thereby activating them. This process leads to the activation of a signaling pathway that stimulates the c-Jun N-terminal kinase, p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways, which results in the regulation of the expression of genes involved in cellular migration, invasion and metastasis (4,5). To date, overexpression of TIAM1 has been reported in various types of tumor tissue, including head and neck (5), esophageal (6), colorectal (7), gallbladder (8), renal cell (9), nasopharyngeal (10), hepatocellular (11) and prostate carcinoma (12). In addition, overexpression of TIAM1 has been suggested to be involved in tumor progression via lymphangiogenesis (13), apoptosis (14,15), invasion and migration (16-18). However, the expression of TIAM1 in ENKTCL FFPE tissue and its association with clinical features of patients with ENKTCL remain unclear.

The immunohistochemistry data of the present study demonstrated that TIAM1 protein was overexpressed in ENKTCL FFPE tissues, compared with normal and reactive lymph node hyperplasia FFPE tissues, which is in agreement with the results of previous studies on various malignancies (4-6,8-13). The overexpression of TIAM1 in the ENKTCL cases analyzed in the present study correlated with the Ann Arbor stage and IPI score of the tumors. However, no significant association was observed between the protein expression levels of TIAM1 and any other demographic and clinical characteristics of the patients, including age, gender, levels of LDH and B symptoms. Overall, the results of the present study suggest that TIAM1 may be involved in the pathogenesis of ENKTCL. However, due to the small sample size of the present study, further studies involving a larger number of cases of ENKTCL are required in order to confirm these findings.

In conclusion, the results of the present study suggest that reduced expression of miR-10a and miR-342-3p and overexpression of TIAM1 protein may be involved in the progression of ENKTCL. Additional *in vitro* and *in vivo* studies are required to further elucidate the potential role and mechanism of action of these molecules in the development of ENKTCL.

Acknowledgements

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