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ORIGINAL ARTICLE

TP73-AS1 promotes malignant progression of NK/T cell lymphoma by regulating DKK1 methylation

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Summary

Purpose: *Long non-coding RNA (lncRNA) TP73-AS1 is abnormally expressed in multiple types of tumors, which is able to mediate tumor cell signals. This study aims to explore the role of TP73-AS1 in affecting biological functions of NK/T-cell lymphoma (NKTCL) and DKK1 methylation.*

Methods: *TP73-AS1 levels in peripheral blood of NKTCL patients and healthy volunteers was detected by quantitative real-time polymerase chain reaction (qRT-PCR). After knockdown of TP73-AS1, proliferative and migratory abilities in SNK-6 and HANK-1 cells were assessed by cell counting kit-8 (CCK-8) and Transwell assay, respectively. Regulatory effect of TP73-AS1 on DKK1 methylation in NKTCL cells was evaluated through methylation-specific PCR (MSP), dual-luciferase reporter assay and RNA Binding Protein Immunoprecipitation (RIP). Rescue experiments were conducted to further validate the interaction between TP73-AS1 and DKK1.*

Results: *TP73-AS1 level was higher in peripheral blood of NKTCL patients than that of healthy volunteers. Knockdown of TP73-AS1 in vitro weakened proliferative and migratory functions of NKTCL cells. TP73-AS1 induced methylation of DKK1 promoter through DNMT1/DNMT3, thus regulating NKTCL cell functions.*

Conclusions: *TP73-AS1 level was higher in peripheral blood of NKTCL patients. Through inducing methylation of DKK1 promoter, TP73-AS1 aggravates the malignant progression of NKTCL.*

Key words: *LncRNA TP73-AS1, NKTCL, DKK1, methylation*

Introduction

Malignant lymphoma (ML) derives from lymph nodes and other lymphoid hematopoietic tissues, which can be classified into Hodgkin lymphoma and non-Hodgkin lymphoma [1,2]. Its incidence in women and men ranks fifth and sixth in global tumors, respectively [3]. NK/T-cell lymphoma (NKTCL) belongs to non-Hodgkin's lymphoma. Its incidence differs from geographical locations. Asian people, including Asian Americans and Asian Europeans, are mainly affected by NKTCL. NKTCL is named because the tumor cells are derived from NK cells or T cells [4,5]. Chronic infection of EB virus has a close relation to the pathogenesis of NKTCL [6]. Clinically, NKTCL is highly invasive, rapidly progressed and poorly responded to active treatment. In addition, its recurrence rate remains high [7,8]. So far, therapeutic standard for NKTCL is lacked [9,10].

Long non-coding RNA (lncRNA) lacks an open reading frame, which is initially screened from a large-scale cDNA library in mice [11,12]. The number of lncRNAs is constantly increasing. Most lncRNAs are transcribed by RNA polymerase II. After splicing, adding 3'poly A tail and a 5'cap, a stable structure of lncRNA enables it to interact with other molecules [12,13]. LncRNA has driven many key biological processes of tumors, like tumor cell growth, invasiveness and metastasis [14,15].

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LncRNAs and protein-encoding genes, synergistically or individually play an important role in the carcinogenesis of NCKTL [16,17]. Previous studies have shown that lncRNA TP73-AS1 participates in glioma development *via* the miR-103a/GALNT7 axis [18,19].

In the present study, we aim to explore the biological function of TP73-AS1 in NKTCL process, and the molecular mechanism, aiming to provide a novel target for alleviating the deterioration of NKTCL.

Methods

Subjects

Peripheral blood samples were collected from 18 NKTCL patients and age-matched healthy volunteers. NKTCL patients did not receive chemotherapy or radiotherapy. Tumor staging of NKTCL was evaluated based on Union for International Cancer Control (UICC) criteria. Clinical data of them were completely recorded. This study was approved by the research Ethics Committee of Guizhou Provincial People's Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell lines and reagents

NKTCL cell lines (NK-92, SNK-1, SNK-6, KHYG-1 and HANK-1) and NK cell line were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultivated in F-12 or Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin.

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-60% density in a 6-well plate, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell proliferation assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 24, 8, 72 and 96 h, optical density at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell migration assay

200 μL of suspension (2×10^5 cells/mL) was applied on the top of a Transwell insert (Corning, Corning, NY, USA), which was placed in each well containing 700 μL of medium and 20% FBS. Cells were allowed to migrate for 48 h, and they were fixed, dyed and captured for counting in five random fields per sample.

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

Peripheral blood samples were processed for isolating RNAs. After purification of cellular RNAs, they were reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) and subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Tokyo, Japan). Relative level of PCR products was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH). TP73-AS1: Forward: 5'-CCCGGGTGTGGATCCCTAGA-3', Reverse: 5'-AAGGCCGGGGACTCTCG-3'; DKK1: Forward: 5'-TGACAACTACCAGCCGTACC-3', Reverse: 5'-CAGGCGAGACAGATTTGCAC-3'; GAPDH: Forward: 5'-ATGGAGAAGGCTGGGGCTC-3', Reverse: 5'-AAGTTGTCATGGATGACCTTG-3'.

Methylation-specific PCR (MSP)

Genomic DNA was extracted from cells and tissues using a genomic DNA purification kit (Qiagen company, Hilden, Germany), followed by DNA modification with sodium bisulfite using Intergen CpGenome DNA modifi-

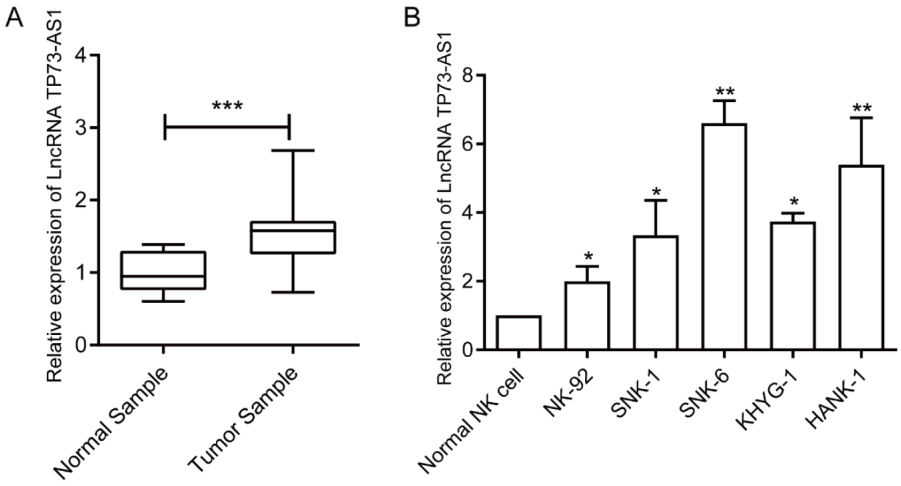

Figure 1. High expression of TP73-AS1 in NKTCL. **A:** Peripheral blood level of TP73-AS1 in NKTCL patients and healthy volunteers. **B:** TP73-AS1 levels in NKTCL cell lines; *p<0.05, **p<0.01, ***p<0.001.

cation kit (Intergen, New York, NY, USA). Unmethylated cytosine was converted to thymine, while the others remained. MSP was conducted using DKK1 methylation specific primers. DNA treated with M.Sssl methylase was used as a positive control, while untreated DNA was used as a non-methylated negative control. To eliminate false positive results, ddH_2O was used as a control instead of the DNA template. PCR products were loaded on TBE and analyzed.

Western blot

Radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) was used to lyse cells on ice, and the lysate was subjected to 14000×g centrifugation at 4°C. Fifteen minutes later, the precipitant was resuspended for measuring protein concentration. Prepared protein samples were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were blocked for non-specific antigens in 5% skim milk for 2 h. Later, membranes were immunoblotted with primary and secondary antibodies for grey value analysis.

RNA Binding Protein Immunoprecipitation (RIP) assay

Magna-RNA binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) was used for RIP. The RNA concentration was detected with a spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the RNA quality was detected with a biological analyzer (Agilent, Santa Clara, CA, USA). The input was the whole cell lysate. Purified RNAs were subjected to qRT-PCR.

Dual-luciferase reporter assay

HEK293T were seeded in a 24-well plate, and co-transfected with sh-TP73-AS1/sh-NC and DKK1-MUT/DKK1-WT vector, respectively. After 48 h, cells were lysed for measuring luciferase activity (Promega, Madison, WI, USA).

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the t-test. The influence of TP73-AS1 on pathological indexes of NKTCL patients were analyzed by Chi-square test. Kaplan-Meier method was introduced

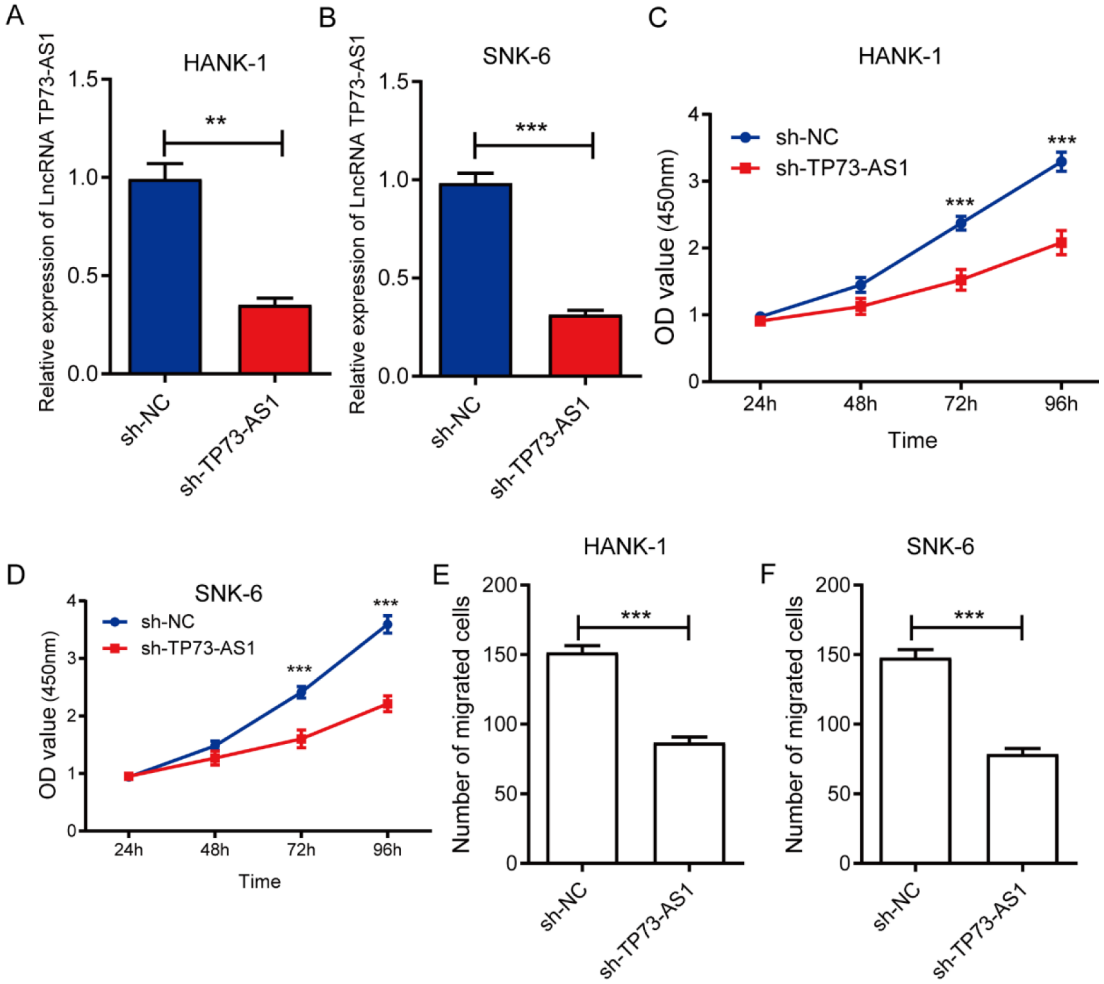

Figure 2. TP73-AS1 promoted proliferative and migratory abilities in NKTCL. **A** and **B:** Transfection efficacy of sh-TP73-AS1 in HANK-1 and SNK-6 cells. **C** and **D:** Viability in HANK-1 and SNK-6 cells transfected with sh-NC or sh-TP73-AS1. **E** and **F:** Migration in HANK-1 and SNK-6 cells transfected with sh-NC or sh-TP73-AS1; **p<0.01, ***p<0.001.

for survival analysis, followed by log-rank test for comparing differences. Correlation between TP73-AS1 and DKK1 levels was assessed by Pearson correlation test. $p<0.05$ was considered as statistically significant.

Results

High expression of TP73-AS1 in NKTCL

In comparison to healthy volunteers, peripheral blood level of TP73-AS1 was much higher in NKTCL patients (Figure 1A). Besides, it was upregulated in NKTCL cells than that of normal NK cells (Figure 1B). SNK-6 and HANK-1 cells were used in the experiments because they expressed the highest level of TP73-AS1 than the other tested cells. It is suggested that TP73-AS1 could be a novel indicator for predicting the malignant process of NKTCL.

TP73-AS1 promoted proliferative and migratory abilities in NKTCL

Transfection efficacy of sh-TP73-AS1 was first examined in HANK-1 and SNK-6 cells (Figure 2A

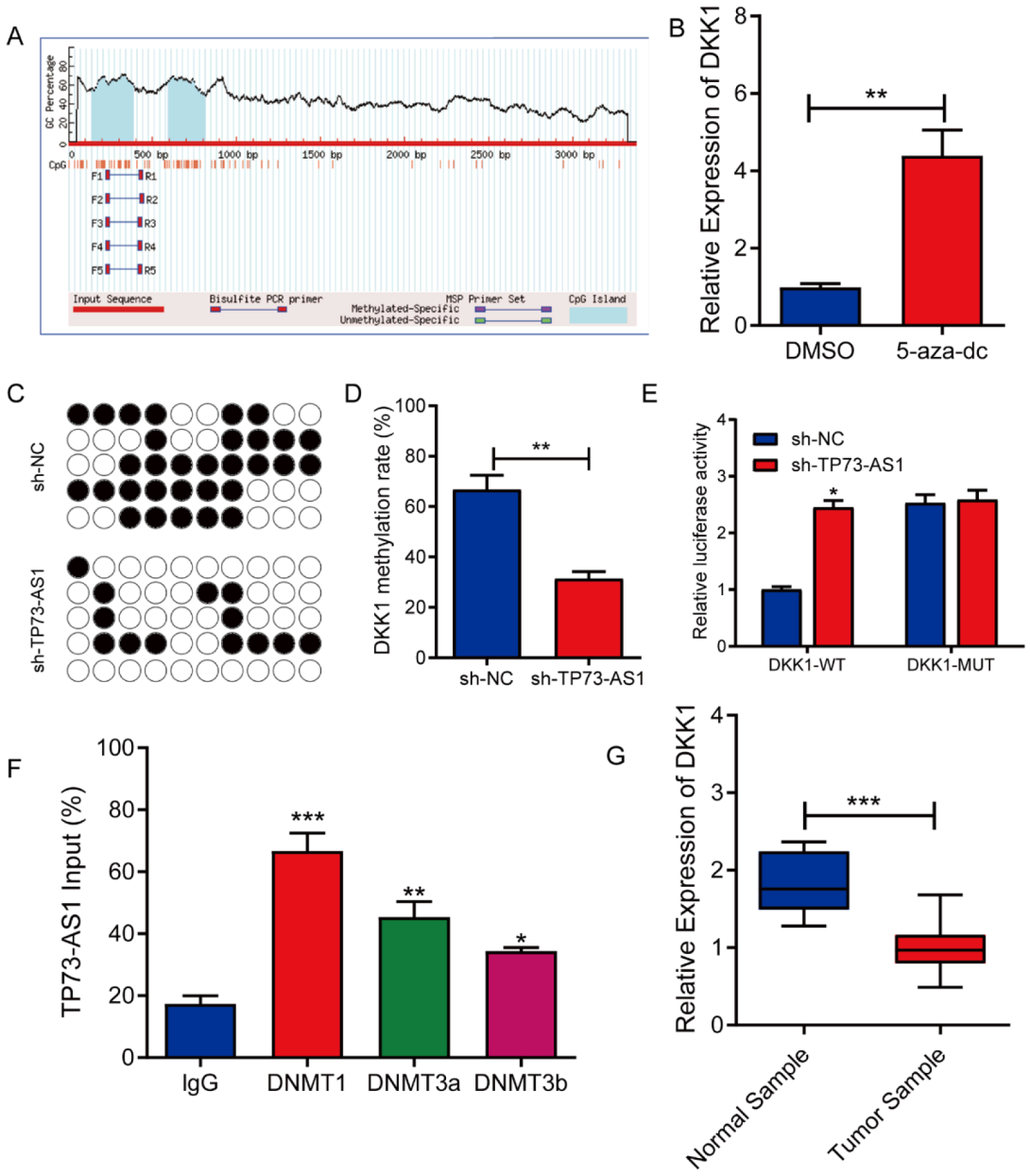

Figure 3. TP73-AS1 inhibited the transcription of DKK1 through mediating the methylation of the DKK1 promoter. **A:** Online search of DKK1 promoter region. **B:** DKK1 levels after 5-Aza-dC induction. **C:** DKK1 promoter methylation level in HANK-1 cells transfected with sh-NC or sh-TP73-AS1. **D:** DKK1 methylation rate in HANK-1 cells transfected with sh-NC or sh-TP73-AS1. **E:** Luciferase activity in HEK293T cells co-regulated by TP73-AS1 and DKK1. **F:** TP73-AS1 input in anti-IgG, anti-DNMT1, anti-DNMT3a and anti-DNMT3b. **G:** Peripheral blood level of DKK1 in NKTCL patients and healthy volunteers; *p<0.05, **p<0.01, ***p<0.001.

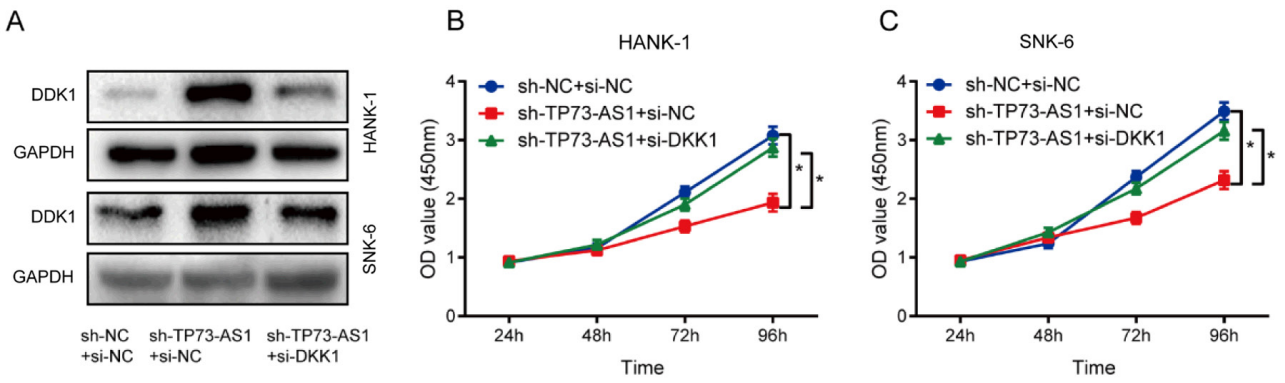

Figure 4. TP73-AS1 regulated NKTCL cell functions by interacting with DKK1. **A:** Transfection efficacy of si-DKK1 in HANK-1 and SNK-6 cells. **B** and **C:** Viability in HANK-1 and SNK-6 cells co-regulated by TP73-AS1 and DKK1; *p<0.05.

and 2B). After knockdown of TP73-AS1, cell viability was markedly reduced in NKTCL cells (Figure 2C and 2D). Transwell assay obtained the conclusion that knockdown of TP73-AS1 decreased migratory rate in NKTCL cells (Figure 2E and 2F).

TP73-AS1 inhibited the transcription of DKK1 through mediating the methylation of the DKK1 promoter

Through database screening, DKK1 was searched as the downstream gene of TP73-AS1. Using the MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), the presence of DKK1 promoter regions was discovered (Figure 3A). Induction of the methyltransferase inhibitor 5-Aza-dC remarkably upregulated DKK1 (Figure 3B). After knockdown of TP73-AS1, methylation level of DKK1 promoter was reduced, indicating that TP73-AS1 was able to activate DKK1 methylation (Figure 3C). In cells transfected with sh-NC, CpG island in the promoter region of DKK1 was highly methylated, which was declined by transfection of sh-TP73-AS1 (Figure 3D). Subsequently, luciferase activity of DKK1-WT vector in HEK293T cells was detected to be inhibited by knockdown of TP73-AS1, and TP73-AS1 did not pose a potential impact on the luciferase activity of DKK1-MUT vector (Figure 3E). It is verified that TP73-AS1 directly bound to DKK1. RIP assay was conducted to confirm the interaction between TP73-AS1 and DNMT1/DNMT3. The results showed that DNMT1, DNMT3A and DNMT3B markedly increased the enrichment of TP73-AS1 compared with IgG treatment (Figure 3F). DKK1 was lowly expressed in peripheral blood samples of NKTCL patients (Figure 3G).

TP73-AS1 regulated NKTCL cell functions by interacting with DKK1

Transfection efficacy of si-DKK1 was examined in NKTCL cells with TP73-AS1 knockdown (Figure 4A). Interestingly, the declined viability in NKTCL cells with TP73-AS1 knockdown were partially reversed by co-knockdown of DKK1 (Figure 4B, 4C).

Discussion

NKTCL is a relatively rare type of lymphoma, which can metastasize to distant organs [4,5]. Local radiotherapy combined chemotherapy is ideal for the treatment of early stage NKTCL. Nevertheless, therapeutic response for advanced NKTCL patients or whom combined hemophagocytic syndrome is relatively poor [6-9]. Abnormally expressed lncRNAs have been abundantly emerged in tumor profile, and they are vital regulators in tumor cell functions [12-15]. The role of lncRNAs in NKTCL is rarely reported [16,17]. Therefore, discovering lncRNAs related to the pathogenesis of NTKTL and exploring their functions are of great significance for the clinical treatment [10,17].

Recent studies have identified that TP73-AS1 is a recognized oncogenic lncRNA, which is upregulated in glioma and acute myeloid leukemia cases [18,19]. In the present study, TP73-AS1 level remained higher in peripheral blood of NKTCL patients than that of healthy volunteers. *In vitro* knockdown model of TP73-AS1 was constructed in HANK-1 and SNK-6 cells. After knockdown of TP73-AS1, proliferative and migratory functions of NKTCL cells were remarkably weakened. It is concluded that TP73-AS1 served as an oncogene in NKTCL.

To further ascertain how TP73-AS1 affects NKTCL process, we predicted its potential target genes. DKK1 may interact with TP73-AS1. The stability of DDK1 protein depends on its post-translational modifications (e.g. phosphorylation, acetylation, ubiquitination, methylation, etc.) [20]. This study found the presence of the DKK1 promoter region. Moreover, induction of the methyltransferase inhibitor 5-Aza-dD remarkably upregulated DKK1.

Results from MSP, RIP and dual-luciferase reporter assay yielded a conclusion that TP73-AS1 inhibited the transcription level of DKK1 by mediating its methylation. Moreover, rescue experiments demonstrated that knockdown of DKK1 could abolish the attenuated proliferative and migratory rates in NKTCL cells with TP73-AS1 knockdown. The above findings proposed a feedback loop that TP73-AS1 promoted the malignant process of NKTCL *via* inducing DKK1 promoter methylation.

Conclusions

TP73-AS1 level was higher in peripheral blood of NKTCL patients. Through inducing methylation of DKK1 promoter, TP73-AS1 aggravates the malignant progression of NKTCL.

Conflict of interests

The authors declare no conflict of interests.

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