Original Article

MiR-363 suppresses the tumor growth of natural killer/T-cell lymphoma via the *SIRT6/PI3K/AKT* axis

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Background: Natural killer/T cell lymphoma (NKTCL) is a rare and aggressive tumor of non-Hodgkin's lymphoma. The role of micro ribonucleic acid (*RNA*) (*miR*)-*363* in NKTCL has not yet been elucidated. The present study aimed to investigate the potential role of *miR-363* in NKTCL.

Methods: The expression of the top five differentially expressed microRNAs (miRNAs) as well as sirtuin 6 (*SIRT6*) in NK normal cells and its tumor cell lines were explored. The clinical tissues of NKTCL patients were collected and analyzed for expression of *miR-363* and *SIRT6*. In addition, human NK/T-cell lymphoma cells (SNK-6) were transfected into different groups to detect cell proliferation and apoptosis abilities through cell counting kit 8 (CCK-8) experiment and flow cytometry analysis. Western blot assay was employed to examine protein expression. NKTCL nude mice models were constructed by subcutaneous injection of stably transfected SNK-6 cells to validate the mechanism of *miR-363* in NKTCL via *SIRT6 in vivo*.

Results: *MiR-363* was down-regulated in NKTCL tissues and cell lines. Overexpression of *miR-363* inhibited cell proliferation and promoted cell apoptosis. In contrast, *SIRT6* was up-regulated in NKTCL and proved to be a downstream target of *miR-363*. *SIRT6* could activate the phosphatidylinositol-3-kinase (*PI3K*)/protein kinase B (*AKT*) signaling pathway. Also, *miR-363* mimic could suppress the proliferation and induce the apoptosis of NKTCL via the *SIRT6/PI3K/AKT* axis both *in vitro* and *in vivo*.

Conclusions: *MiR-363* suppresses the *SIRT6/PI3K/AKT* pathway to restrain cell proliferation and accelerate cell apoptosis during NKTCL progression.

Keywords: Natural killer/T-cell lymphoma (NKTCL); *miR-363; SIRT6; PI3K/AKT*

Submitted Oct 17, 2022. Accepted for publication Dec 05, 2022.
doi: 10.21037/atm-22-5649
View this article at: https://dx.doi.org/10.21037/atm-22-5649

Introduction

Natural killer/T-cell lymphoma (NKTCL) is a rare and aggressive tumor type of non-Hodgkin's lymphoma (1). It appears to have a geographical predilection for Asia (2) and is relatively uncommon in North America and Europe (3). The early treatment of NKTCL usually involves a sequential or simultaneous combination of chemotherapy and radiotherapy (4,5). Clinically, NKTCL is usually accompanied by a poor treatment response and prognosis, and thus, chemotherapy combined with radiotherapy is typically needed to achieve a superior curative effect (6). In spite of the improvement of treatment strategies for NKTCL, the optimal treatment for patients with advanced-stage or recurrent NKTCL is not yet mature and so continues to demonstrate poor results. Therefore, there is an urgent need to explore novel and efficient therapeutic targets.

Micro ribonucleic acids (RNAs) (miRNAs/miRs) are small non-coding RNAs that can regulate gene expression at the post-transcriptional level. Previously, numerous studies have demonstrated the key regulatory role of miRNAs in the pathogenesis of diseases, especially in cancer (7,8). In addition, researchers have also identified a series of abnormally expressed miRNAs, which exert some unknown but vital functions in T-cell differentiation and the development of T-cell malignant tumors (9). To further clarify these unknown roles of miRNAs in NKTCL, researchers established a genome-wide differential miRNAs expression profile of NKTCL, and the findings showed that *miRNA-363* was down-regulated in NKTCL (10). Therefore, *miRNA-363* might be a molecular biomarker of NKTCL. Yet, the biological role of *miRNA-363* in NKTCL progression remains poorly understood although past papers have reported its function in other carcinomas. In previous studies, *miR-363* has been confirmed as a tumor-suppressive miRNA that suppresses the growth and metastasis of a variety of malignant tumors, including gastric cancer (11), breast cancer (12), multiple myeloma (13), colorectal cancer (14), and ovarian cancer (15).

As a nuclear protein of the conserved sirtuins family, sirtuin 6 (*SIRT6*) is also a nicotinamide adenine dinucleotide $(NAD)^+$-dependent deacetylase, which is mainly involved in metabolic homeostasis and stress resistance (16). *SIRT6* has been shown to have a double-sided identity in cancer; it functions as a suppressor or oncogene depending on the tumor type (17,18). For example, the up-regulation of *SIRT6* could predict poor outcomes and promote NSCLC metastasis (19); however, it is also an important tumor suppressor of pancreatic ductal adenocarcinoma (PDAC), and thus, is regarded as a potential therapeutic signal in the molecular-defined PDAC subgroup (20). In addition, in hepatocellular and prostate carcinomas, the suppression of *SIRT6* further emphasizes its sensitivity to chemotherapy and induction of apoptosis (21,22). Previous reports have shown that miRNA regulation alters *SIRT6* expression and participates in the progression of a variety of diseases (23,24). These reports highlight the contribution of *SIRT6* in tumor development, but its role in NKTCL has not been studied.

It is reported that *SIRT6* downregulation in diffuse large B-cell lymphoma (DLBCL) cells suggested enhanced sensitivity to chemotherapy, during which the targets of the phosphatidylinositol-3-kinase (*PI3K*)/protein kinase B (*AKT*) pathway are reduced (25). In other words, the *PI3K/AKT* pathway naturally displays a positive promotion of cell survival (26). Previous literature has shown that the inhibition of *PI3K/AKT* signaling can promote the apoptosis of primary exudative lymphoma cells (27). Also, significant activation of *PI3K/AKT* signaling is associated with carcinogenesis in cancers, especially NKTCL (28,29).

Our study first explored the inhibitory effects of *miR-363* on NKTCL and its molecular mechanism. The *miR-363* mimic could lead to the inhibition of downstream *SIRT6* expression, thereby regulating the *PI3K/AKT* pathway and inducing the decline of malignant proliferation and apoptosis in most NKTCL cell lines. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5649/rc).

Highlight box

Key findings

- *MiR-363* is down-regulated in natural killer/T cell lymphoma (NKTCL) and affects proliferation and apoptosis via *SIRT6/PI3K/AKT* pathway *in vitro* and *in vivo*.

What is known and what is new?

- *MiR-363* is down-regulated but poorly understood in NKTCL. SIRT6 can function as an oncogene depending on the tumor type and it targets *PI3K/AKT* pathway in diffuse large B-cell lymphoma (DLBCL) cells.
- This study firstly examines the expression pattern and mechanism of *MiR-363* in NKTCL tissues and cells. *MiR-363* is significantly decreased in NKTCL and suppresses proliferation and apoptosis of SNK-6 cells upon SIRT6/PI3K/AKT axis.

What is the implication, and what should change now?

- These findings can provide a new target and guidance for the treatment and diagnosis, as well as offer an additional molecular option for future drug development of NKTCL.

Methods

Clinical samples

To verify the expression of *miR-363/SIRT6* in NKTCL, 10 tumor tissue and adjacent normal tissue samples were obtained from NKTCL patients at The Fourth Hospital of Hebei Medical University. All NKTCL patients who only underwent surgery (aged 12 to 77 years) were followed-up until December 2020. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of The Fourth Hospital of Hebei Medical University (No. 2020046) and informed consent was taken from all the

Table 1 Primer sequences used for qRT-PCR

Genes	Primer sequence
MiR-363	F: 5'-GCGGCCAATTGCACGGTAT-3'
	R: 5'-GTGCAGGGTCCGAGGTATTC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-AACGCTTCACGAATTTGCGT-3'
SIRT6	F: 5'-TGTGCCAAGTGTAAGACGCAG-3'
	R: 5'-TTGCCTTAGCCACGGTGCAG-3'
GAPDH	F: 5'-AGACAGCCGCATCTTCTTGT-3'
	R: 5'-TGATGGCAACAATGTCCACT-3'

qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

patients or the patients' guardians.

Isolation of normal NK cells from peripheral blood

As previously described (30), the whole-blood samples of healthy blood donors from The Fourth Hospital of Hebei Medical University were prepared to isolate highly purified (90–99%) normal human NK cells using an NK cell separation Kit (Miltenyi Biotec, Germany). The NK cells were then stimulated and cultured in the presence of human recombinant interleukin (IL)-2 (Miltenyi Biotec).

Cell culture and treatments

NKTCL cell lines (SNK-6 and HANK1) were purchased from Shanghai Yaji Biotechnology Co., Ltd. (China). The culture conditions were set according to a previous study (31). When the SNK-6 cells grew to confluence, the cells in each group were transfected with a *miR-363* mimic, a mimic negative control (NC), *SIRT6*-overexpression (OE), or OE-NC using lipo3000 (Life Technologies, USA) for 48 h. The protein and mRNA were then extracted for subsequent expression analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

A qRT-PCR assay was implemented to estimate gene expression. RNA was isolated using a simple total RNA kit (BioTeke, China) and complementary deoxyribonucleic acid (cDNA) was obtained using the Primescript RT Master Mix (TaKaRa, China). The expressions of *miR-363* and *SIRT6* were quantified by using the SYBR Green Master Mix (TaKaRa). The primer sequences used are listed in *Table 1*.

Western blot

Total proteins were extracted from cells or tissues using a lysis buffer and then quantified using bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA) to detect the protein expression. Proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, USA) by electroblotting. Subsequently, the membranes were blocked and incubated with the following primary antibodies: *SIRT6* (1:1,000, #12486, CST, USA), *PI3K* (19H8) (1:1,000, #4257, CST, USA), *p-PI3K* (Tyr458) (1:500, #17366, CST, USA), *AKT* (C67E7) (1:1,000, #4691, CST, USA), *p-AKT* (Ser473) (1:500, #4060, CST, USA), and *GAPDH* (1:2,000, #5174, CST, USA), as well as a secondary antibody immunoglobulin G (IgG) (1:2,000, #14708, CST, USA). Protein bands were visualized using a BeyoECL Star kit (P0018AS, Beyotime, China).

Dual-luciferase reporter assay

A dual-luciferase reporter assay was implemented to affirm the interaction between *miR-363* and *SIRT6*. Briefly, the sequences between *miR-363* and *SIRT6* (*SIRT6*-WT) and the respective mutated sequences (*SIRT6*-MUT) were cloned into pmirGLO vectors (Promega, USA). The SNK-6 cells were transfected with the above-mentioned plasmids, together with *miR-363* mimic or NC mimic, and then collected and lysed. Finally, the Dual-Luciferase Reporter System (Promega, Madison, WI, USA) was employed to analyze the luciferase activity (32) post-transfection.

Proliferation analysis

A cell counting kit 8 (CCK-8) experiment was conducted to assess the proliferation ability. Firstly, the SNK-6 cells were inoculated in a 96-well plate. Next, cell viability was examined at specific time points (0, 1, 2, 3, 4, and 5 days) by adding 10 μL of CCK-8 solution to each well. After 4 h of incubation, the microplate reader purchased from Bio-Tek Instruments (Hopkinton, MA, USA) was used to monitor

the absorbance at 450 nm.

Apoptosis analysis

To measure cell apoptosis, an AnnexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (C1062M, Beyotime, China) was used to stain the SNK-6 cells. Following removal of the suspended cells, the adherent SNK-6 cells were detached by Trypsin-ethylenediamine tetraacetic acid (EDTA). The SNK-6 cells were suspended in an AnnexinV buffer set and stained with 20 μg/mL propidium iodide (Sigma-Aldrich, USA). The apoptosis of SNK-6 cells was investigated using a flow cytometer (Beckman Coulter, USA), and data were analyzed using the compatible FlowJo software (BD, USA).

Animal experiment

Animal models were established to investigate the effect of *miR-363/SIRT6* on NKTCL development *in vivo*. A total of 28 mice were collected, 24 of which were included in the study due to their healthy status. Four mice were excluded owing to technical or instrumental failures during animal preparation. In the animal experiment, the mice were randomly divided into four groups: mimic NC + OE NC, *miR-363* mimic, OE-*SIRT6*, and *miR-363* mimic + OE-*SIRT6*, with six mice in each group. The 5–6-week-old nude BALB/c male mice (weight 17–20 g) purchased from Charles River Inc. (Beijing) were raised in a specific-pathogen-free (SPF) environment at 26 ℃, with a 12 h day/night cycle at 70% humidity, to eliminate inference.

The subcutaneous transplanted tumor model was established via injection of the transfected SNK-6 (2×10^7) cells into the right flank skin of the mice (n=6 per group). The tumor volume was recorded every week and calculated according to the following equation: V (mm^3) = 0.5 × length × $width^2$, which was constantly monitored during the modeling. Four weeks after inoculation, the mice were sacrificed via carbon dioxide (CO_2) inhalation, and thereafter, the tumors were separated and weighed. Animal experiments were performed under a project license (No. 2020047) granted by ethics board of The Fourth Hospital of Hebei Medical University, in compliance with national guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Immunohistochemistry (IHC)

The NKTCL tissue sections (4-μm) were dewaxed and then washed with Tris-buffered saline (TBS) to examine the expression of *SIRT6*. After soaking and blocking, the sections were incubated with a primary antibody against *SIRT6* (1:500, Cruz, sc-517556) overnight at 4 ℃. The next day, the sections were soaked with TBS and Tween-20 (TBST), then incubated with an anti-rabbit secondary antibody for 45 min at 37 ℃, and stained with hematoxylin. After sealing and drying, the positive *SIRT6* expression was examined and photographed under a microscope (Olympus, BX63, Japan).

Statistical analysis

All data from triplicated assays were statistically analyzed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, USA), and the results of the three experiments were used for standard deviation (SD) detection. The differences between two or multiple groups were compared using the Student's *t*-test or one-way analysis of variation (ANOVA). Data were presented as the mean ± SD as well as the effect size with a 95% confidence interval (CI). $P<0.05$ was considered statistically significant.

Results

MiR-363 is down-regulated in NKTCL and affects cell proliferation and apoptosis

According to Ng *et al.* (10), among the miRNAs differentially expressed between NKTCL patients and normal controls, the top five down-regulated miRNAs were *miR-26b*, *miR-342-5p*, *miR-150*, *miR-363*, and *miR-28-5p*. We verified the expression of these miRNAs in normal NK cells and NK tumor cell lines by qRT-PCR, which showed that *miR-363* was most significantly down-regulated in SNK-6 and HANK1 cells (*Figure 1A*). We found that *miR-363* expression was markedly lowly expressed in NKTCL tissues, which is consistent with previous findings (*Figure 1B*). Further, a mimic NC and *miR-363* mimic were transfected into SNK-6 cells, and the transfection efficiency was detected (*Figure 1C*). CCK-8 and flow cytometry experiments were performed to characterize proliferation and apoptosis of SNK-6 cells in each transfection group. Compared to the control, the *miR-363* mimic group showed a significantly reduced SNK-6 cell proliferation level (*Figure 1D*). As for cell apoptosis, *miR-363* overexpression markedly increased the apoptosis capabilities of SNK-6 cells (*Figure 1E*). These data suggest that *miR-363* plays an important role in NKTCL progression by inhibiting the

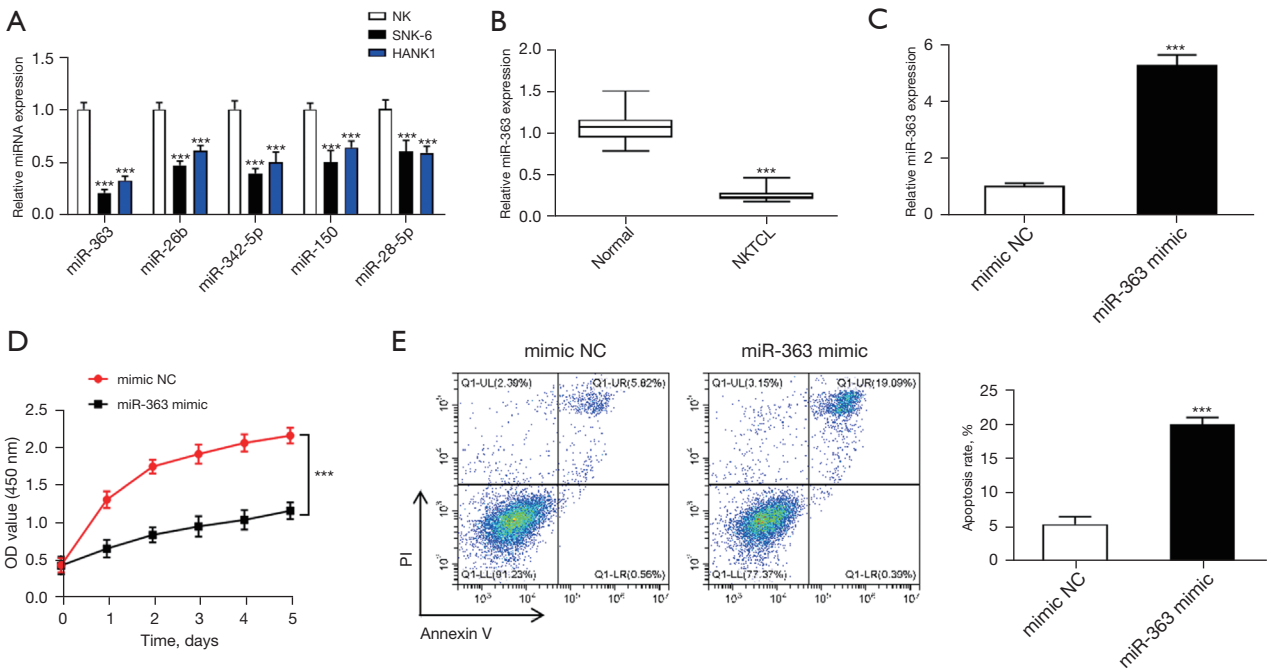

Figure 1 *MiR-363* was down-regulated in NKTCL and affected cell proliferation and apoptosis. Firstly, the clinical tissue samples of NKTCL patients were collected, and normal NK cells and NKTCL cell lines were cultured. SNK-6 cells were transfected with a *miR-363* mimic and a mimic NC. (A) The expression of the top five miRNAs in normal cells and tumor cell lines was detected by qRT-PCR. (B) *MiR-363* expression in 10 clinical samples of NKTCL was detected by qRT-PCR. (C) Transfection efficiency of *miR-363* in SNK-6 cells was detected by qRT-PCR. (D) CCK-8 proliferation assay in SNK-6 cells. (E) Flow cytometry was used to detect the apoptosis capabilities of SNK-6 cells. Data are shown as mean ± SD. ***, P<0.001. NK, natural killer; NKTCL, natural killer/T cell lymphoma; NC, negative control; OD, optical density; PI, propidium iodide; qRT-PCR, quantitative real-time polymerase chain reaction; CCK-8, cell counting kit 8; SD, standard deviation.

proliferation and promoting the apoptosis of NKTCL cells.

MiR-363 targets SIRT6 to activate the PI3K/AKT pathway

There is evidence that *SIRT6* promotes tumorigenesis and drug resistance of DLBCL (25). Herein, the qRT-PCR analysis showed that *SIRT6* was notably overexpressed in NKTCL, indicating its potential role (*Figure 2A*). Also, compared with NK normal cells, we found that *SIRT6* was significantly overexpressed in both NKTCL cell lines (*Figure 2B*). Interestingly, an ENCORI website analysis found that *miR-363* and *SIRT6* had binding sites (*Figure 2C*). The dual-luciferase assay conducted in the present study further verified that *miR-363* directly regulated the expression of *SIRT6* (*Figure 2D*). Moreover, we observed *miR-363* mimic could reduce the mRNA and protein expression of *SIRT6* (*Figure 2E,2F*). Taken together, the above data showed that *SIRT6* was up-regulated in NKTCL as a downstream target of *miR-363*.

Recent reports have demonstrated that *SIRT6* could increase the radiation sensitivity of non-small cell lung cancer (NSCLC) and suppress tumor progression through the *PI3K/AKT* pathway (33). To better analyze the molecular mechanism of *miR-363/SIRT6* participation in NKTCL progression, we further explored the downstream regulatory pathway of *SIRT6*. So, we overexpressed *SIRT6* in SNK-6 cells, and the transfection efficiency was detected (*Figure 2G*). As indicated in *Figure 2H*, following the overexpression of *SIRT6*, the protein expressions of *p-PI3K* and *p-AKT* were significantly up-regulated. Taken together, these results suggest that *miR-363* could target *SIRT6*

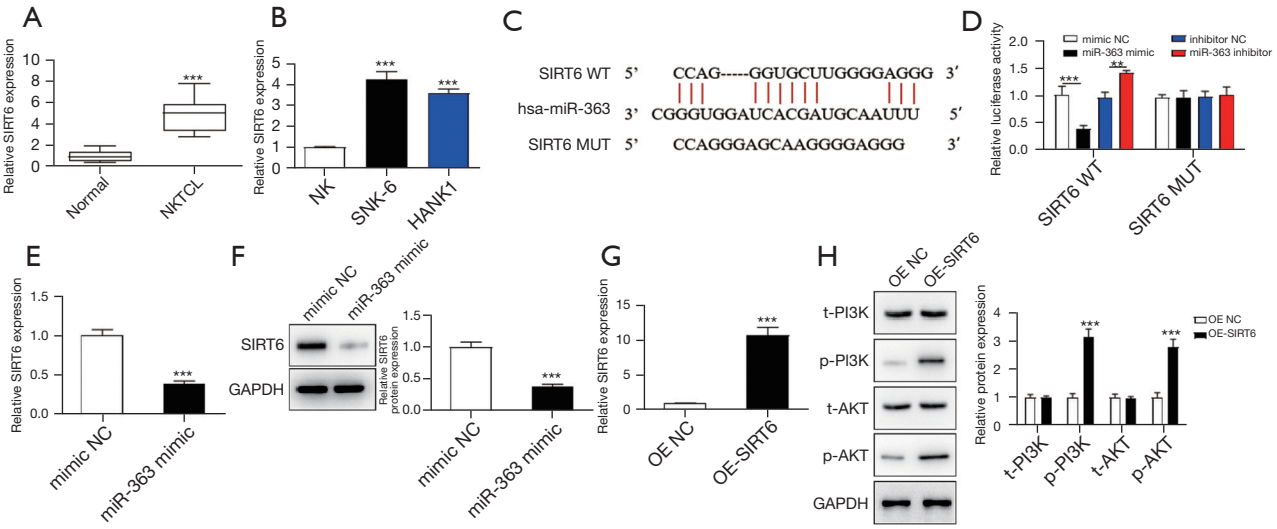

Figure 2 *MiR-363* targets SIRT6 to activate the PI3K/AKT pathway. SNK-6 cells were transfected with mimic NC/*miR-363* mimic and OE-NC/OE-*SIRT6*. The expression of *SIRT6* in (A) 10 NKTCL clinical samples and (B) NKTCL cell lines were detected by qRT-PCR. (C) The binding sites of *miR-363* and *SIRT6* were predicted by a bioinformatics website. (D) Dual-luciferase assay further verified the binding relationship between *miR-363* and *SIRT6*. (E) qRT-PCR and (F) western blot were used to detect the mRNA and protein expressions of *SIRT6* in different transfection groups. (G) Transfection efficiency of *SIRT6* in SNK-6 cells was detected by qRT-PCR. (H) The protein expression of *PI3K-AKT* pathway-related proteins in each transfected group was detected by western blot. Data are shown as mean ± SD. **, P<0.01; ***, P<0.001. NK, natural killer; NKTCL, natural killer/T cell lymphoma; WT, wild type; MUT, mutant type; NC, negative control; OE, overexpression; *t-PI3K*, total *PI3K*; *p-PI3K*, phosphorylated *PI3K; t-AKT*, total *AKT*; *p-AKT*, phosphorylated *AKT*; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.

binding to activate the *PI3K/AKT* pathway.

MiR-363 affects NKTCL proliferation and apoptosis via the SIRT6/PI3K/AKT pathway in vitro

Accordingly, we speculated that the molecular mechanism of *miR-363* affecting the progression of NKTCL acted via the *SIRT6/PI3K/AKT* pathway. To verify this conjecture, we initially transfected SNK-6 cells with a *miR-363* mimic or/and OE-*SIRT6*, and the transfection efficiency was detected by qRT-PCR (*Figure 3A*). The western blot results showed that *p-PI3K* and *p-AKT* were markedly reduced by the *miR-363* mimic, which was reversed by OE-*SIRT6* transfection (*Figure 3B*). In addition, CCK-8 and flow cytometry indicated that the *miR-363* mimic could substantially restrain cell proliferation and elevate apoptosis, while the effect of OE-*SIRT6* on proliferation and apoptosis was opposite to that of the *miR-363* mimic. Also, *SIRT6* overexpression could notably reverse the influence of the *miR-363* mimic on SNK-6 cell proliferation and apoptosis (*Figure 3C,3D*).

MiR-363 affects NKTCL proliferation and apoptosis via the SIRT6/PI3K/AKT pathway in vivo

To further verify the repressive role of *miR-363* on NKTCL *in vivo*, we inoculated stably transfected SNK-6 cells into nude mice to form transplanted tumors. The tumors in each group were then isolated and photographed (*Figure 4A*). After 4 weeks, the mean tumor volumes of the *miR-363* mimic groups were significantly larger than those of the NC groups. OE-*SIRT6* significantly reversed the effect of the *miR-363* mimic (*Figure 4B*). As expected, the tumor weights in each group corresponded to the respective volumes (*Figure 4C*).

The IHC detection results showed that *SIRT6* was substantially decreased in the *miR-363* mimic group but increased in the OE-*SIRT6* group, and this was reversed in *miR-363* + OE-*SIRT6* group (*Figure 4D*). In addition, the protein expression trends of *SIRT6*, *total (t)-PI3K*, *phosphorylated (p)-PI3K*, *t-AKT*, and *p-AKT* were consistent with those *in vitro* (*Figure 4E*). The *in vivo* experiments verified that *miR-363* affected the progression of NKTCL

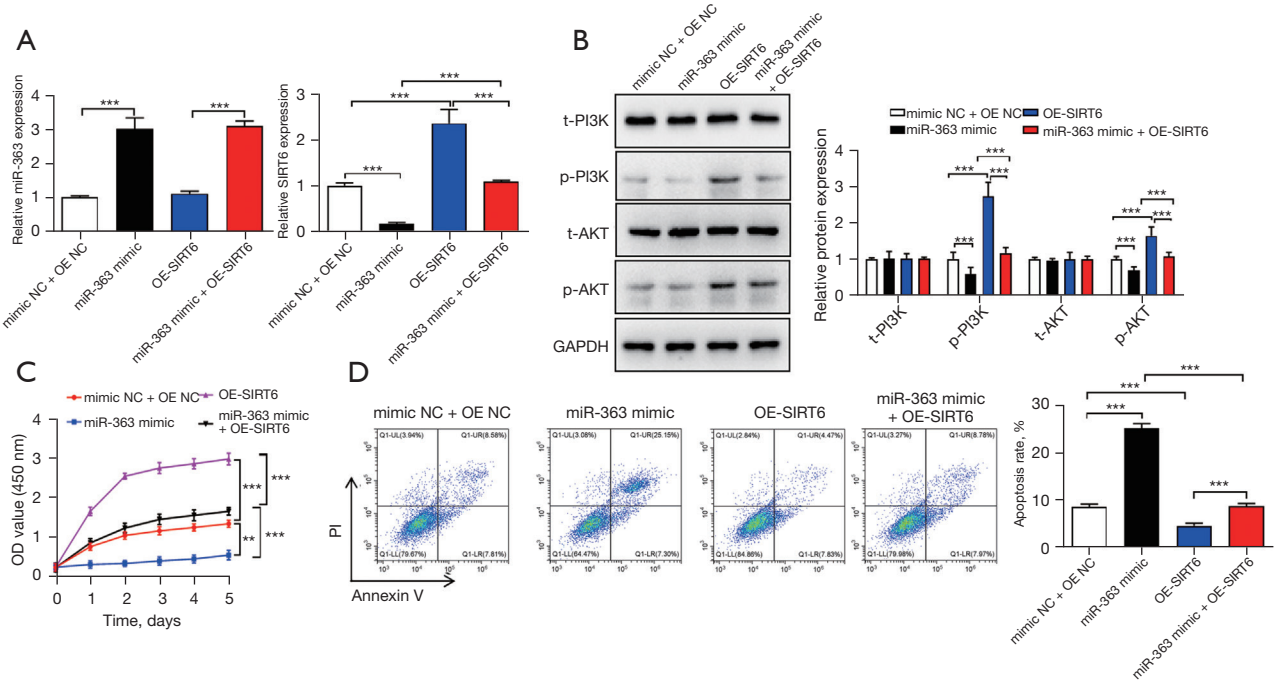

Figure 3 *MiR-363* affects the proliferation and apoptosis of NKTCL via the *SIRT6/PI3K/AKT* pathway *in vitro*. We initially transfected SNK-6 cells with a mimic NC + OE NC, *miR-363* mimic, OE-*SIRT6* and *miR-363* mimic + OE-*SIRT6*, and the transfection efficiency was detected. (A) qRT-PCR was used to detect the expressions of miR-363 and SIRT6 in each transfected group. (B) The expression of *PI3K-AKT* pathway-related proteins in each transfected group was detected by western blot. (C) CCK-8 was applied to test proliferation in each transfection group. (D) Flow cytometry was used to detect the apoptosis capability in each group. Data are shown as mean ± SD. **, P<0.01; ***, P<0.001. NKTCL, natural killer/T cell lymphoma; NC, negative control; OE, overexpression; *t-PI3K*, total *PI3K*; *p-PI3K*, phosphorylated *PI3K; t-AKT*, total *AKT*; *p-AKT*, phosphorylated *AKT*; OD, optical density; PI, propidium iodide; qRT-PCR, quantitative real-time polymerase chain reaction; CCK-8, cell counting kit 8; PI, propidium iodide; SD, standard deviation.

via the *SIRT6/PI3K/AKT* pathway.

Discussion

NKTCL is an Epstein-Barr virus (EBV)-related lymphoproliferative disease, accounting for about 2% of T-cell lymphomas. It can be further divided into lymph node (nNKTCL) or extralymph node (eNKTCL), which vary significantly in terms of their clinical, pathophysiological, and genetic characteristics (34). Usually, most eNKTCLs can be seen in the nasal cavity, which is known as nasal-type eNKTCL, but they can be found anywhere. Localized extranodal NKTCL can be cured, but the prognosis of most advanced patients is poor (35,36).

Innate immune cells such as macrophages, neutrophils and natural killer cells and adaptive immune cells can facilitate carcinoma process in tumor microenvironment, lymphoma contained (37,38). MiRNAs have been demonstrated to regulate microenvironment or function in microenvironment during tumor progression (39,40). Moreover, numerous studies have confirmed that miRNAs possess the ability of multi-gene regulation to directly or indirectly regulate various signaling pathways and thereby affect the occurrence and metastasis of malignant tumors (41). For instance, *miR-155* has been illustrated to target BRG1 to activate *STAT3/VEGFC* signaling and mediate lymphangiogenesis in NKTCL (42). And *miR-188-5p* refrains NKTCL progression by inhibited regulation of *XRCC5* (43). Thus, we attempted to dig out functional miRNAs in NKTCL. After browsing website, we uncovered the top five down-regulated miRNAs, *miR-26b*, *miR-342-5p*, *miR-150*, *miR-363*, and *miR-28-5p* contained, in NKTCL patients (10). After verification, it was observed that *miR-363* exhibited the most significant down-regulation in SNK-6 and HANK1 cells, suggesting the involvement of *miR-363* in NKTCL. According to

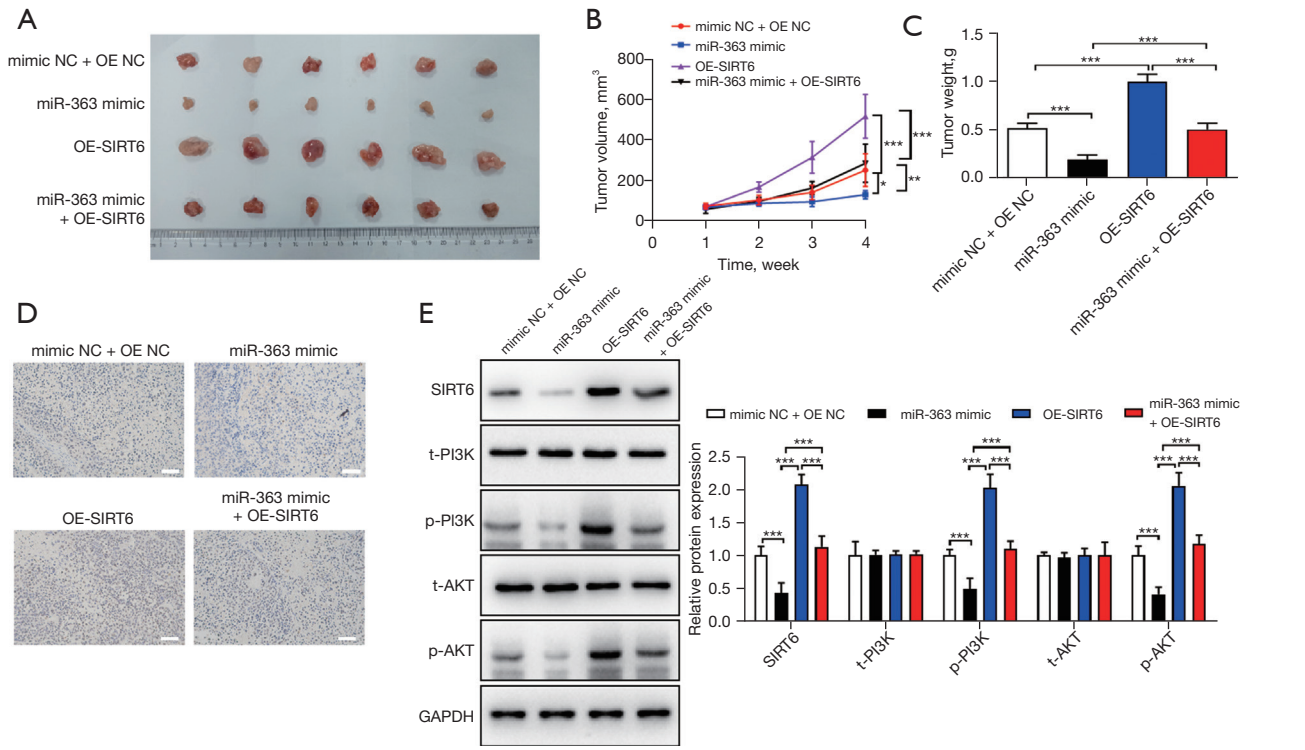

Figure 4 *MiR-363* affects proliferation and apoptosis of NKTCL via the *SIRT6/PI3K/AKT* pathway *in vivo*. We constructed a mouse NKTCL tumor with stably transfected SNK-6 cells: mimic NC + OE NC, *miR-363* mimic, OE-*SIRT6*, *miR-363* mimic + OE-*SIRT6*. (A) The mouse tumors in each group (n=6) were dissected and photographed. The tumor volumes (B) and weights (C) were measured. (D) The expression of SIRT6 in tumor tissues was detected by IHC. Scale bar =400 μm. (E) Western blot was used to detect the expression of related factors in tumor tissues. Data are shown as mean ± SD. *, P<0.05; **, P<0.01; ***, P<0.001. NKTCL, natural killer/T cell lymphoma; NC, negative control; OE, overexpression; *t-PI3K*, total *PI3K; p-PI3K*, phosphorylated *PI3K*; *t-AKT*, total *AKT*; *p-AKT*, phosphorylated *AKT*; IHC, immunohistochemistry; SD, standard deviation.

previous studies, *miR-363* is a typical and effective tumor-suppressor gene in cancer (44,45), which is consistent with our research. Furthermore, *miR-363* has been indicated to regulate *SQLE* that is significantly related with tumor immune cell infiltration and checkpoints in pancreatic adenocarcinoma (46). However, *miR-363* has been rarely reported in NKTCL. Therefore, the present study aimed to reveal the significance and mechanism of *miR-363* in NKTCL for the first time.

Herein, we found that *miR-363* was down-regulated in NKTCL tissues and cell lines, which affected the proliferation and apoptosis of SNK-6 cells. It is well known that miRNAs with tumor-suppressive functions are abnormally lowly expressed in tumors and can regulate the growth and metastasis of cancer cells by targeting and up-regulating the expression of downstream tumor-related genes (47). Given this, we further studied the downstream regulatory mechanism of *miR-363*. Among its many target genes, *SIRT6* attracted our attention.

Abnormal *SIRT6* expression is associated with a variety of malignant tumors, such as gastric cancer (48), ovarian cancer (49), medulloblastoma (50), etc. There is also evidence that *SIRT6* can be regarded as an effective tumor suppressor owing to its effective regulation of aerobic glycolysis of cancer cells, which suppresses tumor metabolism (17). Considering the primary role of *SIRT6* in intracellular homeostasis, it has broad prospects for development into small molecule inhibitors or activators and has emerging therapeutic potential for cancer diagnosis and treatment (51). Yet, the role and mechanism of *SIRT6* in NKTCL have not been reported.

In our study, we firstly elucidated the significant up-regulation of *SIRT6* in NKTCL clinical tissues, and qRT-PCR experiments demonstrated that *SIRT6* was significantly

overexpressed in NKTCL cell lines. Moreover, the rescue experiments showed that *miR-363* could target *SIRT6* binding to activate the *PI3K/AKT* pathway. It is common for constitutively activating signaling pathways to regulate the development of malignant tumors (52). Furthermore, the amplification of *PI3K/AKT* signaling has been shown to mediate the occurrence of a variety of cancers (53).

To verify whether *miR-363* affects the progression of NKTCL via the *SIRT6/PI3K/AKT* pathway axis, cellular and mouse tumor formation experiments were carried out. The results revealed that the differential expression of *miR-363* or *SIRT6* significantly affected the activation of the *PI3K/AKT* pathway, and thus, changed the proliferation and apoptosis levels of NKTCL cell lines. Moreover, the *miR-363/SIRT6/PI3K/AKT* axis mechanism affecting mouse NKTCL progression was also confirmed, and the data trends were consistent with SNK-6 cells.

However, there are several limitations in the current research that should be noted. Firstly, the potential upstream molecule of the *miR-363/SIRT6* axis in NKTCL was not investigated. Also, other biological functions, such as stemness, migration, invasion, etc., were not analyzed. These considerations will be included in our future studies.

Conclusions

Expression of *miR-363* in NKTCL is significantly lower than that in normal tissues or cells. *MiR-363* could repress proliferation but accelerate apoptosis via targeting *SIRT6* to activate *PI3K/AKT* pathway in NKTCL, which is expected to provide a new target and guidance for the treatment and diagnosis of NKTCL, as well as offer an additional molecular option for future drug development of NKTCL.

Acknowledgments

Funding: None.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5649/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5649/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5649/coif). The authors have no conflicts of interest to declare.

Ethics Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal protocols in this study were performed under a project license (No. 2020047) granted by ethics board of The Fourth Hospital of Hebei Medical University, in compliance with national guidelines for the care and use of animals. Experiments involving human participants were approved by the ethics board of The Fourth Hospital of Hebei Medical University (No. 2020046), and was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all the patients or the patients' guardians.

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(English Language Editor: A. Kassem)

Cite this article as: Xu B, Jiang L, Cui JL, Zhu XL, Bai YJ, Chen J, Diao YQ. *MiR-363* suppresses the tumor growth of natural killer/T-cell lymphoma via the *SIRT6/PI3K/AKT* axis. Ann Transl Med 2022;10(23):1276. doi: 10.21037/atm-22-5649

