microRNA-196a-3p inhibits cell proliferation and promotes cell apoptosis by targeting ADP ribosylation factor 4 in diffuse large B-cell lymphoma

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most prevalent type of non-Hodgkin's lymphoma with a heterogeneous molecular pathogenesis and aggressive clinical manifestations. The aim of the present study was to investigate the role of miR-196a-3p and its target gene in the development and progression of DLBCL. RT-qPCR was used to detect the miR-196a-3p expression level in human DLBCL cell lines and DLBCL pathological tissues and compare them with the normal control. The clinical significance of the miR-196a-3p expression was also analyzed in DLBCL patients. Next, the effect of miR-196a-3p overexpression on the cell cycle, apoptosis, and proliferation of DLBCL cells was evaluated. To explore its underlying mechanism, the target gene of miR-196a-3p was predicted and validated using bioinformatics and molecular biological approaches. Finally, the expression of this target gene in clinical specimens and its correlation with clinicopathological characteristics were determined. The decreased expression of miR-196a-3p was validated in DLBCL, with further analysis proving that it was correlated with poor prognosis. It was shown that the overexpression of miR-196a-3p was associated with cell cycle arrest, enhanced apoptosis, and inhibited proliferation in DLBCL cells. Furthermore, ADP ribosylation factor 4 (ARF4) was verified as the downstream target gene of miR-196a-3p. Similar to miR-196a-3p restoration *in vitro*, endogenous ARF4-knockdown was proven to inhibit cell proliferation through cell cycle arrest and elevate apoptosis in DLBCL. The present results indicated that miR-196a-3p downregulation contributed to the tumorigenesis of DLBCL by targeting ARF4 expression, which may be used as a novel prognostic marker or potential molecular therapeutic target for DLBCL management in the future.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most prevalent clinicopathological subtype of lymphoma in adults, accounting for 30-40% of non-Hodgkin's lymphoma (1). Standard immunotherapy and chemotherapy treatment regimens, such as R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone) have led to significant improvements in the clinical outcome of DLBCL, with the 5-year overall survival (OS) rate of DLBCL in the USA reaching 60% (2,3). However, 1/3 of DLBCL patients still experience primary refractoriness or relapse after achieving complete remission, comprising a major challenge in the mortality and morbidity of DLBCL. DLBCL is generally considered a heterogeneous entity in terms of genetics, morphology and biological behavior. According to gene expression profiling, DLBCL can further be classified into three subtypes, germinal center B cell-like (GCB), activated B cell-like (ABC) and an unclassified subtype; however, the mechanism underlying the pathogenesis of DLBCL remains largely elusive (4).

MicroRNAs (miRNAs) are a class of short, single-stranded, non-coding RNAs usually 18-22 nucleotides in length, which regulate target genes post-transcriptionally and result

in either direct mRNA degradation or protein translation inhibition. Compelling evidence has demonstrated that aberrant miRNA expression plays an essential role in the pathogenesis of hematological malignancies, including lymphoma (5,6). Costinean *et al* generated Eμ-miR155 transgenic mice overexpressing miR-155, which were shown to develop a lymphoproliferative disease resembling high-grade lymphoma (7). A study by Mihailovich *et al* found that the miR-17-92 cluster regulates c-myc to ensure optimal B-cell lymphoma growth in an established lymphoma model (8). Several miRNAs have been reported to discriminate between DLBCL subtypes and be correlated with chemosensitivity, while some miRNAs can predict the outcomes of DLBCL patients treated with the R-CHOP regime (9,10). Given the pivotal role of aberrant miRNA expression in the pathogenesis of DLBCL, the correction of abnormal miRNA expression has emerged as a promising therapeutic strategy (11).

Transforming growth factor β1 (TGF-β1) is a versatile cytokine that plays an important role in the pathogenesis of cancer as a tumor suppressor or promoter, depending on the context. Findings of previous studies have shown that TGF-β1 is involved in the development and progression of malignant tumors by regulating the expression of various miRNAs (12,13). From a miRNA microarray profile following the knockdown of TGF-β1 in colorectal cancer cell lines, our group identified a set of TGF-β1-associated differentially expressed miRNAs. RT-qPCR was used to validate the differential expression of these miRNAs in DLBCL cells, and the miR-196a-3p expression was found to decrease in two DLBCL cell lines. The influence of overexpressed miR-196a-3p on the survival of DLBCL cells was then evaluated, and it was experimentally validated that miR-196a-3p directly inhibited the translation of ADP ribosylation factor 4 (ARF4) *in vitro*. The present findings suggested that miR-196a-3p was involved in the tumorigenesis of DLBCL through the downregulation of ARF4.

Materials and methods

Patients and human tissue samples. A total of 68 patients with a confirmed diagnosis of *de novo* DLBCL by biopsy in the First Affiliated Hospital of Soochow University (Suzhou, China) were enrolled in the present study. All 68 DLBCL patients had available formalin-fixed paraffin-embedded (FFPE) tissues (3 μm in thickness) for RNA extraction and had not received radiotherapy or chemotherapy. Patients with DLBCL secondary to other B-cell hematological malignancies, primary CNS B-cell lymphoma and mediastinal large B-cell lymphoma were excluded from the present study. Ten reactive lymph node hyperplasia (RLH) specimens from patients in the First Affiliated Hospital of Soochow University were included as the normal control. According to the Hans algorithm, all DLBCL cases were classified into GCB and non-GCB subtypes, based on immunohistochemical (IHC) markers.

All the patients provided written informed consent and were followed in the outpatient clinic or by telephone every 3 months. This study was approved by the Institutional Review Board of the First Affiliated Hospital of Soochow University.

Cell lines and cell culture. The human DLBCL cell lines used in this study included Farage and OCI-LY3, and were obtained from the American Type Culture Collection. Farage and OCI-LY3 cells were cultured in RPMI-1640 medium (GE Healthcare Life Sciences) in a humidified atmosphere with 5% CO_2 at 37˚C. The culture media was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Following ethics approval from the Institutional Review Board, normal B lymphocytes were isolated from the peripheral blood of 6 healthy volunteers who signed written informed consent (age range 24-39 years, 3 males and 3 females) using the Human B cell Enrichment kit (10954; Stemcell Technologies, Inc.). Volunteers who had a positive pregnancy test or signs of active infection were excluded from the present study.

RNA extraction from cell lines or FFPE tissues, and RT-qPCR. Total RNA was extracted from DLBCL cells using TRIzol reagent (204205; Thermo Fisher Scientific, Inc.). A MagMax™ FFPE DNA/RNA Ultra kit (A31881; Thermo Fisher Scientific, Inc.) was used to extract total RNA from FFPE tissue samples, according to the manufacturer's instructions. After measuring the concentration with a NanoDrop 2000 spectrophotometer (NP80; IMPLEN), all RNA was reverse-transcribed and then stored at -80˚C until the time of assay.

RT-qPCR was used to detect the miRNA expression using the stem-loop method. The cDNA was used as the template for the amplification of the RNA, and RT-qPCR was performed on reverse-transcribed miRNAs and mRNA using Power SYBR™ Green PCR Master Mix (4367659; Thermo Fisher Scientific, Inc.) for quantification following amplification. GAPDH and small nuclear RNA U6 were used as internal controls for mRNA and miRNA RT-qPCR detection, respectively. RT-qPCR was carried out using a previously described procedure (14). The primer sequences of miRNA and candidate target genes were listed in Tables SI and SII. RT-qPCR conditions for miRNA listed as below: Activation 10 min at 95˚C; denaturation for 15 sec at 95˚C; extension 60 sec at 60˚C. RT-qPCR thermocycling conditions for mRNA: Activation 10 min at 95˚C; denaturation 15 sec at 95˚C; extension 60 sec at 60˚C. Relative expression changes were calculated by the $2^{-\Delta\Delta Cq}$ method (14).

Oligonucleotide synthesis and transfection. miR-196a-3p mimic (mimic sequence, 5'-CGGCAACAAGAAACUGCC UGAG-3') and control scrambled oligonucleotide (scrambled sequence, 5'-UUUGUACUACACAAAGUACUG-3') were chemically synthesized by Guangzhou RiboBio Co., Ltd. ARF4 gene-specific small interfering RNA (siRNA) was designed using siCATCH™ siRNA Designer (Guangzhou RiboBio Co., Ltd.). Following confirmation by RT-qPCR (data not shown), optimal ARF4 siRNA (5'-AGACAACCA TTCTGTATAA-3') and scrambled siRNA (5'-UUUTGATCA UTGATGAAA-3') sequences were selected for ARF4 RNA interference and synthesized by Guangzhou RiboBio Co., Ltd (Fig. S3). For miR-196a-3p mimic or ARF4 siRNA transfection, 5 μl Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Inc.) in 200 μl serum-free medium was mixed with 100 pmol oligonucleotides dissolved in 200 μl Opti-MEM

(Thermo Fisher Scientific, Inc.) Scrambled oligonucleotides were transfected to the negative control, and Lipofectamine 3000 without oligonucleotides was added to the mock control.

Cell counting kit-8 (CCK-8) cell proliferation assay. Cell proliferation was analyzed using a CCK-8 assay kit (Guangzhou RiboBio Co., Ltd.). Farage and OCI-LY3 cells ($2x10^4$ cells/ml) were seeded in a 96-well cell culture plate in a volume of 200 μl/well. Prior to detection, 20 μl CCK-8 solution was added to each well and incubated for 4 h in a 37˚C incubator. Cell proliferation was measured at 24, 48 and 72 h after transfection, and 5 replicate test wells were used. The optical density value at an absorbance of 450 nm was determined using a microplate reader (Thermo Fisher Scientific, Inc.).

Flow cytometry for the detection of cell cycle, viability and apoptosis. Farage and OCI-LY3 cells were treated with the same transfection conditions described above. EdU (5-ethynyl-2'-deoxyuridine) staining assays (C10310-1; Guangzhou RiboBio Co., Ltd.) were performed to investigate the influence of transfection on DLBCL cell viability. Cells were stained with EdU reagent 24 h post-transfection and analyzed by flow cytometry. Propidium iodide (PI) staining and flow cytometry were used to analyze the cell cycle. For cell apoptosis analysis by cytometry, the cells were stained with Annexin V and PI for 10 min at room temperature in the dark using the Annexin V-FITC Apoptosis Detection Kit (C1062M; Beyotime Biotechnology Co., Ltd.). All the reactions were analyzed in triplicate by flow cytometry (BD FACSCalibur; BD Biosciences).

Bioinformatics search. Potential miR-196a-3p target genes were predicted through algorithms from four canonical online databases: Targetscan (http://www.targetscan.org), miRDB (http://mirdb.org), microRNA (http://www.microrna.org/microrna) and DIANA-microT (http://diana.imis.athena-innovation.gr/DianaTools/). Overlapping results underwent further functional analysis using the miR-ontology database to screen candidate target genes.

Western blot analysis. Following transfection, DLBCL cells were collected and lysed in RIPA buffer (b100020; ProteinTech Group, Inc.) and total protein was extracted using bicinchoninic acid (BCA) method. Equal aliquots of extracts were electrophoresed on SDS-polyacrylamide gels and electrotransferred onto PVDF membranes. In this experiment, 25 μg proteins were loaded per lane and 8-12% gels were used. Membranes were blocked with TBS containing 5% non-fat dry milk for at least 1 h at room temperature The blots were then incubated with one of the following primary antibodies, against ARF4 (11673-1-AP; dilution 1:500; ProteinTech Group, Inc.), ZNF280 (dilution 1:500; DF9950, Affinity Bioscience), coronin-1C (CORO1C) (14749-1-AP; dilution 1:500; ProteinTech Group, Inc.) or neuropilin-2 (NRP2) (dilution 1:500; DF7032, Affinity Bioscience). The blots were incubated with a peroxidase-conjugated secondary antibody (BA1056; dilution 1:2,000; Boster Biological Technology) at room temperature for 1 h and visualized using Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100; EMD Millipore). β-actin was detected at the same time as the control, and the protein expression was quantified using SkanIt™ Software (Thermo Fisher Scientific).

Dual-luciferase reporter assays. The 3'-untranslated region (3'-UTR) sequence that contained either a wild-type or mutant version of the ARF4 binding sequence was inserted into a psi-Check2 luciferase reporter plasmid (Promega Corporation), and the procedure was performed according to the manufacturer's instructions. 293T cells were seeded in 24-well plates ($3.0x10^5$ cells/well) and the cells were transiently transfected by Lipofectamine 3000 with pGL3-X-baI (Promega Corporation) and 100 ng/well psi-Check2 luciferase reporter (Promega Corporation), and co-transfected with the miR-196a-3p mimic or scrambled sequence. *Renilla* luciferase activity was detected using the Luciferase Reporter System (Promega Corporation) 48 h after transfection.

Immunostaining. The FFPE tissues of DLBCL and RLH were sectioned for immunohistochemistry to determine the protein expression of the 4 candidate target genes. Following endogenous peroxidase blocking and immunostaining with antibodies, the sections were incubated with the following biotinylated anti-rabbit primary antibodies: ARF4, ZNF280B, CORO1C or NRP2 (dilution 1:200; ProteinTech Group, Inc.). DAB color-substrate solution was added following washing and the slides were counterstained with hematoxylin. The total immunoreactive score (IRS) algorithm was used to score the antibody expression by immunohistochemistry (IHC) as follows: 0, no positive cells; 1, ≤25% positive cells; 2, >25 and ≤50% positive cells; 3, >50% positive cells. Staining intensity was categorized as follows: 0, negative; 1, weak; 2, moderate; 3, strong. IRS values of 0-2 and 3-6 were defined as a low and high expression of ARF4 proteins, respectively. Slides were viewed using a Nikon Eclipse E100 microscope (Nikon Corporation) at a magnification of x100 and x400. The immunostaining was reviewed by two independent pathologists.

Statistical analysis. All data are presented as the mean ± SD. The statistical software SPSS version 21.0 for Windows (IBM Corp) was used for data analysis. Independent sample t-tests, ANOVA with Tukey's post hoc test and chi-square tests were used to compare the results between different groups. Survival curves were plotted using the Kaplan-Meier method, and compared using the log-rank test. Results are the mean ± SD of three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Decreased miR-196a-3p expression is associated with poor survival in DLBCL. Previous findings identified the following 5 differentially expressed miRNAs by knocking down TGF-β1 expression in colorectal cancer cell lines: miRNA-3691-3p, miR-4313, miR-196a-3p, miR-411-3p and miR-324-3p (GSE53338) (15). To investigate whether the 5 miRNAs were also differentially expressed between DLBCL cell lines and normal B cells, RT-qPCR was performed to compare the expression level of these miRNAs in DLBCL cell lines and normal

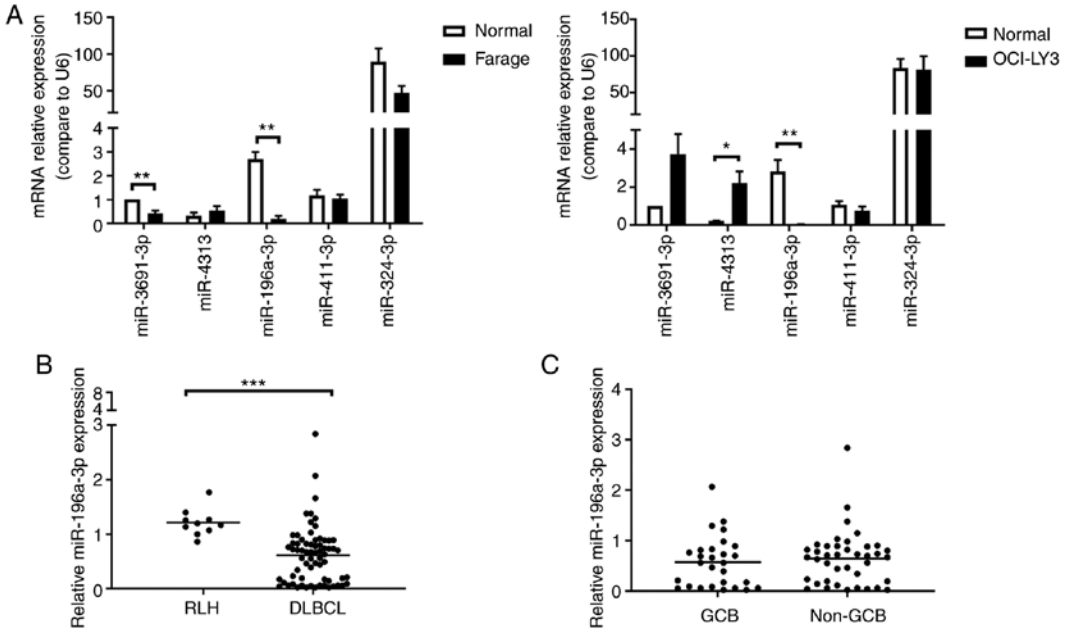

Figure 1. The miR-196a-3p expression is reduced in DLBCL cell lines and clinical specimens. (A) The relative expression levels of different miRNAs in Farage and OCI-LY3 cell lines were compared with those of normal CD19$^+$ B cells by RT-qPCR. (B) The expression levels of miR-196a-3p in 10 RLH and 68 DLBCL tissues were determined by RT-qPCR. (C) The expression levels of miR-196a-3p were compared between the GCB and non-GCB groups. *P<0.05, **P<0.01 and ***P<0.001. DLBCL, diffuse large B-cell lymphoma; RLH, reactive lymph node hyperplasia; GCB, germinal center B cell-like.

B cells. Two DLBCL cell lines were analyzed in these experiments: Farage (GCB-like DLBCL) and OCI-LY3 (ABC-like DLBCL), as described in the Materials and methods section. Normal CD19$^+$ B cells were isolated from healthy individuals, with their consent, by magnetic-activated cell sorting (Fig. S1). Among the 5 miRNAs, only the expression of miR-196a-3p significantly decreased in both Farage and OCI-LY3 cells, as compared with normal CD19$^+$ B cells (Fig. 1A).

To further determine whether miR-196a-3p was also differentially expressed in clinical DLBCL specimens, the expression of miR-196a-3p in 68 DLBCL FFPE samples was analyzed by RT-qPCR. A total of 10 RLH tissues were analyzed as the control. It was found that the miR-196a-3p expression was reduced by 50% in DLBCL, as compared with RLH (P<0.001; Fig. 1B). However, there was no difference in miR-196a-3p expression between the GCB and non-GCB DLBCL groups (Fig. 1C).

To investigate the clinical relevance of the miR-196a-3p expression in DLBCL, 68 DLBCL patients were divided into two groups according to the median level of the miR-196a-3p expression (median=0.66-fold change). Table I summarizes the association between the miR-196a-3p expression and clinicopathological variables of these 68 DLBCL patients. Statistical analysis revealed that a low miR-196a-3p expression level was correlated with Ann Arbor stage (P=0.001), international prognosis index (P=0.049), bone marrow involvement (P=0.017) and number of extranodal sites (P=0.027). However, no correlations were identified between the miR-196a-3p level and age, sex, B symptom, primary site, Eastern Cooperative Oncology Group (ECOG) score, lactate dehydrogenase (LDH) level and Hans classification. The Kaplan-Meier method and log-rank test were then used to compare survival between the two groups with a different miR-196a-3p expression (Fig. 2). The low miR-196a-3p expression group had a significantly shorter OS and disease-free survival (DFS) than did the high miR-196a-3p expression group (P<0.01 and P<0.001, respectively).

Ectopic overexpression of miR-196a-3p induces cell cycle arrest and apoptosis and reduces cell proliferation in DLBCL. In the previous experiment, a notably decreased expression of miR-196a-3p was confirmed in both DLBCL cell lines and pathological specimens, as compared with normal controls. To further evaluate the biological role of miR-196a-3p in the development and progression of DLBCL, and whether miR-196a-3p-overexpression influenced DLBCL cell cycle, apoptosis and proliferation were next investigated. Synthesized miR-196a-3p mimic and scrambled mimic oligonucleotide as negative controls were transiently transfected into Farage and OCI-LY3 cells, and the transfection efficiency of the miR-196a-3p mimic was validated by RT-qPCR (data not shown, Fig. S2). Next, multiple functional assays, including cell cycle analysis, EdU staining, cell apoptosis and CCK-8 proliferation assays were performed to evaluate cell survival dysfunction of DLBCL *in vitro*. As shown in Fig. 3A, cell cycle analysis revealed a reduced percentage of S-phase cells in Farage and OCI-LY3 cells following miR-196a-3p mimic transfection (P<0.05 and P<0.01, respectively), and G1-phase cells increased in OCI-LY3 cells (P<0.01). The EdU-positive percentage of Farage and OCI-LY3 cells in the mimic-transfected group was significantly lower than that of the control group (Fig. 3B). In the apoptosis assays, the overexpression of miR-196a-3p led to a markedly increased cell apoptosis by 14.3-fold in Farage cells and 5.6-fold in OCI-LY3 cells (P<0.001; Fig. 3C). Finally, a CCK-8 assay was performed to evaluate the collective effect of miR-196a-3p on the growth and survival of DLBCL cells. Proliferation rates 48 and 72 h after mimic transfection of both Farage and OCI-LY3 cells

Table I. Correlation of expression of miR-196a-3p with clinicopathologic features of DLBCL patients.

	miR-196a-3p expression		
Clinicopathological parameter	High (34)	Low (34)	P-value
Age (years)			
<60	25	21	0.300
≥60	9	13	
Sex			
Male	19	17	0.627
Female	15	17	
Ann Arbor stage			
I and II	18	5	0.001[b]
III and IV	16	29	
B symptom			
No	23	20	0.451
Yes	11	14	
Primary site			
Nodal	20	21	0.804
Extranodal	14	13	
Performance status (ECOG)			
0-1	27	22	0.177
≥2	7	12	
LDH			
Normal	23	19	0.318
Elevated	11	15	
BM involvement			
Absent	31	22	0.017[a]
Present	3	12	
IPI group			
Low (0-2)	24	16	0.049[a]
High (3-5)	10	18	
Hans classification			
GCB	11	17	0.139
Non-GCB	23	17	
No. of extranodal sites			
0-1	19	10	0.027[a]
≥2	15	24	

[a]P<0.05, [b]P<0.01. BM, bone marrow; IPI, International Prognosis Index.

decreased significantly, as compared with the negative control group (P<0.01). Furthermore, the 72-h proliferation rate was reduced to 56.5 and 71.5% in Farage and OCI-LY3 cells, respectively (P<0.001; Fig. 3D).

ARF4 is the downstream target gene of miR-196a-3p in DLBCL. To investigate the molecular mechanisms underlying the influence of miR-196a-3p on the DLBCL cell phenotype, a bioinformatics approach was used to explore the putative targets of miR-196a-3p. From online prediction algorithms of TargetScan, miRDB, microRNA and DIANA-microT, 8 candidate genes were identified based on intersecting search results from these 4 bioinformatics databases. After a functional analysis by the miR-Ontology database, ARF4, Zinc finger protein (ZNF280B), CORO1C and NRP2 were identified as 4 potential target genes (Fig. 4A).

Our next aim was to further confirm the effect of miRNA-196a-3 on these targets in Farage and OCI-LY3 cells. The protein expression levels of the 4 target genes in both DLBCL cell lines were examined by western blot analysis following miR-196a-3p mimic transfection, as compared with the negative control. Significantly decreased protein expression levels of AFR4 and ZNF280B were observed in mimic-transfected Farage and OCI-LY3 cells,

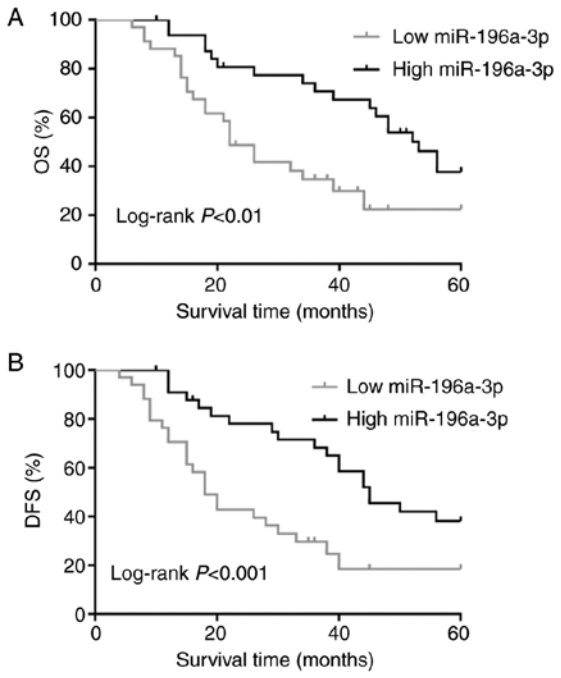

Figure 2. The reduced miR-196a-3p level is correlated with poor clinical outcome in DLBCL patients. (A) OS and (B) DFS were compared using Kaplan-Meier survival curves for high and low miR-196a-3p expression groups. OS, overall survival; DFS, disease-free survival; DLBCL, diffuse large B-cell lymphoma.

as compared with negative controls (Fig. 4B). RT-qPCR was used to detect the level of target gene mRNA, which revealed that only the ARF4 mRNA level was significantly decreased in both DLBCL cell lines, as compared with control cells (Fig. 4C). Based on the above results, ARF4 was selected as our target gene for further experimental verification.

Dual-luciferase gene reporter assays were then used to determine whether miR-196a-3p regulated ARF4 expression directly through specific base pairing *in vitro*. ARF4 3'-UTR harboring the potential miR-196a-3p-binding site or a mutant sequence were cloned and inserted into a luciferase reporter vector. The relative luciferase activity in cells containing the wild-type ARF4 3'-UTR was reduced by 35.3%, as compared with the negative control, while no change was observed in cells containing the mutated reporters (Fig. 4D). These findings suggested that miR-196a-3p directly binds to the 3'-UTR of ARF4 mRNA to suppress ARF4 protein translation.

ARF4 induces cell proliferation and inhibits apoptosis in DLBCL. To explore whether the downregulation of ARF4 produced a similar effect to that of the miR-196a-3p mimic on the phenotype of DLBCL cells, siRNA technology was used to silence the expression of ARF4 *in vitro*. Following ARF4 siRNA transfection, functional assays evaluating DLBCL cell growth and survival were performed individually.

Transfection of Farage and OCI-LY3 cells with ARF4 siRNA resulted in an increased accumulation of G1-phase cells, as compared with control transfections (P<0.01), while no difference in the number of S-phase cells was observed between the two groups (Fig. 5A). EdU staining and cytometric analysis indicated that the percentage of EdU-positive cells significantly decreased, as compared with the control (Fig. 5B). Similarly, by annexin-PI double staining cytometric analysis, ARF4 siRNA transfection induced an increase in the percentage of apoptosis in both Farage and OCI-LY3 cells, as compared with nonsense control transfection (P<0.01 and P<0.05, respectively; Fig. 5C). As determined by the CCK-8 assay, the two DLBCL cell lines exhibited a significantly reduced cell proliferation 24, 48 and 72 h after ARF4 siRNA inhibition (Fig. 5D). The 72-h proliferation inhibition percentage following ARF4 knockdown was 62.8 and 56.7% in Farage and OCI-LY3 cells, respectively (P<0.001). From these results, it was concluded that ARF4 contributes to cell growth and survival of DLBCL as an oncogene, which can be downregulated by miR-196a-3p.

Upregulation of ARF4 is correlated with clinicopathologic variables in DLBCL. By gain and loss of function tests and luciferase reporter assays *in vitro*, it was validated that ARF4 contributes to DLBCL progression under the direct regulation of miR-196a-3p. The protein expression of the 4 candidate target genes was measured in 32 DLBCL FFPE tissues by immunohistochemical staining, as compared with 10 RLH FFPE tissues (Fig. 6A). The IRS score was used to compare IHC results of target proteins. Compared with RLH, the ARF4 protein expression levels were significantly higher in the DLBCL tissues (P<0.001), while the ZNF280B protein expression was slightly higher (P<0.05; Fig. 6B). Furthermore, the high ARF4 proteinexpression group had a lower miR-196a-3p expression level than the low ARF4 expression group in the DLBCL specimens. (P<0.01; Fig. 6C).

To further elucidate the clinical significance of ARF4 expression, the association between the ARF4 protein expression level and clinicopathologic variables in 68 DLBCL patients was analyzed. The ARF4 IHC staining was performed on the FFPE slides of the DLBCL patients, and all patients were divided into two groups, according to the ARF4 expression level determined by the IRS score (≥3 and <3). High ARF4 expression levels were significantly associated with Ann Arbor stage (P=0.040), LDH (P=0.022) and the number of extranodal sites (P=0.013). However, there was no correlation between the ARF4 level and age, sex, B symptom, primary site, ECOG score, IPI, bone marrow involvement and Hans classification (Table II).

Discussion

The miRNA-196 family, located in the homeobox gene clusters, consisted of 3 miR-196 genes: miR-196a-1, miR-196a-2 and miR-196b. It has been extensively reported that miR-196 participates in various fundamental biological processes including development, inflammation, immunity, and cancer pathogenesis (16,17). Various miRNA profiling studies reported overexpressed miR-196 levels in malignant tumors (18-20). miRNA-196a-5p levels were found to be inversely correlated with survival in pancreatic duct adenocarcinoma patients (21). Lu *et al* found that miR-196 mediated an invasive phenotype in oral cancer through the JNK signaling pathway (22). One recent study from our group validated the decreased expression of miR-196a-3p in breast cancer, and the downregulation of miR-196a-3p was

Table II. Association of expression of ARF4 IRS with clinicopathological characteristics of DLBCL patients.

Parameter	High ARF4 (IRS ≥3) 43 patients	Low ARF4 (IRS <3) 25 patients	P-value
Age (years)			
<60	28	18	0.559
≥60	15	7	
Sex			
Male	22	14	0.700
Female	21	11	
Ann Arbor stage			
I and II	20	3	0.04[a]
III and IV	23	22	
B symptom			
No	30	13	0.143
Yes	13	12	
Primary site			
Nodal	29	21	0.804
Extranodal	14	14	
Performance status (ECOG)			
0-1	29	12	0.114
≥2	14	13	
LDH			
Normal	31	11	0.022[a]
Elevated	12	14	
BM involvement			
Absent	37	16	0.066
Present	6	9	
IPI group			
Low (0-2)	31	10	0.09
High (3-5)	12	15	
Hans classification			
GCB	16	12	0.383
Non-GCB	27	13	
No. of extranodal sites			
0-1	6	24	0.013[a]
≥2	19	19	

[a]P<0.05. BM, bone marrow; IPI, International Prognosis Index.

found to promote the metastasis of breast cancer by targeting NRP2 (23). Several miRNAs are involved in the pathogenesis of DLBCL by regulating downstream target genes. However, the biological role of miR-196, particularly miR-196a-3p, in the development and progression of DLBCL remains to be elucidated.

As in the case of other malignant tumors, the dysfunction of cell survival is a hallmark of DLBCL pathogenesis. Several mechanisms have been attributed to survival dysfunction, including intrinsic signaling aberrations, tumor environmental dysfunction and viral factors (24). The dysfunction of multiple cellular pathways, including the BCR signaling, BCL2 and p53 pathways, was found to contribute to aberrant DLBCL cell survival (25). Various novel drugs targeting abnormal cell survival pathways have been developed for DLBCL. The BTK inhibitor ibrutinib, a novel drug targeting the BCR pathway, has shown promising efficiency in relapsed and refractory DLBCL patients (25). In recent years, several miRNAs have been reported to play essential roles in cell growth and DLBCL progression. A report by Zhu *et al* found that the downregulation of miR-155 inhibited DLBCL cell growth by inducing cell cycle arrest and promoting cell apoptosis, and luciferase reporter assays demonstrated where TGFBR2 is a target of miR-155 in lymphoma cell lines (26). Another study by Fan *et al* found that miR-10a can suppress the proliferation and promote the

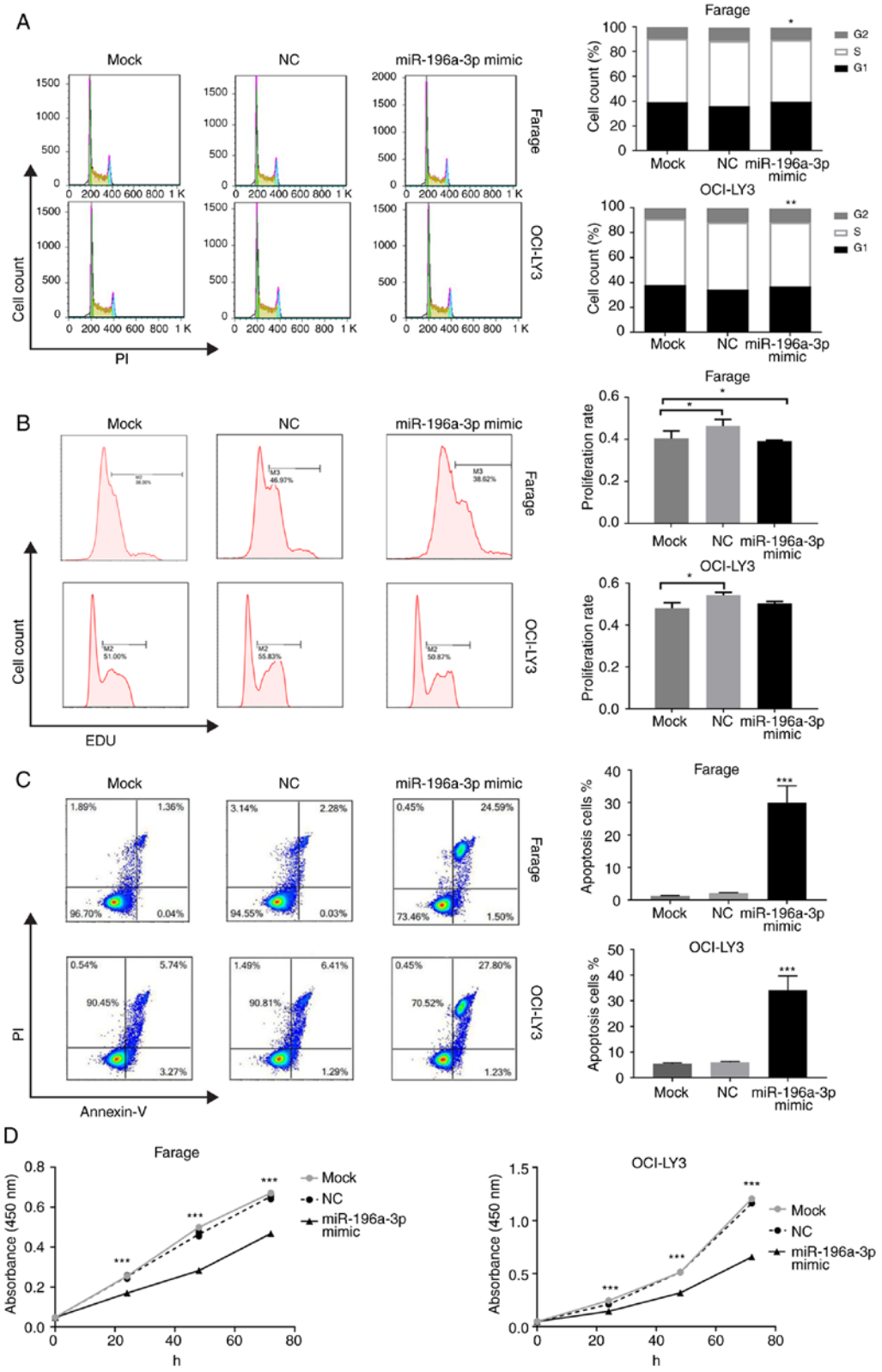

Figure 3. Effect of miR-196a-3p on proliferation and apoptosis in DLBCL cell lines *in vitro*. Farage and OCI-LY3 cells were transfected with miR-196a-3p mimic or a scrambled sequence as the negative control. (A) Cell cycle analysis was performed by PI-staining flow cytometry. (B) Viability of Farage and OCI-LY3 cells was determined by EdU staining. (C) Cell apoptosis was determined by Annexin V and PI staining and flow cytometry. (D) Cell proliferation was analyzed by CCK-8 assay. *P<0.05, **P<0.01 and ***P<0.001. OD, optical density; DLBCL, diffuse large B-cell lymphoma; CCK-8, Cell Counting Kit-8.

apoptosis of DLBCL cells by targeting BCL6 (27). Jia *et al* found that miR-27b suppressed DLBCL cell viability and proliferation by targeting the MET/PI3K/AKT pathway, which is regulated by HDAC6. Moreover, the decreased expression of miR-27b was associated with poor survival in DLBCL (28).

Findings of the present study showed that the expression level of miR-196a-3p was reduced in DLBCL, and

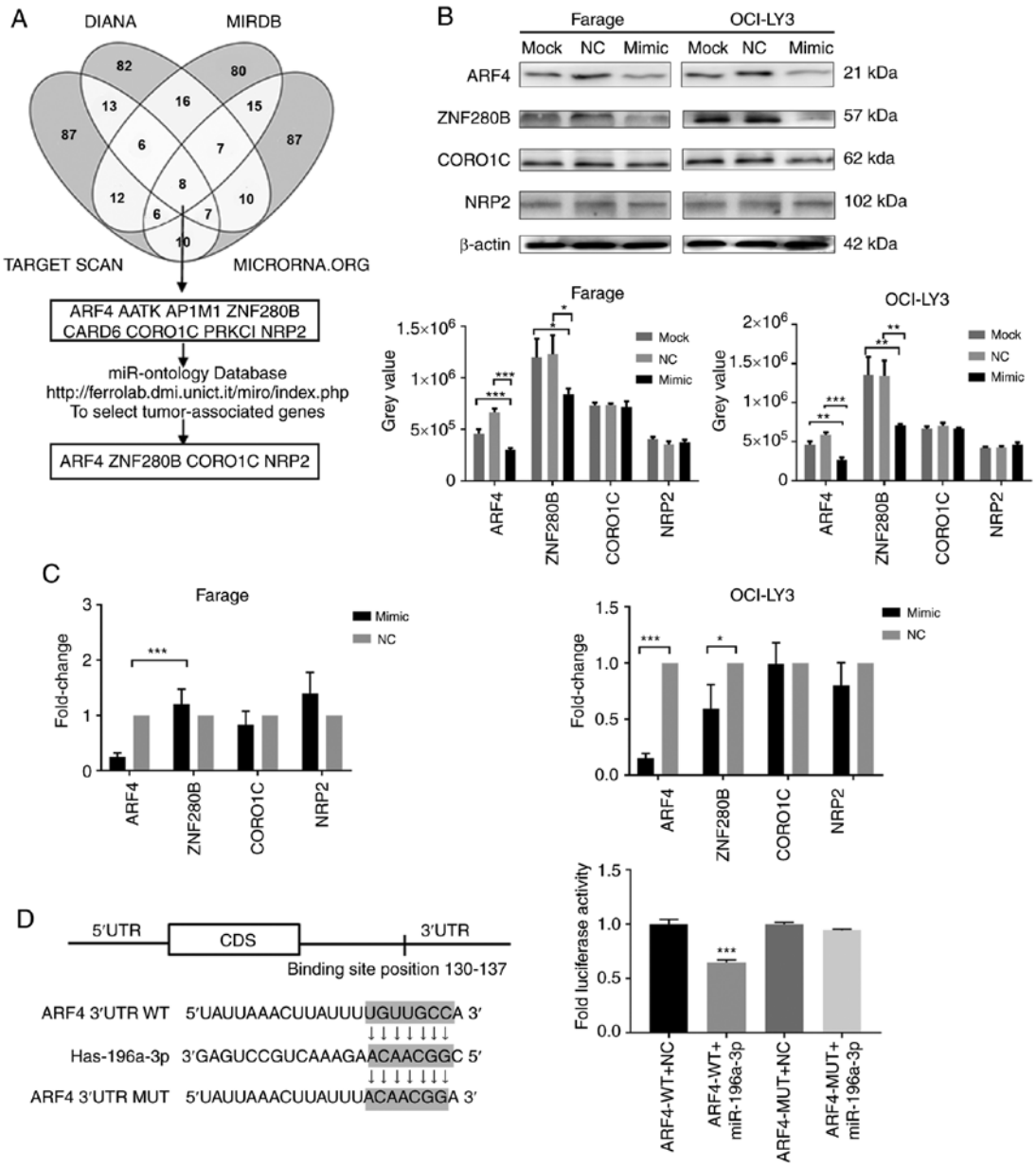

Figure 4. Prediction and identification of miR-196a-3p target genes in DLBCL cells. (A) miRNA targets were predicted using TargetScan, microRNA, miRDB and DIANA-microT. (B) The protein levels of 4 miR-196a-3p candidate target genes were analyzed by western blot analysis. Up, protein bands; down, grey value. (C) mRNA levels of 4 miR-196a-3p candidate target genes were analyzed by RT-qPCR. (D) 293T cells were transiently transfected with *Renilla* luciferase reporter vectors containing either wild-type or mutant ARF4 3'-UTR seed sequence, and luciferase activity was measured following co-transfection with miR-196a-3p or NC. *P<0.05, **P<0.01 and ***P<0.001. DLBCL, diffuse large B-cell lymphoma; miRNA, microRNA; ARF4, ADP ribosylation factor 4; 3'-UTR, 3'-untranslated region; NC, negative control.

that the downregulation of miR-196a-3p plays an important role in cell survival dysfunction in DLBCL. Subsequent survival analysis revealed that the decreased expression of miR-196a-3p was associated with Ann Arbor stage, BM involvement, IPI, number of extranodal sites and poor survival in DLBCL patients. To explore the influence of miR-196a-3p on the DLBCL phenotype and the underlying mechanism, we first transfected miR-196a-3p mimic and target gene siRNA in gain and loss of function tests. Next, the growth and survival of DLBCL cells were evaluated via 4 different functional assays, including cell cycle, EdU staining, cell apoptosis and CCK-8 assays. In addition to testing the effect of miR-196a-3p on target gene expression, ARF4 was verified as the target gene of miR-196a-3p by a bioinformatics approach and luciferase reporter assay. It is well established that one single miRNA can regulate multiple genes and multiple miRNAs can target one gene. miR-196a-3p may be involved in DLBCL tumorigenesis through a network of various target genes. However, as with miR-196a-3p mimic restoration in DLBCL cells, silencing of the ARF4 expression induced cell cycle arrest and apoptosis, and inhibited cell proliferation. Furthermore, using IHC staining analysis, it was confirmed that the ARF4 protein was overexpressed in DLBCL samples and inversely associated with the miR-196a-3p level. These findings consistently suggested that the targeting of ARF4 is an important mechanism through which miR-196a-3p plays its tumor-suppressor role in DLBCL.

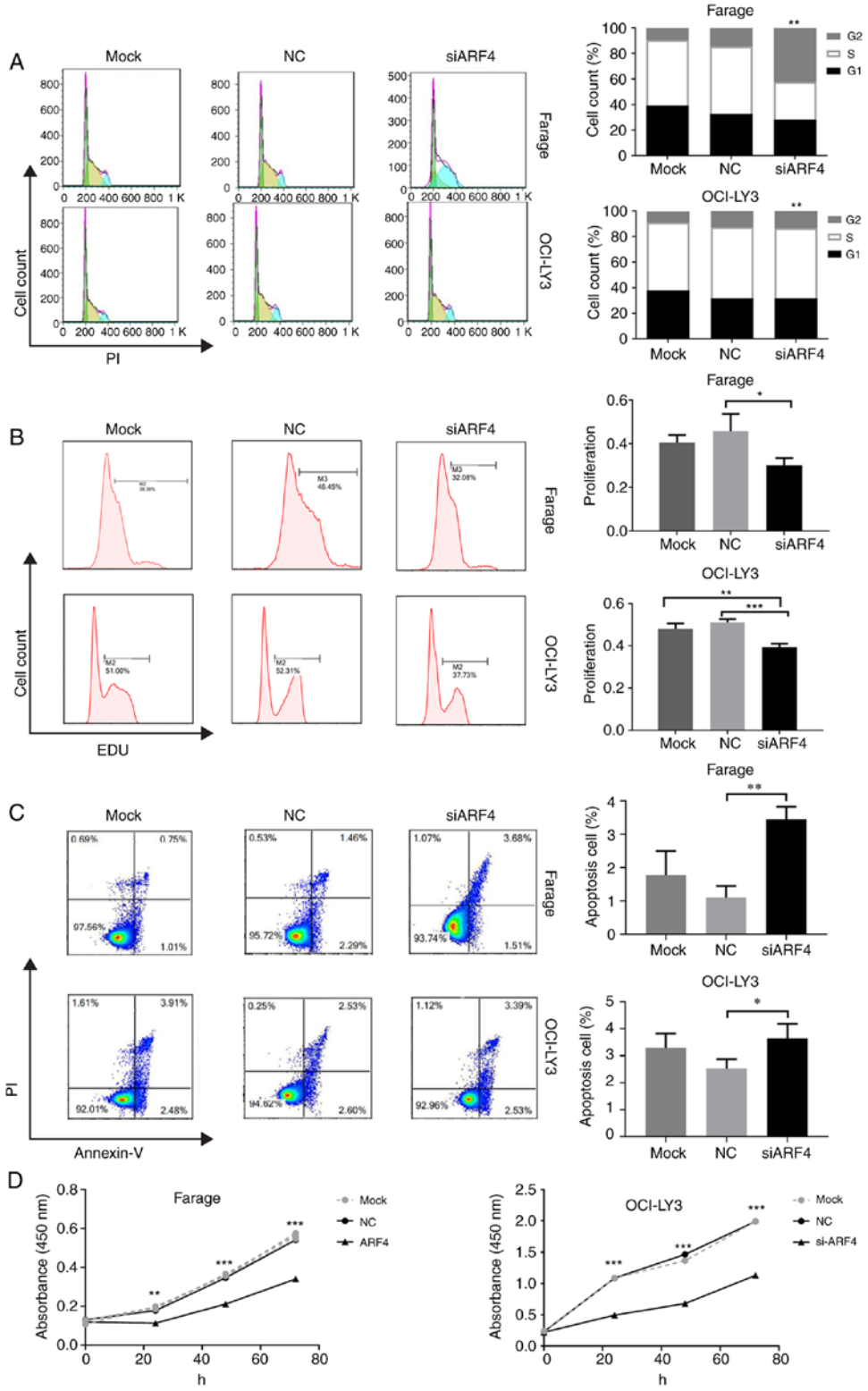

Figure 5. Role of ARF4 in the regulation of proliferation, apoptosis and cell cycle in DLBCL cells. Farage and OCI-LY3 cells were transfected with ARF4 siRNA or a scrambled sequence as the negative control. (A) Cell cycle analysis was performed by PI-staining flow cytometry. (B) Cell viability was determined by the EdU staining assay. (C) Apoptosis of cells was determined by Annexin V and PI staining and flow cytometry. (D) DLBCL cell proliferation was detected by CCK-8 assay. *P<0.05, **P<0.01 and ***P<0.001. OD, optical density; ARF4, ADP ribosylation factor 4; siARF4, ARF4 siRNA; DLBCL, diffuse large B-cell lymphoma; siRNA, small interfering RNA; PI, propidium iodide; CCK-8, Cell Counting Kit-8.

ARF4 is a member of the Ras superfamily of small guanine nucleotide-binding proteins. ARFs, which consist of 6 Arf isoforms (Arf1-Arf6), participate in several cellular processes, including vesicular trafficking, cytokinesis, cell adhesion and tumor cell invasion (29). Several reports have suggested that ARF4 plays a pivotal role in tumorigenesis. Woo *et al* found that ARF4 could function as an anti-apoptotic protein and reduce the generation of reactive oxygen species

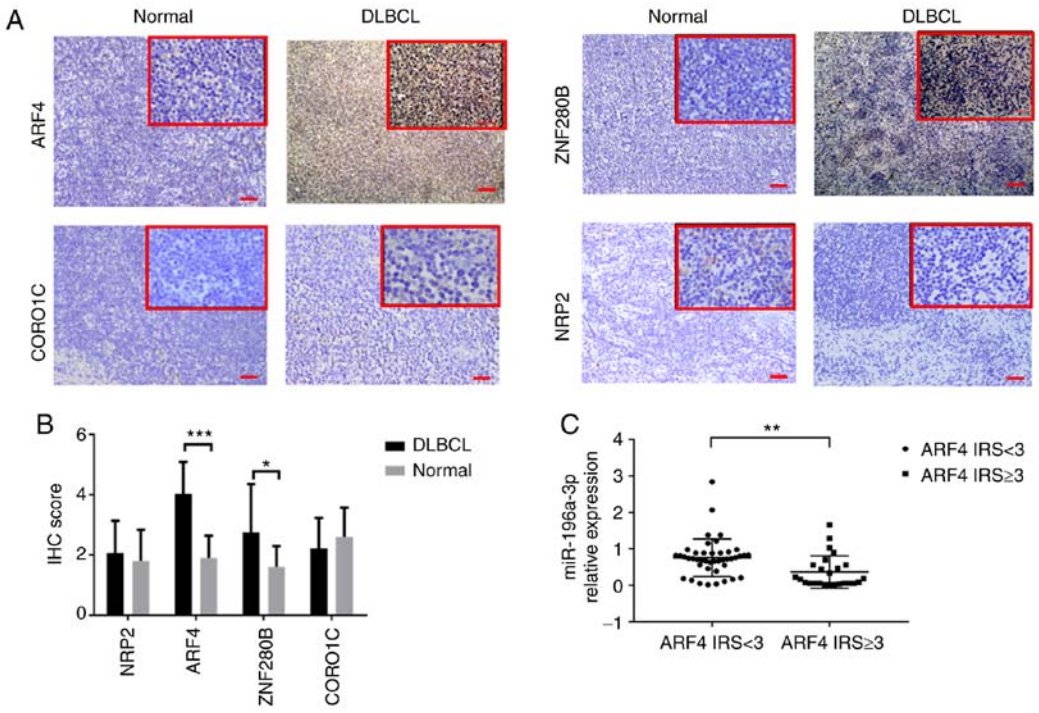

Figure 6. Expression of miR-196a-3p and its candidate target genes in DLBCL as compared with RLH clinical specimens. (A) Representative immunohistochemical staining for ARF4, ZNF280B, CORO1C and NRP2. (B) Protein expression was compared between DLBCL and RLH specimens using the IRS value. (C) The expression of miR-196a-3p was compared in two different groups based on the ARF4 protein level. DLBCL, diffuse large B-cell lymphoma; RLH, reactive lymph node hyperplasia; ARF4, ADP ribosylation factor 4; ZNF280B, Zinc finger protein; CORO1C, coronin-1C; NRP2, neuropilin-2; IRS, immunoreactive score.

in response to either B-cell lymphoma 2-associated X protein or N-(4-hydroxyphenyl) retinamide (30). In another study, miR-221-3p was shown to inhibit the proliferation and migration of epithelial ovarian cancer cells by targeting ARF4 (31). The present study revealed that the ARF4 expression was increased in DLBCL, and that this increase was associated with an advanced Ann Arbor stage (III, IV), elevated LDH and increased number of extranodal sites (>2). Moreover, the knockdown of ARF4 *in vitro* exhibited a pro-apoptotic and anti-proliferative effect on DLBCL cells. These results indicated that ARF4 may act as an oncogene mediating aberrant DLBCL cell apoptosis and proliferation.

Two main limitations of the present study should be addressed. First, this is a single-center retrospective study and only 68 DLBCL patients were enrolled, and a prospective research including multiple centers will further prove the value of miR-196a-3p and ARF4. Second, we did not perform the ARF4 rescue experiment which probably would alleviate the effect of overexpressed miR-196a-3p on DLBCL cells.

In conclusion, the present study found a significant decrease of miR-196a-3p in DLBCL, which was associated with poor clinical prognosis. It was demonstrated that the downregulation of the ARF4 expression by miR-196a-3p resulted in the accumulation of cells in the G1 phase cell cycle, accompanied by an inhibited cell proliferation and elevated apoptosis in DLBCL. The present findings delineated a novel molecular regulatory network of miR-196a-3p and ARF4 in DLBCL cell proliferation and apoptosis, which may have a potential therapeutic or prognostic value for the management of DLBCL in the future.

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Availability of data and materials

The dataset supporting the conclusions of this article is included within this article.

Authors' contributions

JF and XL designed and performed the experiments, statistical analysis and writing of the manuscript. DW and SWan participated in experiment design and direction, and reviewed the manuscript. ZC, XZ and MZ participated in the collection of clinical specimens and patient follow-up. SWang and LG were involved in the conception of the study and revising the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All patients and volunteers recruited have signed informed consent and this research was approved by the Institutional

Review Board of the First Affiliated Hospital of Soochow University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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