

Restoration of microRNA-212 causes a G0/G1 cell cycle arrest and apoptosis in adult T-cell leukemia/lymphoma cells by repressing CCND3 expression

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATL) is a highly aggressive T-cell malignancy. This study was designed to explore the expression and functional significance of microRNA (miR)-212 in ATL. The expression of miR-212 in human ATL tissues and cell lines were investigated. Gain-of-function experiments were carried out to determine the roles of miR-212 in cell proliferation, tumorigenesis, cell cycle progression, and apoptosis. We also identified and functionally characterized the target genes of miR-212 in ATL cells. Compared with normal lymph node biopsies, lymphoma samples from ATL patients displayed underexpression of miR-212 ($p=0.0032$). Consistently, miR-212 was downregulated in human ATL cell lines, compared with normal T lymphocytes. Restoration of miR-212 significantly ($p<0.05$) inhibited ATL cell proliferation and tumorigenesis in mice. Overexpression of miR-212 led to an accumulation of G0/G1-phase cells and a concomitant reduction of S-phase cells. Moreover, enforced expression of miR-212-induced significant apoptosis in ATL cells. CCND3, which encodes a cell cycle regulator cyclin D3, was identified as a direct target of miR-212 in ATL cells. Rescue experiments with a miR-212-resistant variant of CCND3 demonstrated that overexpression of CCND3 restored cell-cycle progression and attenuated apoptotic response in miR-212-overexpressing ATL cells. Taken together, miR-212 exerts growth-suppressive effects in ATL cells largely by targeting CCND3 and may have therapeutic potential in ATL.

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) is a highly aggressive malignancy of peripheral T cells. Human T-cell leukemia virus type 1 infection is a primary cause of this malignancy.¹ At present, no curative treatment is available for this disease. The median overall survival for patients with ATL is only about 1 year.^{2–3} Therefore, it is of importance to uncover the pathogenesis of ATL.

microRNAs (miRs) are short non-coding RNAs implicated in a variety of biological processes, including differentiation, growth, and tumorigenesis.⁴ Via imperfect base pairing with the 3'-untranslated region (3'-UTR) of target

Significance of this study

What is already known about this subject?

- ▶ miRs play important roles in tumor growth and progression.
- ▶ miR-212 is dysregulated in many human malignancies.
- ▶ miR-212 overexpression can impair B-cell cancer development induced by c-Myc.

What are the new findings?

- ▶ miR-212 was downregulated in adult T-cell leukemia/lymphoma (ATL).
- ▶ Restoration of miR-212 inhibited ATL cell proliferation and tumorigenesis.
- ▶ Overexpression of miR-212 induced a cell cycle arrest and apoptosis in ATL cells.
- ▶ CCND3 is a novel target of miR-212.

How might these results change the focus of research or clinical practice?

- ▶ Restoration of miR-212 may represent a promising therapeutic approach to control ATL growth.

mRNAs, miRs negatively regulates gene expression. miR-212 is dysregulated in many human malignancies such as cervical cancer,⁵ glioblastoma,⁶ and prostate cancer.⁷ In several preclinical cancer models, miR-212 shows the ability to suppress solid tumor growth.^{6–8} It has been documented that miR-212 is involved in B-cell development⁹ and interleukin-17-producing T helper cell differentiation.¹⁰ Most interestingly, miR-212 overexpression can inhibit B-cell cancer development induced by oncogenes such as c-Myc.⁹ However, the link between miR-212 expression and T-cell lymphoma development is still elusive.

In the present study, we examined miR-212 expression in human ATL tissues and cell lines and sought to clarify its functional significance in ATL growth and tumorigenesis. The potential target genes that may mediate the biological activities of miR-212 in ATL were further characterized.



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MATERIALS AND METHODS

Patients and tissue samples

A series of lymphoma tissue samples were collected from 34 patients with ATL who underwent lymph node surgical biopsy at Tongji Hospital (Wuhan, China). Histological diagnosis of each case was performed. As a non-malignant control, seven normal lymph node biopsies were included. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C for future gene expression analysis. All the participants had signed an informed consent. The use of patient samples was approved by the Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China).

Cell culture

Human ATL cell lines (Jurkat, Loucy, MOLT-3, and CCRF-CEM) were purchased from the American Type Culture Collection (Manassas, Virginia, USA), and KOPTK1 cells from the Shanghai Institute of Cell Biology of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Fisher/Thermo Scientific, Pittsburgh, Pennsylvania, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA).

Normal T lymphocytes were magnetically isolated from peripheral blood of four healthy volunteers using anti-CD4 and anti-CD8 monoclonal antibodies (Miltenyi Biotec, Auburn, California, USA). Cells were maintained in RPMI-1640 medium containing 10% fetal calf serum.

Measurements of miR-212 expression by quantitative real-time PCR analysis

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen). Quantitative real-time PCR analysis of miR-212 expression was carried out using the TaqMan miRNA assay system (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. miR-212 expression levels were normalized to the values of RNU6B (an internal control) using the $2^{-\Delta\Delta\text{Ct}}$ method.¹¹

Plasmid construction, recombinant lentivirus production

Human miR-212 precursor was amplified from genomic DNA and cloned into the pCDH lentiviral vector. To construct CCND3 3'-UTR reporters, a fragment containing the CCND3 3'-UTR was inserted into the pGL3 vector downstream of the firefly luciferase gene. Mutations of the putative binding site for miR-212 were achieved using the Site-directed Mutagenesis kit (Stratagene, La Jolla, California, USA). The mutated CCND3 3'-UTR fragment was also inserted into the pGL3 vector. For overexpression of a miRNA-resistant variant of CCND3, the complete human CCND3 open reading frame (GenePharma, Shanghai, China) was cloned into the pcDNA3.1(+) vector. All the plasmids were verified by DNA sequencing.

pCDH-miR-212 vector was transfected together with packaging vectors into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA). At 48 hours post-transfection, recombinant lentivirus was harvested and used to transduce Jurkat and KOPTK1 cells. The transduced cells were selected for 5 days in the

presence of puromycin (2 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) to generate stable clones.

Cell transfection

Jurkat and KOPTK1 cells were transfected with 50 nM miR-212 mimic or negative control miRNA (GenePharma.) using Lipofectamine 2000 reagent. The cells were tested for cyclin D3 protein expression at 24 hours post-transfection. In rescue experiments, miR-212-overexpressing Jurkat cells were transfected with the pcDNA3.1-CCND3 plasmid or empty vector and subjected to gene expression, cell proliferation, and apoptosis analysis 24 after transfection.

Cell proliferation assay

Cell proliferation was determined by the MTS assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's protocol (Promega, Madison, Wisconsin, USA). In brief, cells were plated into 96-well plates (3×10^3 cells per well) and allowed to grow for 48 or 72 hours. The AQueous One Solution Reagent was added to each well and incubated at 37°C for 4 hours. Absorbance were recorded at 490 nm.

Cell cycle and apoptosis analysis by flow cytometry

For analysis of cell cycle distribution, cells were fixed in ice-cold 70% ethanol, and incubated for 30 min with propidium iodide (PI; Sigma-Aldrich) solution added with RNaseA (50 $\mu\text{g}/\text{mL}$). Cell apoptosis were detected using the Annexin V-FITC apoptosis detection kit (Invitrogen). Stained cells were analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Caspase-3 activity assay

Caspase-3 activity was measured using a colorimetric assay kit (BioVision, Mountain View, California, USA). Absorbance was read at 405 nm.

Luciferase reporter assay

HEK293T cells were co-transfected with CCND3 3'-UTR reporters (0.3 μg) and miR-212 mimic or control miRNA (50 nM) using Lipofectamine 2000 reagent. The pRL-TK plasmid encoding *Renilla* luciferase (20 ng; Promega) was transfected to control for transfection efficiency. Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) at 24 hours after transfection.

Western blot analysis

Protein was extracted from cells in lysis buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Protein samples were separated on 12% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. The membrane was incubated with rabbit anti-cyclin D3 (1:500; Cell Signaling Technology, Danvers, Massachusetts, USA) and β -actin (1:1000; Sigma-Aldrich). After washing, the membrane was hybridized with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA). Protein signals were developed using a chemiluminescence detection kit (Pierce, Rockford, Illinois, USA). Densitometry was performed using the Quantity One software (Bio-Rad, Hercules, California, USA).

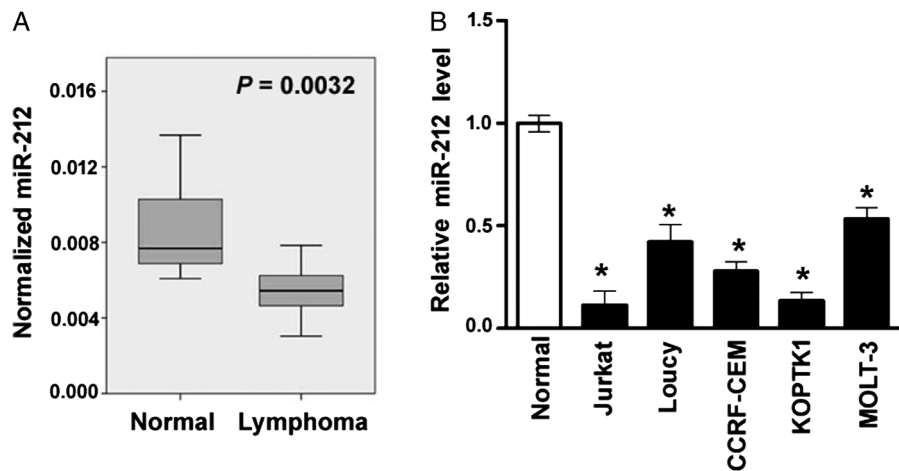


Figure 1 miR-212 is underexpressed in ATL tissues and cells. (A) Analysis of miR-212 in lymphomas and normal lymph node biopsies from 34 patients with ATL. (B) Detection of miR-212 in human ATL cell lines and normal T lymphocytes isolated from peripheral blood of four healthy volunteers. The expression level of miR-212 in normal T lymphocytes was arbitrarily assigned a value of 1.0. * $p < 0.05$ vs normal T lymphocytes ATL, adult T-cell leukemia/lymphoma.

Tumorigenicity assay

Female NOD/SCID mice (4–5-week old) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Jurkat cells stably expressing miR-212 and control cells were subcutaneously injected into both flanks of mice (3×10^6 cells per mouse). Tumor growth was monitored for four consecutive weeks. Afterwards, animals were sacrificed and xenograft tumors were weighed. The experiments involving animals were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology.

Statistical analysis

Data are expressed as mean \pm SEM and were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) with the Tukey test. A *p* value of < 0.05 was considered as a significant difference.

RESULTS

miR-212 is underexpressed in ATL tissues and cells

Compared with normal lymph node biopsies, lymphoma samples from patients with ATL displayed a significant decline in the expression of miR-212 ($p = 0.0032$; [figure 1A](#)). Consistently, miR-212 was underexpressed in a panel of human ATL cell lines, relative to normal T lymphocytes ([figure 1B](#)). These observations suggest that miR-212 may be implicated in the progression of ATL.

Restoration of miR-212 suppresses ATL cell growth and tumorigenesis

To determine the biological relevance of miR-212 underexpression in ATL, we performed gain-of-function experiments. As shown in [figure 2A](#), restoration of miR-212 expression in Jurkat cells resulted in a $\sim 50\%$ decrease in proliferation after culturing for 72 hours ($p = 0.0095$ relative to control). Similarly, KOPTK1 cell proliferation was significantly impaired by ectopic expression of miR-212 ($p = 0.0134$ relative to control; [figure 2B](#)). We extended the observations to in vivo xenograft models. Tumors derived

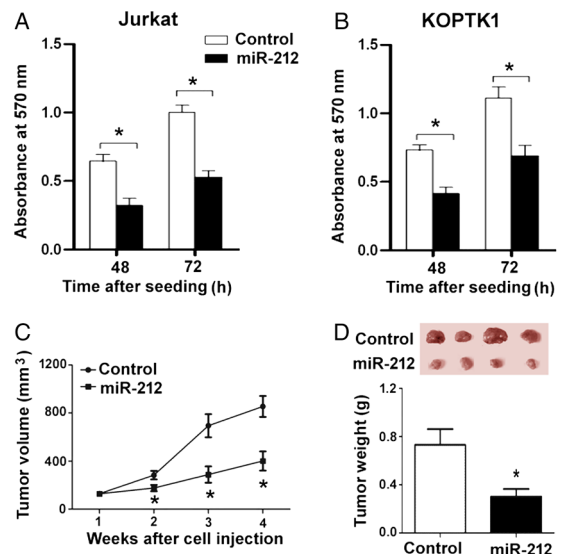


Figure 2 Restoration of miR-212 suppresses ATL cell growth and tumorigenesis. (A) Jurkat and (B) KOPTK1 cells stably expressing miR-212 and negative control miRNA were cultured for 48 or 72 hours and tested for cell viability by the MTS assay. Bar graphs represent the data from three independent experiments. (C and D) miR-212-overexpressed or control Jurkat cells were subcutaneously injected into nude mice and tumor volumes were measured weekly. (C) Tumor growth curves were plotted for each group. (D) Xenograft tumors (top panels) were removed at 4 weeks after cell inoculation and weighed. * $p < 0.05$ vs control group. ATL, adult T-cell leukemia/lymphoma.

from miR-212-overexpressing Jurkat cells grew significantly slower than control counterparts ([figure 2C](#)). At 4 weeks after cell implantation, animals were sacrificed and tumors were weighed. Mean tumor weight was significantly reduced in the miR-212-overexpressing group ($p = 0.0126$; [figure 2D](#)). These data indicate that miR-212 exerts growth suppression in ATL.

miR-212 overexpression causes a G0/G1 cell cycle arrest and apoptotic death

Next, we asked whether the growth-suppressive activity of miR-212 is attributable to induction of cell cycle arrest and/or apoptosis. Flow cytometric analysis demonstrated that delivery of miR-212 mimic significantly raised the percentage of G0/G1-phase cells and reduced the percentage of S-phase cells, indicating a G0/G1 cell cycle arrest (figure 3A). Moreover, enforced expression of miR-212 triggered significant apoptotic response in Jurkat and KOPTK1 cells ($p=0.0004$ and $p=0.0007$, respectively; figure 3B). Direct measurement of caspase-3 activity further demonstrated that there was a significant increase in caspase-3 activity in miR-212-transfected cells (figure 3C).

miR-212 represses the expression of CCND3 by binding to its 3'-UTR

To get more insight into the mechanism for miR-212-mediated growth suppression, we sought to identify its downstream target genes. Bioinformatic analysis suggested that the 3'-UTR of CCND3 gene (which encodes cyclin D3) contained a potential target site for miR-212 (figure 4A). To check if miR-212 can repress CCND3 expression by targeting this putative target site, we performed luciferase reporter assays based on luciferase constructs carrying wild-type or mutant CCND3 3'-UTR.

The results showed that co-transfection with miR-212 mimic significantly inhibited the luciferase activity encoded by wild-type CCND3 3'-UTR reporters ($p=0.0012$; figure 4B). However, the luciferase activity of the mutant CCND3 3'-UTR reporter was not altered by co-transfection with miR-212 mimic. To confirm the regulation of CCND3 expression by miR-212, we transfected miR-212 mimic into lymphoma cells and measured the expression changes of CCND3 protein. The amount of cyclin D3 protein was significantly lower in miR-212-overexpressing Jurkat and KOPTK1 cells than in corresponding control cells ($p=0.0015$ and $p=0.0029$, respectively; figure 4C). Taken together, miR-212 negatively regulates the expression of CCND3 in ATL cells by binding to the 3'-UTR of CCND3 mRNA.

Ectopic expression of CCND3 reverses miR-212-mediated growth suppression

To address whether miR-212-mediated growth suppression in ATL cells can be rescued by ectopic expression of CCND3, we transfected a miR-212-resistant variant of CCND3 into miR-212-overexpressing Jurkat cells and examined cell proliferation, cell cycle progression, and apoptosis. Western blot analysis showed the marked expression of cyclin D3 protein in miR-212-overexpressing Jurkat

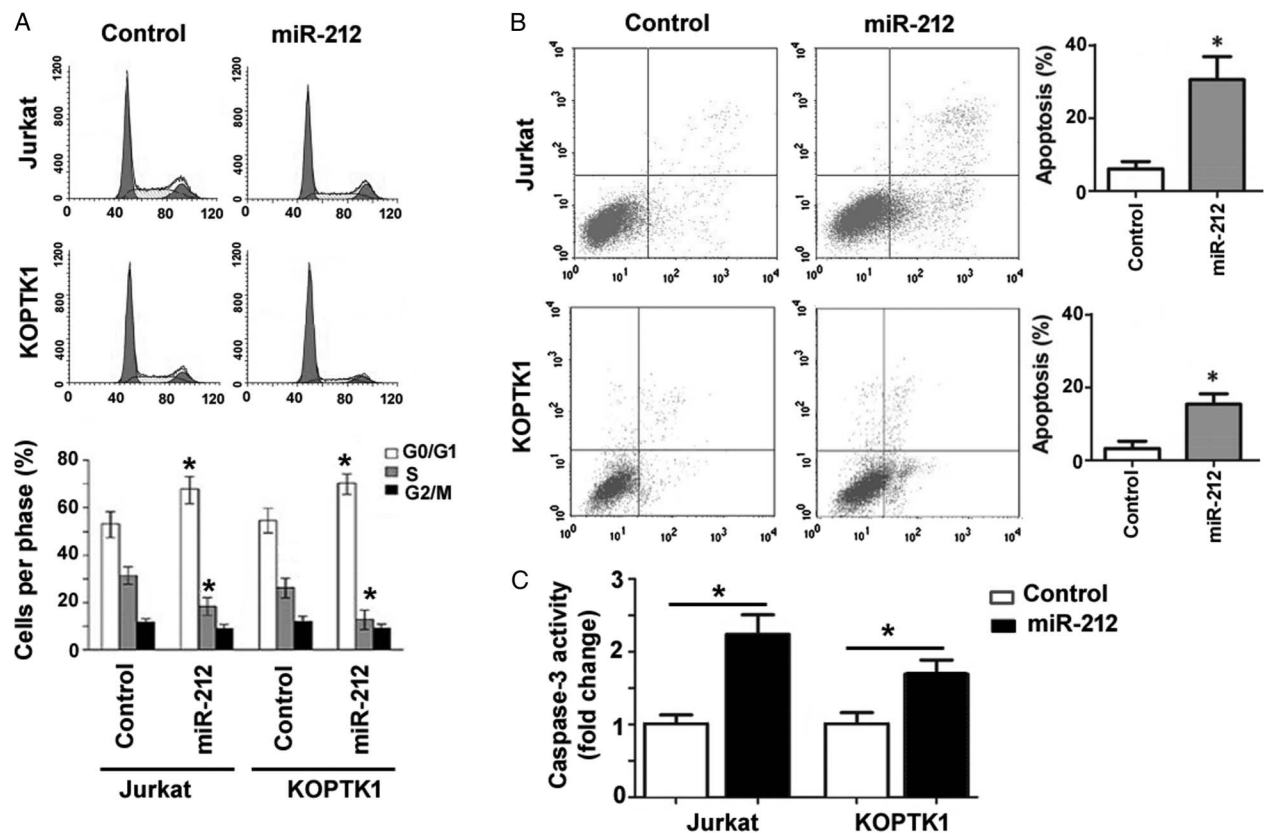


Figure 3 miR-212 overexpression causes a G0/G1 cell cycle arrest and apoptotic death. (A) Flow cytometric analysis of cell cycle distribution in PI-stained Jurkat and KOPTK1 cells. Top, representative flow cytometric histogram. Bottom, quantitative data from three independent experiments. (B) Apoptosis was detected by flow cytometry after Annexin-V/PI staining. Left, representative dot plots. Right, quantitative data from three independent experiments. (C) Measurement of caspase-3 activity. Bar graphs represent the data from three independent experiments. * $p<0.05$ vs control group.

Figure 4 CCND3 is a direct target gene for miR-212. (A) Bioinformatic analysis revealed a potential miR-212 binding site in the 3'-UTR of CCND3. Mutation of the predicted site was achieved as described in 'Materials and methods'. (B) Measurement of luciferase activities in HEK293T cells co-transfected with indicated constructs at 24 hours after transfection. Firefly luciferase activity was normalized to that of *Renilla* luciferase. (C) Western blot analysis of CCND3 protein in Jurkat and KOPTK1 cells transfected with miR-212 mimic or control miRNA. * $p < 0.05$ vs control group.

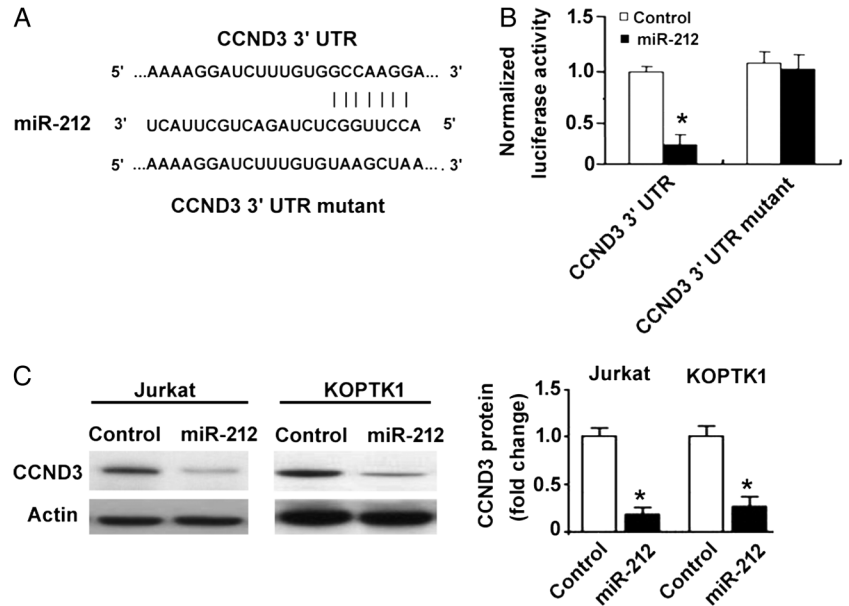
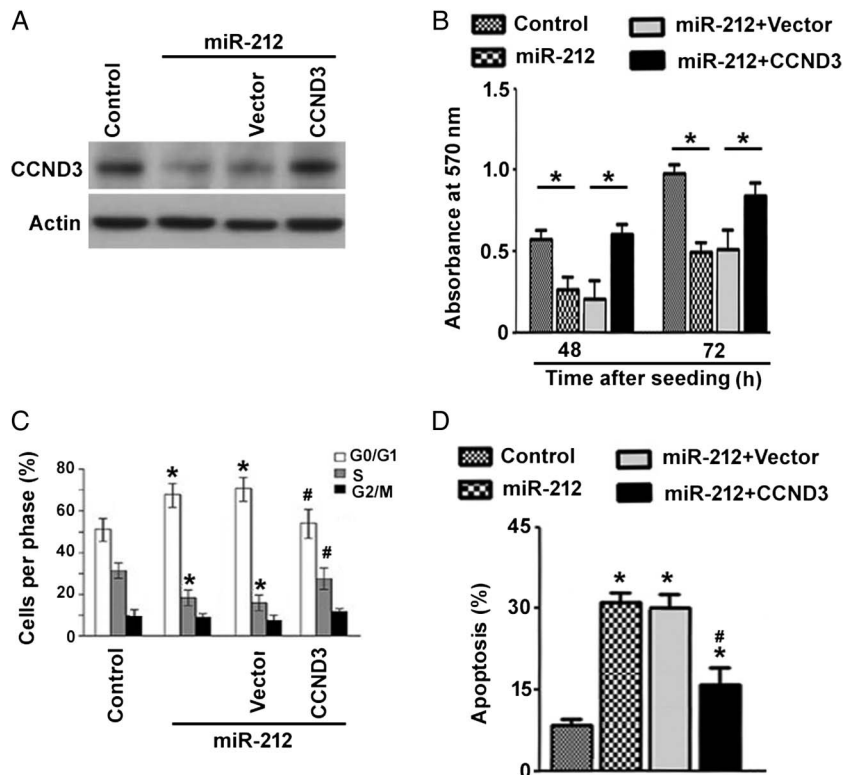


Figure 5 Ectopic expression of CCND3 reverses miR-212-mediated growth suppression. (A) Western blot analysis of CCND3 protein in control and miR-212-overexpressing Jurkat cells transfected with a CCND3-expressing plasmid or vector. (B) Control and Jurkat cells transfected with indicated constructs were cultured for 48 or 72 hours and the viability was measured by the MTS assay. * $p < 0.05$. (C) Flow cytometric analysis of cell cycle distribution in control and Jurkat cells transfected with indicated constructs. (D) Apoptosis assay using Annexin-V/PI staining. Bar graphs represent the results from three independent experiments. * $p < 0.05$ vs control group; # $p < 0.05$ vs vector-transfected cells.



cells after transfection with CCND3-expressing plasmid (figure 5A). Of note, miR-212-mediated inhibition of cell proliferation was significantly reversed by enforced expression of CCND3 (figure 5B). Flow cytometric analysis further demonstrated that overexpression of CCND3 completely restored cell-cycle progression (figure 5C) and prevented apoptotic death (figure 5D) in miR-212-overexpressing Jurkat cells. These results suggest that downregulation of CCND3 is causally linked to growth suppression induced by miR-212 in ATL cells.

DISCUSSION

miR-212 acts as a tumor suppressor in many cancers.^{5 6 8} For instance, overexpression of miR-212 has been found to inhibit cell proliferation and cause apoptotic death in hepatocellular carcinoma cells.⁸ This work revealed that miR-212 was underexpressed in human ATL tissues and cell lines, suggesting its potential role in governing the development of ATL. Gain-of-function experiments confirmed that ectopic expression of miR-212 significantly impaired ATL cell proliferation in vitro and tumorigenesis

in vivo. The growth-suppressive activity of miR-212 in ATL cells provides an explanation for its underexpression in this disease. Hypermethylation of the promoter region of miR-212 has been suggested to be an important mechanism for its downregulation.^{12,13} Loss of heterozygosity also contributes to the reduced expression of miR-212.¹⁴ However, it should be noted that in some types of cancers, miR-212 can be upregulated. For instance, miR-212 upregulation occurs in pancreatic cancer and facilitates cancer cell growth and invasion.^{15,16} Overexpression of miR-212 was found to contribute to more aggressive phenotypes in non-small cell lung cancer cells.¹⁷ Therefore, the functional outcomes of miR-212 expression is dependent on the cellular context.

Several studies have shown that miR-212 is capable of modulating cell cycle progression.^{5,17} miR-212 overexpression was found to lead to a delay in the G1/S phase transition in cervical cancer cells.⁵ Consistently, our data revealed that restoration of miR-212 arrested Jurkat and KOPTK1 cells at the G0/G1 phase, as evidenced by an increased fraction of G0/G1-phase cells and decreased fraction of S-phase cells. Besides inducing cell cycle arrest, ectopic expression of miR-212 was found to trigger apoptosis in the ATL cells. These data provide additional evidence for miR-212-mediated growth suppression in ATL.

Mechanistic studies revealed that miR-212 had the ability to target the 3'-UTR of CCND3 gene and that overexpression of miR-212 led to a significant suppression of CCND3 protein expression. CCND3-encoding protein cyclin D3 is an important regulator in the progression through the G0/G1 phase of cell cycle.¹⁸ Downregulation of cyclin D3 contributes to induction of cell cycle arrest in a variety of cancer cells.^{19,20} CCND3-knockout mouse studies have shown that loss of cyclin D3 suppresses T lymphocyte development and confers resistance to oncogene-induced T-cell transformation.²¹ Similarly, another study reported that ablation of cyclin D3 causes tumor cell apoptosis in mice bearing *Notch1*-driven T-cell lymphoma.²² These studies provide direct evidence that cyclin D3 is required for the development and maintenance of T-cell lymphoma. Therefore, we checked whether downregulation of cyclin D3 is causally linked to miR-212-mediated growth suppression in ATL cells. Interestingly, we noted that overexpression of cyclin D3 significantly promoted cell growth in miR-212-overexpressing ATL cells, which was accompanied by restoration of cell cycle progression and reduction of apoptotic death. Taken together, our data suggest that miR-212 exerts its growth suppression in ATL cells largely by repressing cyclin D3 expression. However, it is also possible that miR-212 may modulate other target genes besides cyclin D3 to suppress ATL cell growth. Indeed, it has been documented that miR-212 targets FOXA1 in hepatocellular carcinoma cells,⁸ SGK3 in glioblastoma cells,⁶ and Sox4 in osteosarcoma cells,²³ leading to reduced cell proliferation.

In conclusion, our data show that miR-212 acts as a tumor suppressor in ATL cells and that the growth-suppressive activity of miR-212 is mediated by downregulation of cyclin D3. Re-expression of miR-212 may have therapeutic potential for ATL.

Contributors QG and PY participated in study design in vitro experiments. HW and GL participated in study design, in vitro experiments, and drafting of the manuscript.

Competing interests None declared.

Patient consent Obtained.

Ethics approval The use of patient samples was approved by the Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China).

Provenance and peer review Not commissioned; externally peer reviewed.

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