European Review for Medical and Pharmacological Sciences 2020; 24: 4404-4411

MiRNA-584 suppresses the progression of NK/T-cell lymphoma by targeting FOXO1

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Abstract. – OBJECTIVE: This study aims to clarify the influences of miRNA-584 on proliferative and invasive abilities in NK/T-cell lymphoma, and to illustrate the underlying mechanism.

PATIENTS AND METHODS: MiRNA-584 levels in peripheral blood of NK/T-cell lymphoma patients and healthy controls were detected by qRT-PCR. Potential relationship between miRNA-584 level and clinical indicators of NK/T-cell lymphoma patients was analyzed. Kaplan-Meier curves were depicted for assessing the prognostic value of miRNA-584 in NK/T-cell lymphoma patients. After overexpression or knockdown of miRNA-584, changes in proliferative and invasive abilities in KHYG-1 and SNK-6 cells were assessed by CCK-8 and transwell assay, respectively. The potential mechanism of miRNA-584 and its downstream gene FOXO1 in regulating the development of NK/T-cell lymphoma was explored by Dual-Luciferase reporter gene assay and rescue experiments.

RESULTS: Peripheral blood levels of miRNA-584 were lower in NK/T-cell lymphoma patients than those of healthy control. Compared with NK/T-cell lymphoma patients expressing high level of miRNA-584, those expressing a low level suffered worse tumor staging, higher rate of bone marrow invasion, and worse prognosis. Overexpression of miRNA-584 suppressed proliferative and invasive abilities in KHYG-1 and SNK-6 cells. On the contrary, knockdown of miRNA-584 yielded the opposite results. FOXO1 was showed to be the direct target binding miRNA-584, and its level was negatively regulated by miRNA-584. Notably, FOXO1 could partially abolish the regulatory effects of miRNA-584 on proliferative and invasive abilities in NK/T-cell lymphoma.

CONCLUSIONS: Low level of miRNA-584 is closely linked to advanced stage, susceptibility to bone marrow invasion, and poor prognosis in NK/T-cell lymphoma. MiRNA-584 suppresses proliferative and invasive abilities in NK/T-cell lymphoma by targeting FOXO1.

Key Words:
MiRNA-584, FOXO1, NK/T-cell lymphoma.

Introduction

Lymphoma is a common malignancy of the hematopoietic system. Based on the characteristics of lymphoma cells, it is classified into Hodgkin's lymphoma and non-Hodgkin's lymphoma (NHL), and the latter accounts for about 90% of lymphoma cases[1,2]. Chemotherapy combined radiotherapy, hematopoietic stem cell transplantation or others is the preferred therapeutic approach for lymphoma. Most subtypes of lymphoma could be relieved after active treatment, while some of them are prone to relapse in a short period. The incidence of extranodal NK/T-cell lymphoma is much higher in China than that in Western countries, accounting for 2-10% of NHL[3-6]. NK/T-cell lymphoma is mainly characterized by tumor necrosis in the lesion, high malignancy, strong aggressiveness, radiotherapy, and chemotherapy sensitivity but is prone to resistance, high rate of recurrence, and poor prognosis. EB virus infection is a leading cause that is responsible for the occurrence of NK/T-cell lymphoma[7-9]. Individualized therapy for NK/T-cell lymphoma is well concerned nowadays[10,11].

MiRNAs are non-coding RNAs with 18-25 nt long. They are widely distributed in eukaryotes[12,13]. MiRNAs are single-stranded and lack an open reading frame (ORF), but they have a unique ability to degrade or downregulate the target gene[14,15]. In addition, miRNAs post-transcriptionally or translationally regulate target gene expressions, thus influencing downstream gene functions[16]. In the whole genomes, miRNAs only account for 1-4%. However, they are able to regulate expressions of about 30% genes, serving as critical mediators[17]. MiRNA-584 is differentially expressed in tumor species and normal tissues. It is reported that miRNA-584 is capable of suppressing the malignant progression of bladder cancer[18,19]. It is important to clarify

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target genes and pathways regulated by miRNAs in tumor diseases[14-16].

Bioinformatics analysis uncovered the binding sequences in 3'UTR of miRNA-584 and FOXO1. FOXO1 is considered to be a product of a tumor-suppressor gene and involved in abnormal cell division during tumorigenesis. Decreased activity of FOXO1 leads to decreased repair ability of DNA damage, thus resulting in genome instability and susceptibility to tumorigenesis[20,21]. This study mainly explored the regulatory effects of miRNA-584/FOXO1 on the development of NK/T-cell lymphoma, which provided new directions in diagnosis and treatment.

Patients and Methods

NK/T-Cell Lymphoma Patients

Peripheral blood samples were collected from 27 NK/T-cell lymphoma patients and 27 healthy subjects. None of enrolled NK/T-cell lymphoma patients received preoperative anti-tumor treatment. Clinical data and follow-up data of enrolled patients were recorded. Tumor staging was assessed based on the guideline proposed by UICC. Patients and their families in this study have been fully informed. This investigation was approved by the Ethics Committee of Affiliated Zhongshan Hospital of Dalian University. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

NK cells and lymphoma cells (NK-92, HANK-1, SNK-1, KHYG-1, and SNK-6) provided by American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in F-12K medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Transfection

Cells were inoculated in a 6-well plate. Transfection was conducted at 70% confluence using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate (2×10^3 cells/well). At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Invasion Assay

Cells were inoculated in a 24-well plate with 2.0×10^5/ml. 200 μL of suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, cells invaded to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and captured using a microscope. Invasive cell number was counted in 5 randomly selected fields per sample (magnification 20×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were as follows: miRNA-584: forward: 5'-TGCAATGTGTGTGTTAGCCA-3', reverse: 5'-ATCATTGCTCCTTGGATGGT-3'; U6: forward: 5'-CTCGCTTCGGCAGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; FOXO1: forward: 5'-CAGCAAATCAAGTTATGGAGGA-3', reverse: 5'-TATCATTGTGGGGAGGAGAGTC-3'; GAPDH: forward: 5'-ATGGAGAAGGCTGGGGCTC-3', reverse: 5'-AAGTTGTCATGGATGACCTTG-3'.

Western Blot

Cellular protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked in 5% skim milk for 1 hour. The specific primary antibody was used to incubate with the membrane overnight at 4°C, followed by secondary antibody incubation for 2 h at room temperature. After Tris-Buffered Saline and Tween-20 (TBST) washing for 1 min, the enhanced chemiluminescent (ECL) substrate

kit (Pierce, Rockville, MD, USA) was used for exposure of the protein band.

Dual-Luciferase Reporter Gene Assay

HEK293T cells were inoculated in a 24-well plate and co-transfected with miR-NC/miRNA-584 mimic and wild-type/mutant-type FOXO1, respectively. 48 hours later, cells were lysed for determining relative luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (SPSS IBM, Armonk, NY USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Kaplan-Meier curves were depicted for survival analysis. Spearman correlation test was performed to assess the relationship between peripheral blood levels of miRNA-584 and FOXO1 in NK/T-cell lymphoma patients. $p<0.05$ was considered as statistically significant.

Results

Low Expression of MiRNA-584 in NK/T-Cell Lymphoma

MiRNA-584 levels in peripheral blood of NK/T-cell lymphoma patients and healthy controls were detected by qRT-PCR. The data showed lower level of miRNA-584 in NK/T-cell lymphoma patients than those of controls (Figure 1A). In addition, miRNA-584 was downregulated in lymphoma cell lines than that of NK cells (Figure 1B). Among the 5 tested lymphoma cell lines, KHYG-1 and SNK-6 cells expressed the lowest abundance of miRNA-584, and they were selected for the following experiments.

MiRNA-584 Expression was Correlated with Tumor Staging, Bone Marrow Invasion and Prognosis in NK/T-Cell Lymphoma Patients

Clinical data of enrolled NK/T-cell lymphoma patients were collected. Our analysis showed that miRNA-584 level was negatively correlated to

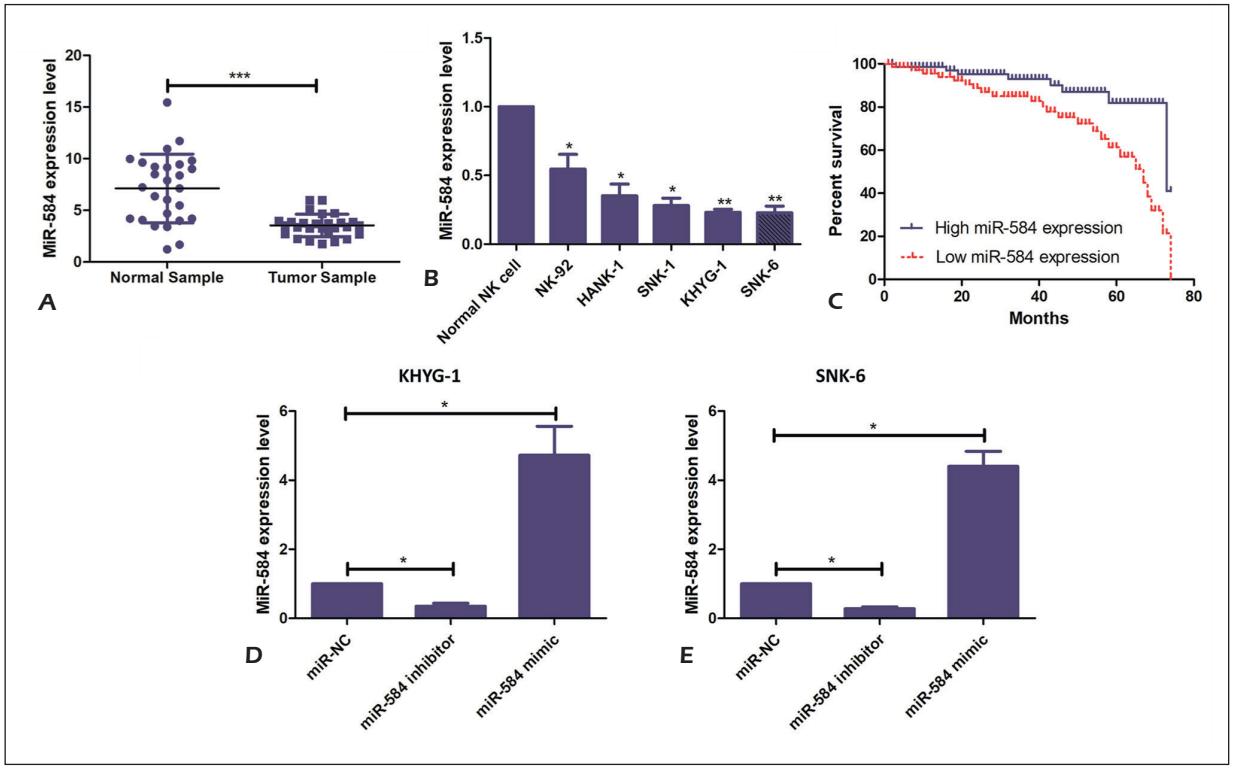

Figure 1. Low expression of miRNA-584 in NK/T-cell lymphoma. **A**, Peripheral blood levels of miRNA-584 in NK/T-cell lymphoma patients and healthy subjects. **B**, MiRNA-584 levels in NK cells and lymphoma cells (NK-92, HANK-1, SNK-1, KHYG-1, and SNK-6). **C**, Overall survival in NK/T-cell lymphoma patients expressing high or low level of miRNA-584. **D**, Transfection efficacies of miRNA-584 inhibitor and mimic in HKYG-1 and SNK-6 cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miR-584 expression with clinicopathologic characteristics of NK/T-cell lymphoma.

Parameters	No. of cases	miR-584 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.822
<60	21	15	6	
≥60	6	4	2	
Gender				0.187
Male	15	9	6	
Female	12	10	2	
T stage				0.008
T1-T2	17	15	2	
T3-T4	10	4	6	
Bone marrow				0.038
No	15	13	2	
Yes	12	6	6	

tumor staging and bone marrow invasion rate in NK/T-cell lymphoma patients, rather than age and gender (Table I). Moreover, Kaplan-Meier method was introduced for assessing the prognostic value of miRNA-584 in NK/T-cell lymphoma patients. Low level of miRNA-584 predicted worse survival in NK/T-cell lymphoma (Figure 1C).

MiRNA-584 Suppressed Proliferative and Invasive Abilities in NK/T-Cell Lymphoma

To elucidate the biological functions of miRNA-584 in NK/T-cell lymphoma, miRNA-584 mimic and inhibitor were prepared. Their transfection efficacies were tested in KHYG-1 and SNK-6 cells, respectively (Figure 1D). Transfection of miRNA-584 mimic markedly reduced viability in NK/T-cell lymphoma cells, and overexpression of miRNA-584 yielded the opposite results (Figure 2A, 2B). In addition, invasive cell number increased after knockdown of miRNA-584 in KHYG-1 and SNK-6 cells. Conversely, it was reduced by overexpression of miRNA-584 (Figure 2C, 2D).

FOXO1 Was the Direct Target of MiRNA-584

Protein level of FOXO1 was downregulated in KHYG-1 and SNK-6 cells overexpressing miRNA-584. Conversely, transfection of miRNA-584 inhibitor upregulated FOXO1 level (Figure 3A, 3B). Through bioinformatics prediction, binding sequences were found in the promoter regions of FOXO1 and miRNA-584 (Figure 3C). It is speculated that there may be a potential interaction between FOXO1 and miRNA-584. Dual-Luciferase reporter gene assay illustrated decreased luciferase activity after co-transfection of miRNA-584 mimic and wild-type FOXO1 vector, verifying that FOXO1 was the direct target binding miRNA-584 (Figure 3D). Of note, in peripheral blood samples of NK/T-cell lymphoma patients, expression levels of FOXO1 and miRNA-584 were negatively correlated (Figure 3E).

FOXO1 Was Responsible for NK/T-Cell Lymphoma Cell Regulation Mediated by miRNA-584

Based on the above findings, we speculated that FOXO1 may be involved in phenotype regulation of NK/T-cell lymphoma mediated by miRNA-584. First of all, transfection efficacies of si-FOXO1 and pcDNA-FOXO1 were tested (Figure 4A, 4B). CCK-8 assay revealed that overexpression of miRNA-584 decreased viability in KHYG-1 cells, which was partially reversed by co-overexpression of FOXO1 (Figure 4C). Transfection of miRNA-584 inhibitor markedly elevated viability in SNK-6 cells, but it was further reduced after co-transfection of si-FOXO1 (Figure 4D). Similarly, the inhibitory effect of miRNA-584 on invasiveness of KHYG-1 cells was partially abolished by overexpression of FOXO1. Conversely, knockdown of miRNA-584 stimulated invasiveness, but the increased trend was further alleviated by co-transfection of si-FOXO1 (Figure 4E). It is concluded that FOXO1 was necessary for miRNA-584 to regulate proliferative and invasive abilities in NK/T-cell lymphoma.

Discussion

Malignant lymphoma is one of the fastest growing malignant tumors. In Western countries, the incidence of malignant lymphoma represents the

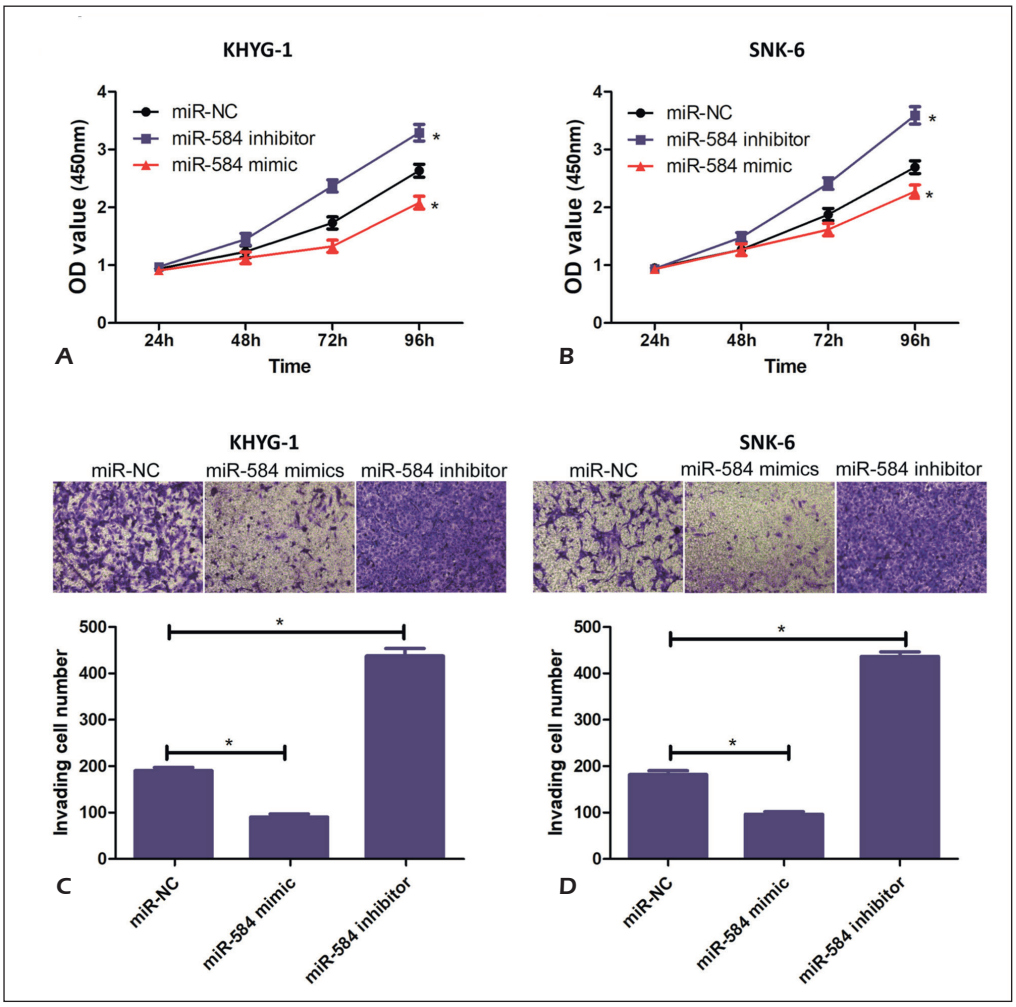

Figure 2. MiRNA-584 suppressed proliferative and invasive abilities in NK/T-cell lymphoma. **A-B**, Viabilities in KHYG-1 (**A**) and SNK-6 cells (**B**) transfected with miR-NC, miRNA-584 inhibitor or miRNA-584 mimic at 24, 48, 72 and 96 h, respectively. **C-D**, Invasive cell number in KHYG-1 (**C**) and SNK-6 cells (**D**) transfected with miR-NC, miRNA-584 inhibitor or miRNA-584 mimic, respectively (magnification 40×). *p<0.05.

eighth place in all malignancies, which is the ninth in Chinese males and the eleventh in Chinese females[1-4]. NK/T-cell lymphoma is a subtype of NHL, and it is the number one subtype in T-cell lymphoma[7]. It presents unique morphology, immunophenotype, and biological behaviors. The clinical manifestations of NK/T-cell lymphoma include soft tissue masses of nasal cavity and paranasal sinus, tumor invasion in bones, swelling, numbness, and pain of the external nose and face, nasal septum and/or hard palate perforation[8,9]. EB virus infection is a leading cause of NK/T-cell lymphoma. More than 90% of NK/T-cell lymphoma (nasal type) cases are EB-positive, while the positive rate of EB virus infection is relatively low in other types[9-11].

MiRNA-584 is downregulated in hepatocellular and medulloblastoma[18,19]. A feedback loop of miRNA-584 and its downstream genes forms a complex regulatory network[14,15]. Our study detected peripheral blood levels of miRNA-584 in NK/T-cell lymphoma patients and healthy controls. It is found that miRNA-584 was lowly expressed in peripheral blood of NK/T-cell lymphoma patients, and its level was closely linked to tumor staging, susceptibility to bone marrow invasion and poor prognosis. Furthermore, *in vitro* experiments demonstrated the inhibitory effects of miRNA-584 on proliferative and invasive abilities of NK/T-cell lymphoma.

MiRNAs exert their biological functions through complementary base pairing to the target genes[12,13]. A single miRNA is able to target multiple genes, and a single gene could be in-

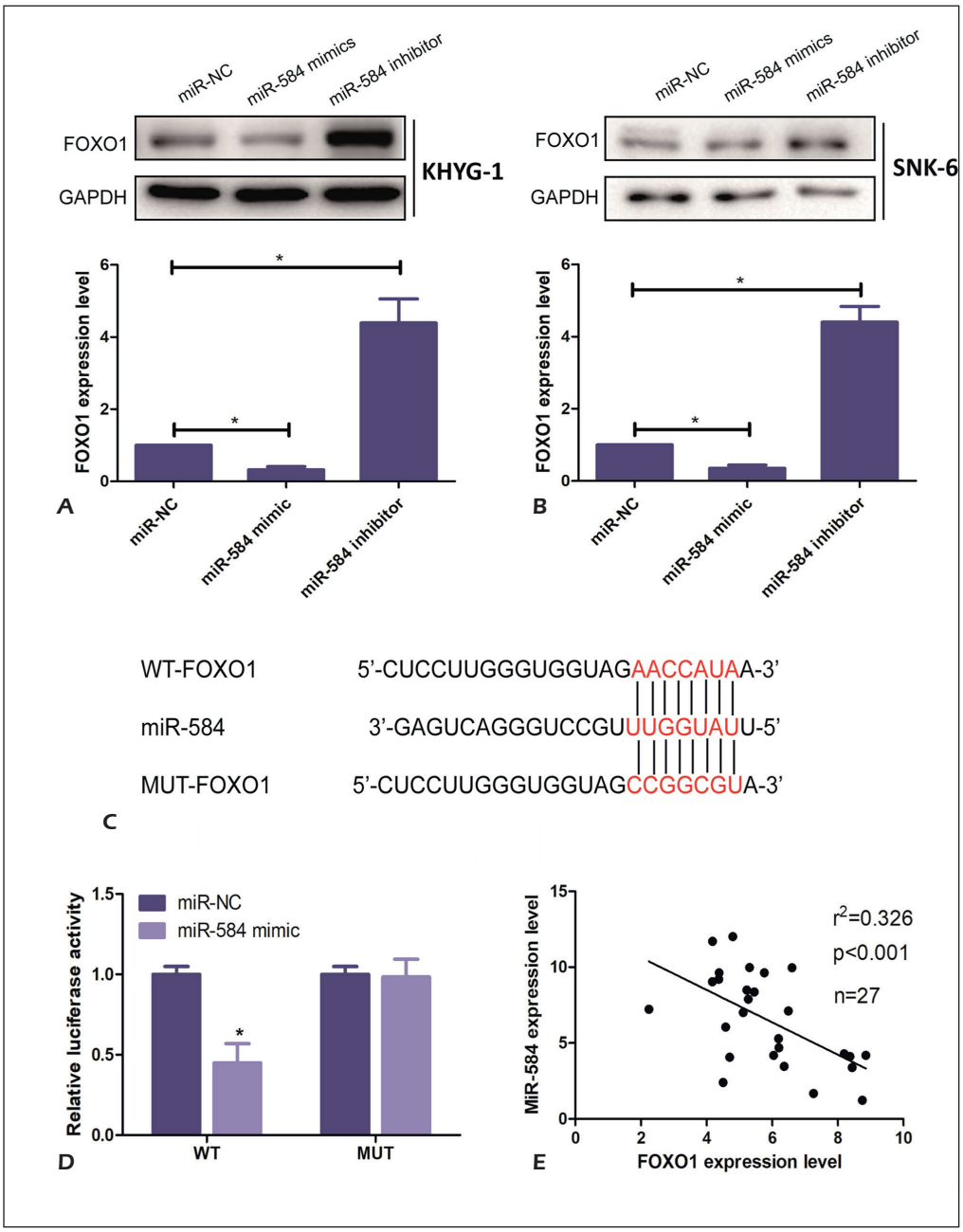

Figure 3. FOXO1 was the direct target of miRNA-584. **A-B**, Protein level of FOXO1 in KHYG-1 (**A**) and SNK-6 cells (**B**) transfected with miR-NC, miRNA-584 inhibitor or miRNA-584 mimic. **C**, Binding sequences between FOXO1 and miRNA-584. **D**, Luciferase activity in cells co-transfected with miR-NC/miRNA-584 mimic and wild-type/mutant-type FOXO1. E, A negative correlation between peripheral blood levels of FOXO1 and miRNA-584 in NK/T-cell lymphoma patients. *p<0.05.

teracted with multiple miRNAs as well. Such a complex regulatory network contributes to influence tumor progression[14-17]. In this paper, FOXO1 was predicted to be the direct target binding miRNA-584 through bioinformatics analysis. Dysregulated FOXO1 is closely related to the development of various epithelial-derived and hematological tumors. Overexpressed FOXO1 is capable of promoting the infiltration and metastasis of tumor cells[18,19].

Our findings detected the binding relationship between miRNA-584 and FOXO1. Moreover, FOXO1 level was negatively regulated by miRNA-584. Rescue experiments illustrated that

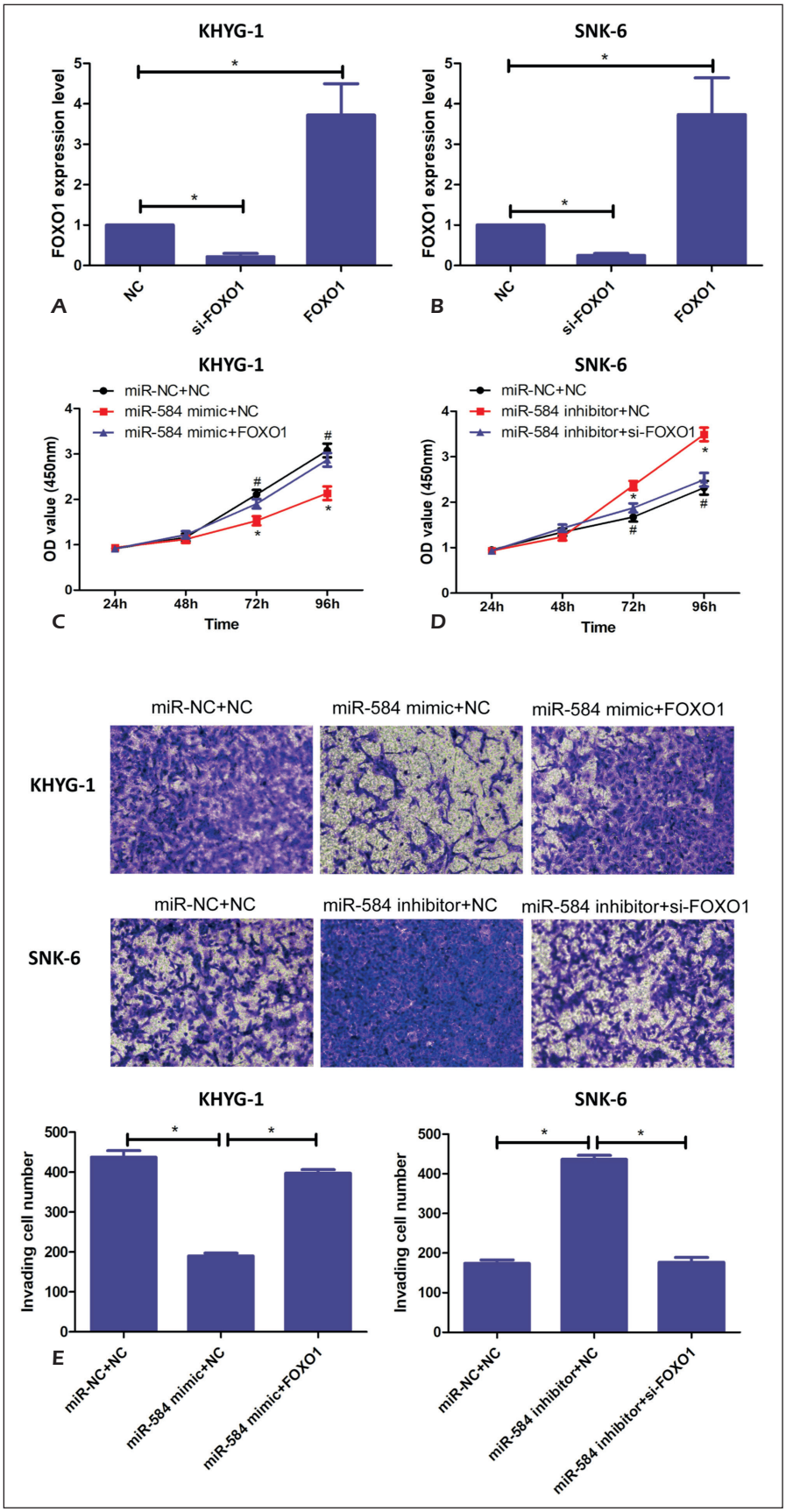

Figure 4. FOXO1 was responsible for NK/T-cell lymphoma cell regulation mediated by miRNA-584. (A, B) Transfection efficacies of si-FOXO1 and pcDNA-FOXO1 in KHYG-1 (**A**) and SNK-6 cells. (**B**). **C**, Viability in KHYG-1 cells transfected with miR-NC+NC, miRNA-584 mimic+NC or miRNA-584 mimic+pcDNA-FOXO1. **D**, Viability in SNK-6 cells transfected with miR-NC+NC, miRNA-584 inhibitor+NC or miRNA-584 inhibitor+si-FOXO1. **E**, Invasive cell number in KHYG-1 cells transfected with miR-NC+NC, miRNA-584 mimic+NC or miRNA-584 mimic+pcDNA-FOXO1, and SNK-6 cells transfected with miR-NC+NC, miRNA-584 inhibitor+NC or miRNA-584 inhibitor+si-FOXO1 (magnification 40×). *p<0.05.

FOXO1 was responsible for miRNA-584-mediated progression of NK/T-cell lymphoma.

Conclusions

Shortly, low level of miRNA-584 is closely linked to advanced stage, bone marrow invasion, and poor prognosis in NK/T-cell lymphoma. MiRNA-584 suppresses proliferative and invasive abilities in NK/T-cell lymphoma by targeting FOXO1.

Conflict of Interests

The authors declare that they have no conflict of interests.

References

1) Jiang M, Bennani NN, Feldman AL. Lymphoma classification update: T-cell lymphomas, Hodgkin lymphomas, and histiocytic/dendritic cell neoplasms. Expert Rev Hematol 2017; 10: 239-249.
2) Mugnaini EN, Ghosh N. Lymphoma. Prim Care 2016; 43: 661-675.
3) Ikoma N, Badgwell BD, Mansfield PF. Multimodality treatment of gastric lymphoma. Surg Clin North Am 2017; 97: 405-420.
4) Marron TU, Kalac M, Brody J. An update on the use of immunotherapy in the treatment of lymphoma. Curr Hematol Malig Rep 2017; 12: 282-289.
5) Shi Y. Current status and progress of lymphoma management in China. Int J Hematol 2018; 107: 405-412.
6) Meng J, Chang C, Pan H, Zhu F, Xiao Y, Liu T, Nie X, Wu G, Zhang L. Epidemiologic characteristics of malignant lymphoma in Hubei, China: a single-center 5-year retrospective study. Medicine (Baltimore) 2018; 97: e12120.
7) Yamaguchi M, Miyazaki K. Current treatment approaches for NK/T-cell lymphoma. J Clin Exp Hematop 2017; 57: 98-108.
8) Tse E, Kwong YL. Diagnosis and management of extranodal NK/T cell lymphoma nasal type. Expert Rev Hematol 2016; 9: 861-871.
9) Tse E, Kwong YL. The diagnosis and management of NK/T-cell lymphomas. J Hematol Oncol 2017; 10: 85.
10) Harabuchi Y, Takahara M, Kishibe K, Nagato T, Kumai T. Extranodal natural killer/T-cell lymphoma, nasal type: basic science and clinical progress. Front Pediatr 2019; 7: 141.
11) de Mel S, Hue SS, Jeyasekharan AD, Chng WJ, Ng SB. Molecular pathogenic pathways in extranodal NK/T cell lymphoma. J Hematol Oncol 2019; 12: 33.
12) Robins H, Li Y, Padgett RW. Incorporating structure to predict microRNA targets. Proc Natl Acad Sci U S A 2005; 102: 4006-4009.
13) Tutar Y. MiRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol 2014; 15: 429.
14) Andres-Leon E, Cases I, Alonso S, Rojas AM. Novel miRNA-mRNA interactions conserved in essential cancer pathways. Sci Rep 2017; 7: 46101.
15) Vos PD, Leedman PJ, Filipovska A, Rackham O. Modulation of miRNA function by natural and synthetic RNA-binding proteins in cancer. Cell Mol Life Sci 2019; 76: 3745-3752.
16) Iswariya GT, Paital B, Padma PR, Nirmaladevi R. MicroRNAs: epigenetic players in cancer and aging. Front Biosci (Schol Ed) 2019; 11: 29-55.
17) Tiwari A, Mukherjee B, Dixit M. MicroRNA key to angiogenesis regulation: miRNA biology and therapy. Curr Cancer Drug Targets 2018; 18: 266-277.
18) Wei H, Wang J, Xu Z, Lu Y, Wu X, Zhuo C, Tan C, Tang Q, Pu J. MiR-584-5p regulates hepatocellular carcinoma cell migration and invasion through targeting KCNE2. Mol Genet Genomic Med 2019; 7: e702.
19) Abdelfattah N, Rajamanickam S, Panneerdoss S, Timilsina S, Yadav P, Onyeagucha BC, Garcia M, Vadlamudi R, Chen Y, Brenner A, Houghton P, Rao MK. MiR-584-5p potentiates vincristine and radiation response by inducing spindle defects and DNA damage in medulloblastoma. Nat Commun 2018; 9: 4541.
20) Chay DB, Han GH, Nam S, Cho H, Chung JY, Hewitt SM. Forkhead box protein O1 (FOXO1) and paired box gene 3 (PAX3) overexpression is associated with poor prognosis in patients with cervical cancer. Int J Clin Oncol 2019; 24: 1429-1439.
21) Chen J, Lu Y, Tian M, Huang Q. Molecular mechanisms of FOXO1 in adipocyte differentiation. J Mol Endocrinol 2019; 62: R239-R253.