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Restoration of microRNA-373 suppresses growth of human T-cell lymphoma cells by repressing CCND1

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Abstract. – OBJECTIVE: Adult T cell lymphoma is a highly aggressive T-cell malignancy. This study was designed to explore the expression and functional significance of microRNA (miR)-373 in T cell lymphoma.

PATIENTS AND METHODS: We analyzed the levels of CCND1 and miR-373 in T cell lymphoma tissue and the relationship of miR-373 levels with patients' prognosis. We then overexpressed miR-373 by miRNA mimics transfection and inhibited miR-373 by miRNA antisense transfection in T cell lymphoma cells. Cell survival and growth were analyzed by CCK-8 assay and MTT assay, respectively. Cell proliferation was analyzed by flow cytometry. Bioinformatics analyses were applied to predict miR-373 targets, which were then confirmed by luciferase reporter assay.

RESULTS: We detected significantly higher levels of CCND1, and significantly lower levels of miR-373 in T cell lymphoma tissue, compared to the adjacent non-tumor tissue. Moreover, the low miR-373 levels were associated with poor survival of the patients. Overexpression of miR-373 significantly inhibited cell growth, while depletion of miR-373 increased cell growth in T cell lymphoma cells. Moreover, the effects of miR-373 on cell growth appeared to result from an alteration in cell proliferation. Finally, miR-373 was found to bind to the 3'-UTR of CCND1 mRNA to inhibit its translation in T cell lymphoma cells.

CONCLUSIONS: Our study suggests that reduced miR-373 levels in T cell lymphoma tissue may promote T cell lymphoma growth, possibly through CCND1-mediated cell proliferation.

Key Words:

T cell lymphoma; miR-373; cancer cell proliferation; CCND1.

Introduction

Lymphoma is the most common blood cancer, which occurs when lymphocytes aberrantly outgrow, followed by metastasis into lymph nodes, spleen, bone marrow, blood, or other organs, and form tumor[1-3]. The two main forms of lymphoma are Hodgkin lymphoma and non-Hodgkin lymphoma. Both B and T lymphocytes can develop into lymphomas. T cell lymphomas account for approximately 15 percent of all non-Hodgkin lymphoma in the United States[3-5]. There are a variety of different types of T cell lymphoma, and thus the standard lymphoma therapies include chemotherapy, radiation, stem cell transplantation and surgery[6].

Cyclin D1, also called CCND1, is a cell-cycle activator. Regulatory component of the CCND1-CDK4 complex phosphorylates and inhibits members of the retinoblastoma (RB) protein family including RB1 and regulates the cell-cycle during G1/S transition[7-9]. Component of the ternary complex, CCND1/CDK4/CDKN1B, is required for nuclear translocation and activity of the CCND1-CDK4 complex[10-13]. CCND1-CDK4 complexes are major integrators of various mitogenic and antimitogenic signals[14-18]. CCND1/CDK4 complex is often over-activated in cancer cells and is a potent tumor enhancer[19,20].

MicroRNA (miRNA) is a group of non-coding small RNAs of roughly 22 nucleotides, which regulate many genes post-transcriptionally, through regulation of gene expression by repressing translation or directing sequence-specific degradation of complementary mRNA by 3 -untranslated region (3 -UTR) binding[21,22]. MiRNAs play a critical role in carcinogenesis of various tumors, as either tumor suppressor or enhancer[23-30]. Among all miRNAs, miR-373 has been rarely studied. The first report on miR-373 showed a putative miR-373 target site in the promoter of E-cadherin. Transfection of miR-373 and its precursor hairpin RNA (pre-miR-373) readily induced

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E-cadherin expression. Knockdown experiments confirmed that induction of E-cadherin by pre-miR-373 required the miRNA maturation protein Dicer[31]. Later on, miR-373 has been shown to be involved in the tumorigenesis of gastric cancer[32], breast cancer[33], lung cancer[34], colon cancer[35] and hepatocellular carcinoma[36]. However, the targets of miR-373 in these cancers are very different, and thus the effects of miR-373 may be cancer type-specific. Moreover, a role of miR-373 in T cell lymphoma has not been reported.

Here, we analyzed the levels of CCND1 and miR-373 in T cell lymphoma tissues, and studied the association of miR-373 with the prognosis of the patients. We then overexpressed miR-373 or inhibited miR-373 in 2 T cell lymphoma cell lines and studied their effects on CCND1, cancer cell growth, survival, and proliferation.

Patients and Methods

Patient Specimens

Surgical specimens from 30 T cell lymphoma patients and matched tumor-adjacent normal tissues (NT) were obtained postoperatively in the Harbin Medical University Cancer Hospital from 2011 to 2015. All patients gave signed, informed consent for the tissue to be used for scientific research. Ethical approval for the study was obtained from the Harbin Medical University Cancer Hospital. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. All patients had been followed-up for 30 months. Complete clinical data was electronically recorded.

Cell Line Culture and Transfection

H9 and HuT102 are two commonly human T cell lymphoma lines used for research, and were both purchased from ATTC (American Type Culture Collection, Manassas, VA, USA). Both cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO_2 at 37°C. MiRNAs mimics (miR-373) and miRNAs antisense oligonucleotides (as-miR-373) were obtained from Origene (Beijing, China). A null sequence was used as a control (null). The plasmids were transfected into cells at a concentration of 50 nmol/l using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen).

MiRNA Target Prediction and 3'-UTR Luciferase-Reporter Assay

MiRNAs targets were predicted using the algorithms TargetSan online software. The CCND1 3'-UTR reporter plasmid (pRL-CCND1) and the CCND1 3'-UTR reporter plasmid with a mutant at the miR-373 binding site (pRL-CCND1-mut) were purchased from Creative Biogene (Shirley, NY, USA). TT cells were co-transfected with pRL-CCND1/pRL-CCND1-mut and miR-373/as-miR-373/null by Lipofectamine 2000 (5×10^4 cells per well). Cells were collected 48 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from resected specimens or cultured cells with the miRNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was randomly primed from 2 g of total RNA using the Omniscript reverse transcription kit (Qiagen). Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed, using 2-ΔΔCt method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin and, then, compared to the experimental controls.

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well microplate at a density of 5×10^4/ml. After 24h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20 ml/well) was added and the plate was incubated at 37°C for 2 h. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

MTT Assay

For assay of cell growth, 5×10^3 cell per well were seeded into 96 well-plate and subjected to a Cell Proliferation Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm in a microtiter plate reader (Promega, Madison, WI, USA). Experiments were performed 5 times.

Proliferation Assay

For analysis of apoptosis, cultured cells were dissociated and resuspended at a density of 10^6 cells/ml in PBS. After staining with FIT cell lymphoma-conjugated BrdU antibody (FIT cell lymphoma BrdU Flow Kit, Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA), cells were analyzed using FACScan flow cytometer (Becton-Dickinson Biosciences) equipped with Cell Quest software (Becton-Dickinson Biosciences) for determination of FIT cell lymphoma+ S-phase proliferating cells.

Western Blot

Total Protein was extracted from the patients' specimens or cultured cells by RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). An equal amount of proteins was loaded in the gel. Primary antibodies for Western Blot are rabbit anti-CCND1 and anti-α-tubulin (all purchased from Cell Signaling, St. Jose, LA, USA). The secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). The protein levels were first normalized to α-tubulin and, then, normalized to control. Images shown in the figure were representatives from 3 repeats. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MD, USA).

Statistical Analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA). All values in cell and animal studies are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher' Exact Test for comparison of two groups. Patients' survival was determined by Kaplan-Meier analysis.

Results

Low miR-373 Levels in T Cell Lymphoma Specimens Associate with Poor Prognosis

The levels of CCND1 and miR-373 in 30 pairs of T cell lymphoma tissues and matched tumor-adjacent normal tissues (NT) were measured by Western blot and RT-qPCR, respectively. We found that T cell lymphoma specimens expressed significantly higher levels of CCND1 (Figure 1A), and significantly lower levels of miR-373, shown by individual values (Figure 1B), and by mean ± SD (Figure 1C). To examine the clinical significance of low miR-373 levels in T cell lymphoma, the 30 T cell lymphoma patients were followed-up for 30 months. The median value of all 30 cases was chosen as the cutoff point for separating miR-373 high-expression cases (n=15) from miR-373 low-expression cases (n=15). Kaplan-Meier curves indicated that T cell lymphoma patients with low miR-373 levels had a significantly worse prognosis than those with low miR-373 levels (Figure 1D).

Overexpression of miR-373 Inhibits T Cell Lymphoma Cell Growth

Next, the role of miR-373 in the growth of cultured T cell lymphoma cells was investigated. We used 2 human T cell lymphoma cell lines, H9 and HuT102, and transfected those cells with miR-373 mimics (miR-373), or antisense for miR-373 (as-miR-373), or a control null sequence as a control (null). First, the levels of miR-373 in these modified cells were assayed by RT-qPCR, 72 hours after transfection. We found that the miR-373 levels in H9 cells significantly increased by miR-373, while the miR-373 levels in H9 cells significantly decreased by miR-373 suppression (Figure 2A). The cell survival and growth were then assayed by CCK-8 assay and by MTT assay, respectively. We found that the survival of H9 cells was not affected by miR-373 modification (Figure 2B). However, overexpression of miR-373 significantly inhibited the cellular growth in H9 cells, while inhibition of miR-373 significantly increased the cellular growth in H9 cells, in an MTT assay (Figure 2C). Similar results were obtained from HuT102 cells (Figure 2D-F). Thus, miR-373 inhibits T cell lymphoma cell growth *in vitro*.

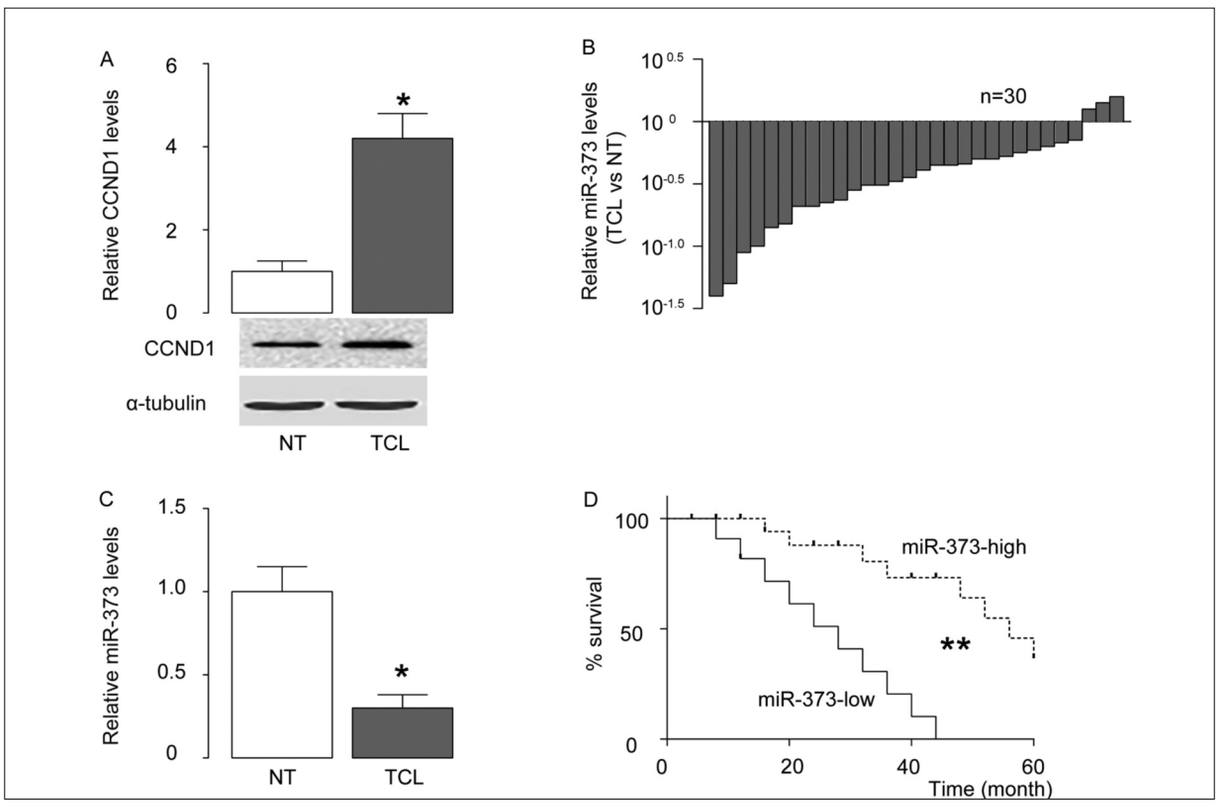


Figure 1. Low miR-373 level in T cell lymphoma tissue associates with poor survival. ***A,*** The levels of CCND1 in 30 T cell lymphoma tissues and matched tumor-adjacent normal tissue (NT) by Western blot. ***B,-C,*** The levels of miR-373 in 30 T cell lymphoma tissues and matched NT by RT-qPCR, shown by individual values ***(B)***, and by mean ± SD ***(C)***. ***D,*** The 30 T cell lymphoma patients were followed-up for 60 months. The median value of all 30 cases was chosen as the cutoff point for separating miR-373 high-expression cases (n=15) from miR-373 low-expression cases (n = 15). Kaplan-Meier curves were shown. $*p < 0.05$. $**p < 0.01$. N = 30.

T Cell Lymphoma Cell Proliferation is Regulated by miR-373

Since cell survival was not altered by miR-373 modification, we hypothesized that miR-373 might alter cell proliferation. Thus, we analyzed proliferating S-phase cells based on BrdU incorporation. We found that overexpression of miR-373 significantly inhibited H9 cell proliferation, while inhibition of miR-373 significantly increased H9 cell proliferation, shown by quantification (Figure 3A), and by representative flow charts (Figure 3B). Similar results were obtained from HuT102 (Figure 3C-D). Hence, miR-373 inhibits T cell lymphoma cell growth through suppressing cell proliferation.

MiR-373 Targets CCND1 to Regulate T Cell Lymphoma Cell Proliferation

Next, we predicted the potential targeted genes of miR-373 by bioinformatics algorithms. Among all predicted genes, we found that CCND1 was the one that altered its value by miR-373 modification. The 3'-UTR of CCND1 mRNA was found to be a target for miR-373 (Figure 4A). The intact 3'-UTR of CCND1 mRNA, together with a 3'-UTR with mutant at the miR-373-binding site of CCND1 mRNA, was then cloned into Luciferase reporter plasmids, and used for co-transfection with miR-373-modified plasmids into H9 cells. The Luciferase activities were quantified in these cells, suggesting that miR-373 specifically targets 3'-UTR of CCND1 mRNA to inhibit its translation in T cell lymphoma (Figure 4B).

MiR-373 Decreases CCND1 Protein but not mRNA

We then evaluated the effects of miR-373 on CCND1 levels in T cell lymphoma cells. We found that although the CCND1 transcripts did not change by miR-373 levels (Figure 5A), the protein levels of CCND1 in miR-373-

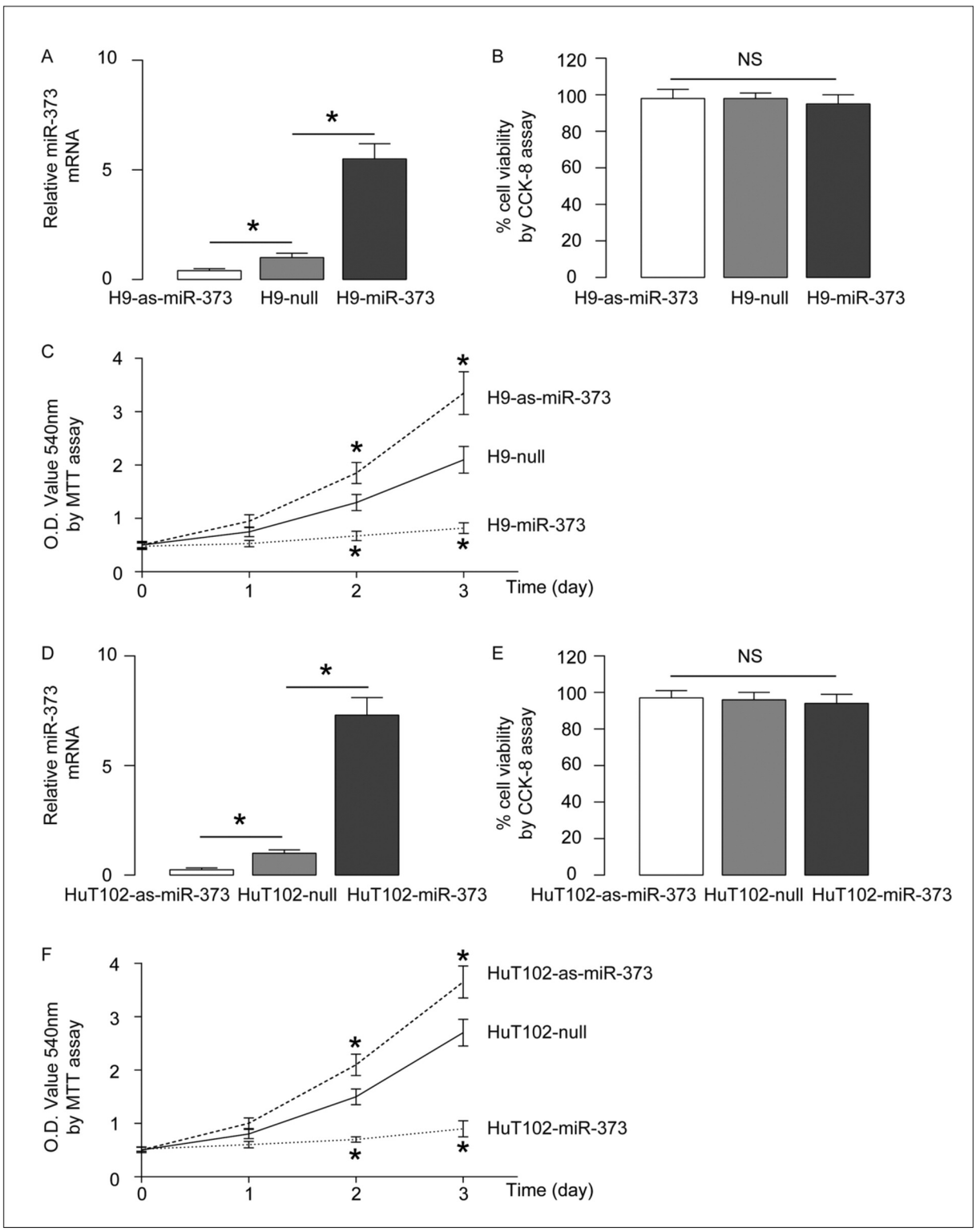


Figure 2. Overexpression of miR-373 inhibits T cell lymphoma cell growth. We transfected two human cell lymphoma cell lines with miR-373 mimics (miR-373), or antisense for miR-373 (as-miR-373), or a control null sequence (null). ***A,*** The levels of miR-373 transcripts in miR-373-modified H9 cells were assayed by RT-qPCR, 72 hours after transfection. ***B,*** The H9 cell survival was assayed by CCK-8 assay. ***C,*** The H9 cell growth was assayed by MTT assay. ***D,*** The levels of miR-373 transcripts in miR-373-modified HuT102 cells were assayed by RT-qPCR, 72 hours after transfection. ***E,*** The HuT102 cell survival was assayed by CCK-8 assay. ***F,*** The HuT102 cell growth was assayed by MTT assay. *$p < 0.05$. NS: non-significant. N = 5.

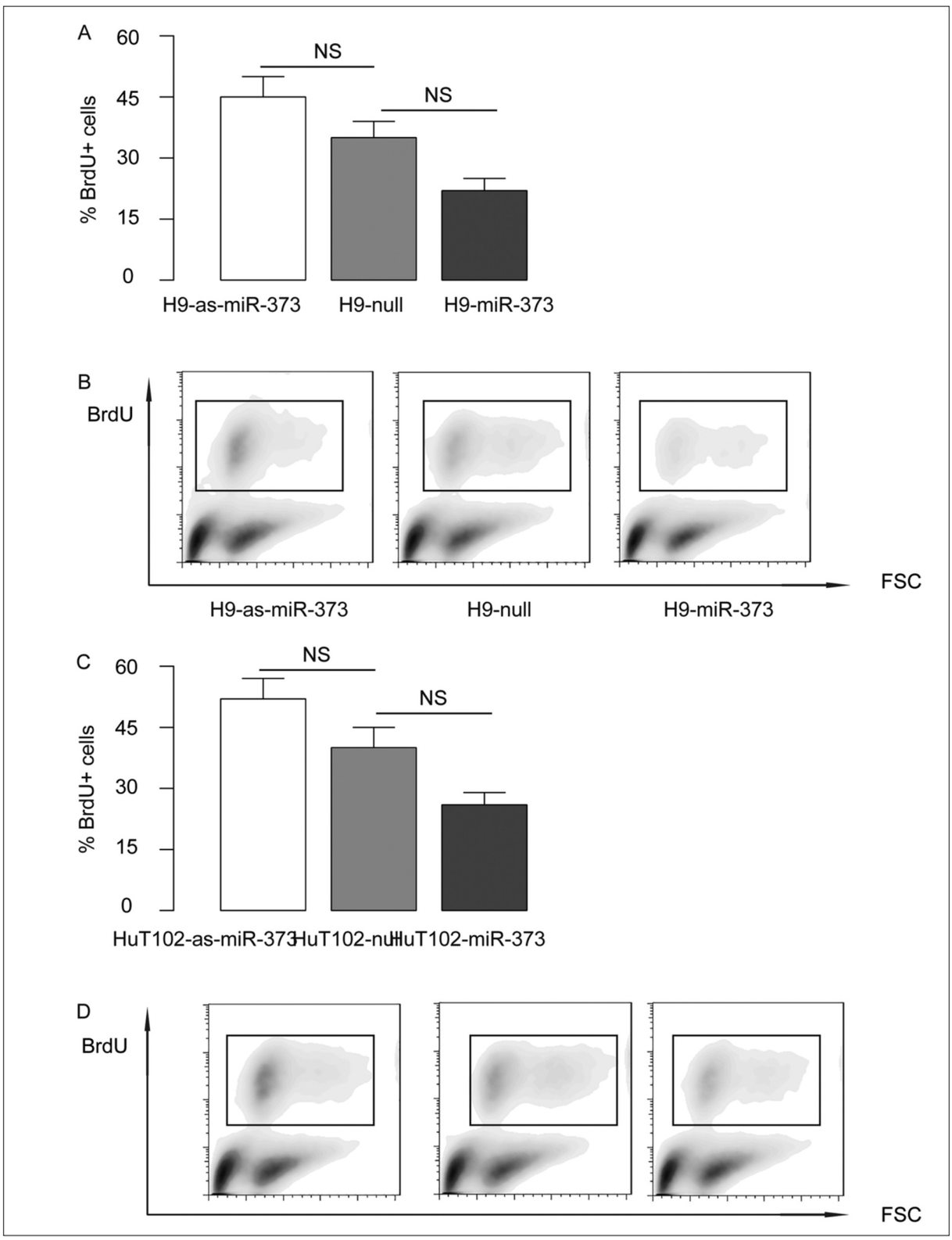
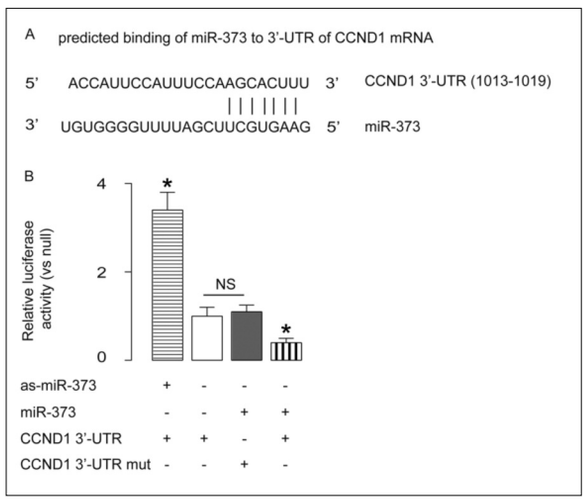


Figure 3. Levels of miR-373 regulate T cell lymphoma cell proliferation. ***A,-B,*** Cell proliferation was analyzed by flow cytometry in miR-373-modified H9 cells, shown by quantification ***(A)***, and by representative flow charts ***(B)***. ***C,-D,*** Cell proliferation was analyzed by flow cytometry in miR-373-modified HuT102 cells, shown by quantification ***(C)***, and by representative flow charts ***(D)***. *$p < 0.05$. N = 5.

Figure 4. MiR-373 targets CCND1 to regulate T cell lymphoma cell proliferation. ***A,*** Bioinformatics algorithms analysis shows that the 3'-UTR of CCND1 mRNA is a target for miR-373. ***B,*** The intact 3'-UTR of CCND1 mRNA, together with a 3'-UTR with mutant at miR-373-binding site of CCND1 mRNA, was then cloned into Luciferase reporter plasmids, and used for co-transfection with miR-373-modified plasmids into H9 cells. The Luciferase activities were quantified in these cells. *$p < 0.05$. NS: non-significant. N = 5.



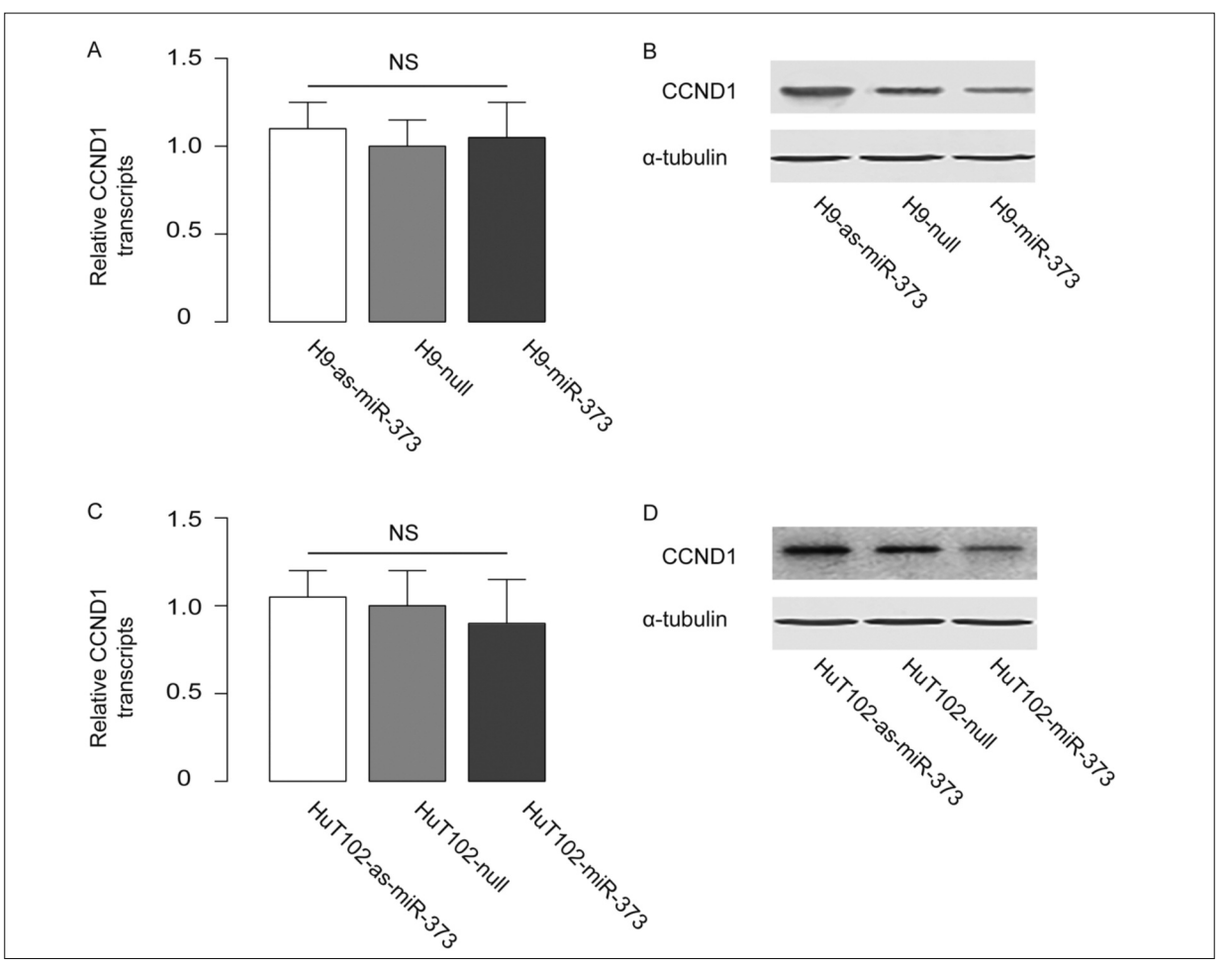


Figure 5. MiR-373 decreases CCND1 protein but not mRNA. ***A,-B,*** We then evaluated the effects of miR-373 on CCND1 levels in H9 cells, by mRNA ***(A)***, and by Western blot ***(B)***. ***C-D,*** We then evaluated the effects of miR-373 on CCND1 levels in HuT102 cells, by mRNA ***(C)***, and by Western blot ***(D)***. *$p < 0.05$. NS: non-significant. N = 5.

overexpressing H9 cells was significantly decreased, while the protein levels of CCND1 in miR-373-depleted H9 cells was significantly increased (Figure 5B). Similar results were obtained from HuT102 cells (Figure 6C-D). These data suggest that the translation of CCND1 in TT cells is suppressed by miR-373. Together, our study demonstrates a role of miR-373 in control of T cell lymphoma cell proliferation, through CCND1 modulation (Figure 6).

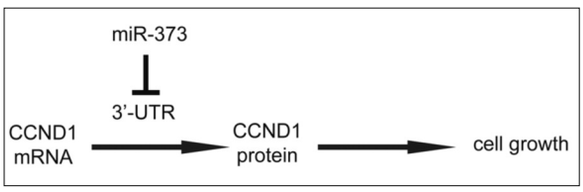


Figure 6. Schematic of the model. MiR-373 regulates T cell lymphoma cell proliferation, through translational suppression of CCND1.

Discussion

The participation of miRNAs in the T cell lymphoma initiation and progression is not adequately investigated. Hence, elucidation of the aberrant expression of miRNAs in T cell lymphoma carcinogenesis will help the physician and tumor biologists to better characterize the molecular regulation of the tumorigenesis of T cell lymphoma and may allow them to identify novel targets to improve the levels of the current therapy.

In this study, we showed that low level of miR-373 in T cell lymphoma tissues were both associated with a low survival rate in T cell lymphoma patients. Then, we showed that miR-373 levels negatively regulated the cell growth in 2 commonly used T cell lymphoma cell lines, through cell proliferation suppression, rather than through interference with cell survival.

Next, we examined how cell growth may be regulated by miR-373 levels in T cell lymphoma cells. Since the changes in cell number may result from a summary of cell death and cell replication but apoptosis seems unchanged in the current system, we thus hypothesize that the signal transductions that regulate cell proliferation may be involved. Among all candidates, we found that CCND1 is a target for miR-373, and its value increased in T cell lymphoma and altered with the adaption of CCND1.

Specifically, we found that the 3'-UTR of CCND1 mRNA was targeted by miR-373, suggesting a strong effect on the translational regulation of CCND1 mRNA by miR-373. Moreover, we further proved that the binding of miR-373 to the mRNA of CCND1 is functional in T cell lymphoma cells, in which it suppressed the protein level, but not mRNA level, consistent with the structural analyses.

Of note, when a miRNA molecule is attached as a perfect match to a target mRNA, it causes the mRNA degradation, resulting in a decrease in mRNA levels[21-25]. Here, it appears that there is a partial interaction between the miR-373 and the 3'-UTR of the CCND1 mRNA. Thus, the miRNA does not form a perfect match with the target mRNA. Therefore, the translation process stops at that point and hence the protein production is reduced, as described here.

Apart from effects of CCND1 on cell growth, it might affect also cell invasiveness. We did not examine cell invasion in the current study. Besides regulation of CCND1 by miRNA, CCND1 protein levels may be also affected by modulation of its degradation, e.g. through ubiquitination. In future, it may be interesting to address these questions.

Conclusions

In T cell lymphoma, the levels of miR-373 appeared to be very low. Hence, miR-373 may be a specific regulator of the tumor cell growth in T cell lymphoma, and its loss may allow the tumor to grow. Further researches may address the molecular mechanisms underlying activation of miR-373 in T cell lymphoma, and these approaches may provide additional evidence for using miR-373 as a novel target for treating T cell lymphoma.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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