

CDK6 overexpression resulted from microRNA-320d downregulation promotes cell proliferation in diffuse large B-cell lymphoma

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Abstract. The molecular and clinical heterogeneity in diffuse large B-cell lymphoma (DLBCL) has raised a need for the investigation of potential therapeutic biomarkers. MicroRNA-320 (miR-320) and CDK6 are both involved in the regulation of cell proliferation in various types of cancer. To investigate the clinical role of CDK6 in DLBCL, CDK6 expression was assessed using immunohistochemistry on formalin-fixed, paraffin-embedded sections. Furthermore, to investigate the relationship between CDK6 and miR-320d, as well as their roles in cell proliferation, a series of experimentally functional validations was performed in DLBCL cell lines. Bioinformatics software and Dual-Luciferase reporter assay were used to predict and validate the potential target of miR-320d. In DLBCL cells transfected with miR-320d lentiviral vector, CDK6 expression at the protein and mRNA levels was detected using western blotting and qRT-PCR, respectively. Overexpression and small-hairpin RNA knockdown of CDK6 were performed by lentiviral transduction. The results of these experiments revealed that CDK6 was overexpressed and predictive of poor prognosis in DLBCL patients. Moreover, CDK6 was revealed to be directly targeted by miR-320d and either overexpression of miR-320d or knockdown of CDK6 inhibited proliferation. In conclusion, CDK6 overexpression in DLBCL may result from miRNA-320d downregulation and subsequent loss of inhibition of cell proliferation, suggesting that miRNA-320d may be a potential therapeutic target for the treatment of DLBCL with high CDK6 expression.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL) in adults, accounting for 30-40% of NHL cases. DLBCL is a heterogeneous disease characterized by morphologic, phenotypic and molecular diversity (1,2). Gene expression profiling studies classify DLBCL into three subgroups, namely germinal center B-cell-like (GCB), activated B-cell-like (ABC), and type 3 DLBCL and these types differ in the cell of origin and survival parameters (3-5). Approximately 50% of DLBCL patients treated with the combination of rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) combination regimen exhibited long-term remission (6); however, patients who are resistant to R-CHOP have a poor prognosis, underscoring the need to develop novel targeted therapies. In addition, the etiology of DLBCL still remains unknown. Therefore, a better understanding of the molecular mechanisms underlying the pathogenesis of DLBCL is critical to improve survival.

MicroRNAs (miRNAs) are small non-coding RNAs that play roles in cell differentiation, proliferation, apoptosis, metabolism and development (7-9). miRNAs bind partially to complementary sequences in the target mRNA, modulating gene expression at the post-transcriptional level and silencing the mRNA target in at least two ways (7). miRNA microarray assays performed on 106 DLBCL and 30 reactive hyperplasia lymph node tissue samples, revealed that miR-320d was significantly downregulated in DLBCL tissues. White and Giffard revealed that miR-320 promoted neuronal regeneration by inhibiting the expression of its target gene cAMP-regulated phosphoprotein-19 (10). Hsieh *et al* revealed that miR-320 prevents the stem cell-like characteristics of prostate cancer cells by inhibiting the Wnt/ β -catenin signaling pathway (11). In stromal fibroblasts, miR-320 is important for the phosphatase and tensin homolog (PTEN) tumor suppressor axis (12). Zhi *et al* demonstrated that miRNA-320d was frequently expressed at high levels in acute myeloid leukemia (AML) (13), whereas Yan *et al* pointed out that overexpression of miR-320d inhibited HSV-1-induced Kaposi's sarcoma-associated herpesvirus replication by targeting transcription activator (14). These findings suggest that miR-320d exerts anticancer effects

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in DLBCL. Although the anticancer functions of miR-320d have been confirmed, the underlying anticancer mechanisms of action are not fully understood. It is worth noting that Zhu *et al* revealed that miR-320 negatively regulated the expression of NRP-1 by binding to the 3' untranslated region (3'UTR) of the NRP-1 promoter, and NRP-1 depletion inhibited cell proliferation by upregulating p27, and downregulating cyclin E and cyclin-dependent kinase (CDK)-2 (15). Therefore, we speculated that miR-320d may affect the DLBCL process by regulating CDK family members.

CDK6 is a member of the CDK family that functions along with cyclins to modulate the phosphorylation of key cell cycle proteins, thereby regulating cell cycle progression. Recent studies have revealed that CDK6 is expressed at high levels and is systematically correlated with poor prognosis in many types of tumors (16,17). For example, CDK6 upregulation was revealed to be positively correlated with the stage and invasive behavior of bladder cancer (18). CDK6 overexpression, which is driven by gene amplification on chromosome 7q21.2, was associated with an adverse prognosis in medulloblastoma and myxofibrosarcoma (19,20). These data indicated that CDK6 may act as an oncogene and play a critical role in tumor development and progression. The molecular mechanisms underlying the response to R-CHOP treatment in DLBCL remain unclear.

In a previous study from our group, it was revealed that miR-320d was downregulated in DLBCL patients with a poor outcome, and overexpression of miR-320d was able to inhibit DLBCL cell proliferation (21). In the present study, we investigated the role of miR-320d in DLBCL and the underlying molecular mechanism and determined whether CDK6 acts as a target gene of miR-320d.

Materials and methods

Patients. A total of 106 samples were obtained from patients from the Department of Pathology of Shanxi Cancer Hospital (Taiyuan, Shanxi, China) who were diagnosed with primary DLBCL and accepted an RCHOP or CHOP-like initial treatment program +/- second-line treatment between January 2010 and December 2015. All patients agreed to be followed up for sampling. Finally, 85 cases with complete follow-up data who met the requirements were included. The controls consisted of 19 lymph node reactive hyperplasia samples obtained from the Department of Pathology of Shanxi Cancer Hospital. The present study was approved by the Medical Ethics Committee of Shanxi Medical University.

Immunohistochemistry. Formalin-fixed and paraffin-embedded (FFPE) tissue samples from DLBCL and the controls were collected and immunohistochemical staining was performed using the EnVision method for CDK6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Positive signals of CDK6 were located at the membrane/cytoplasm. The CDK6 staining results based on the staining intensity were independently evaluated by two experienced pathologists as follows: No staining (score=0), weak (score=1), moderate (score=2) and strong (score=3). The percentage of CDK6 positive cells was 0% (score=0), 1-30% (score=1), 31-60% (score=2), and >60% (score=3). The product of these values provided the final CDK6 score.

Cell lines and cell culture. Human DLBCL cell lines, including OCI-LY1 (GCB subtype) and NU-DUL-1 (ABC subtype) were provided by professor Xiaoyan Zhou (Cancer Hospital, Shanghai Fudan University, Shanghai, China), maintained at our laboratory, were cultured as previously described (21).

Lentivirus production and stable transfection. Lentiviral vector production for pri-miR-320d was performed as previously described (21). A CDK6-shRNA lentiviral vector and its respective negative control vector (universal vector) were generated (Shanghai GeneChem Co., Ltd., Shanghai, China). OCI-LY1 and NU-DUL-1 cells at 60-80% confluency were injected with lentiviral vectors at a multiplicity of infection (MOI) of 50 in 24-well plates (initial seeding at 10^5 cells/well, 1.5 ml/well). Three days after transfection, the cell lines stably expressing miR-320d and CDK6-shRNA were generated and the infected cells were visualized under a fluorescence microscope. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to determine miR-320d and sh-CDK6 efficiency.

qRT-PCR. Total RNA was extracted from cell lines transfected by lentiviral vectors using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. CDK6 mRNA expression was quantified using the SYBR PrimeScript RT-PCR Kit II (Takara Bio, Inc., Shiga, Japan) and normalized to GAPDH expression using the $2^{-\Delta\Delta C_q}$ method (22). The PCR was performed as follows: 95°C for 30 sec, 40 cycles at 95°C for 5 sec, 60°C for 30 sec; and the dissociation stage at 72°C for 30 sec, 60°C for 30 sec and 95°C for 15 sec. The PCR primers used were as follows: GAPDH forward, 5'-CCATCAATGACCCCTTCATTG-3' and reverse, 5'-CATGGGTGGAATCATATTGGAAC-3'; CDK6 forward, 5'-CTGAATGCTCTTGCTCCTTT-3' and reverse, 5'-AAAGTTTGGTGGTCCTTGA-3'. All qRT-PCR amplifications were performed in triplicate.

Protein extraction and western blotting. DLBCL cells transduced with lentivirus-miR-320d or lentivirus-control were lysed in a RIPA lysis buffer (Beyotime Institute of Biotechnology, Beijing, China) and protein concentration was measured using the BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Lysates were denatured with SDS sample buffer at 100°C for 10 min. Protein samples (30 µg) were separated on 10% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon®-P). The membranes were blocked and then probed with antibodies against CDK6 (mouse polyclonal to CDK6; dilution 1:500; cat. no. sc-7961; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C, followed by incubation with secondary antibodies (goat anti-mouse IgG horseradish peroxidase conjugate; dilution 1:1,000; cat. no. AP124F; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Loading was normalized with GAPDH (dilution 1:3,000; Sigma-Aldrich; Merck KGaA). Band signals were visualized using ECL (Pierce; Thermo Fisher Scientific, Inc.). The band density was evaluated by Bio-Rad Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

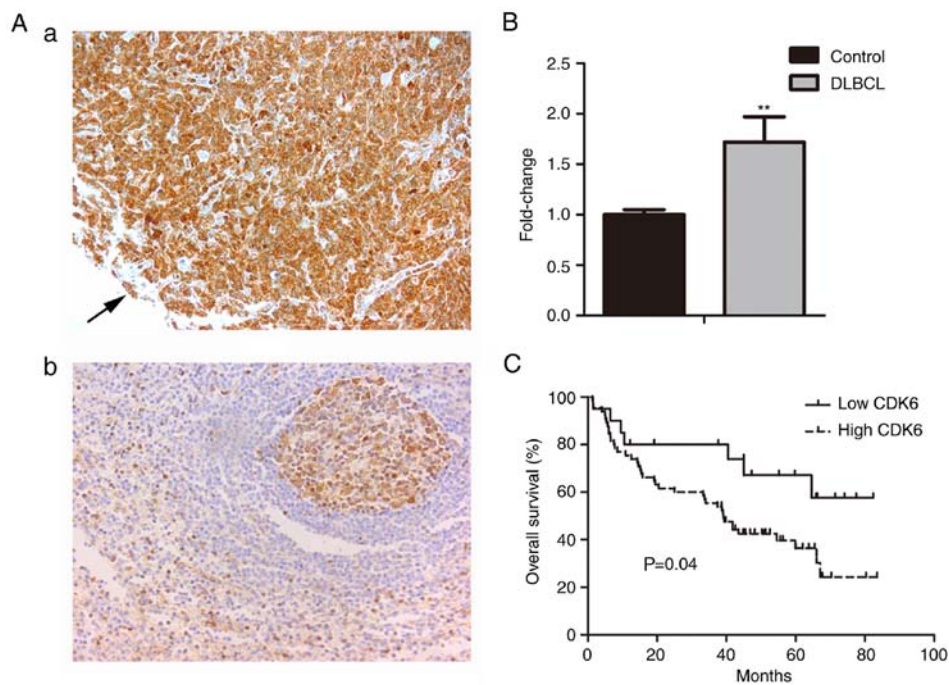


Figure 1. CDK6 is upregulated in DLBCL patients with poor prognosis. (A) Representative microphotographs of DLBCL and lymph node reactive hyperplasia tissue sections stained for CDK6. a, DLBCL; b, lymph node reactive hyperplasia. Scale bar, 100 μ m. (B) Confirmation of frequent upregulation of CDK6 in 85 FFPE samples by qRT-PCR. Nineteen reactive hyperplasia lymph node tissues were included as controls (**P<0.01). (C) Kaplan-Meier analysis using the median abundance of CDK6 as the cut-off revealing that high expression of the CDK6 was associated with shorter OS of DLBCL patients. DLBCL, diffuse large B-cell lymphoma; FFPE, formalin-fixed and paraffin-embedded; OS, overall survival.

Cell proliferation assay. DLBCL cells transfected with lentivirus carrying pri-miR-320d, CDK6-shRNA, and the respective control cells transfected with control vectors were seeded in 96-well plates (1×10^4 cells/well). After transfection for 24 h, cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. CCK-8 reagent (20 μ l) was added to the cells in each well at 24, 48, 72 and 96 h after transfection followed by incubation for 2 h at 37°C, in a 5% CO₂ humidified incubator and measurement of the absorbance at 450 nm was performed with a microplate reader (Thermo Fisher Scientific, Inc.).

Dual-luciferase reporter assay. The following online software programs were used to identify miRNA target genes: TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/microrna/home.do>), and miRDB (<http://www.mirdb.org/mirdb/>). CDK6 was identified as a possible target of miR-320d. The wild-type sequence was NM_001145306-3'UTR: ATTCAGCTTTATGTT, and the mutant-type sequence was NM_001145306-3'UTR: ATTGGTTAAGACTGTT.

DLBCL cells were seeded into 24-well plates at a density of 3×10^4 /well and cultured for 24 h before the addition of 100 ng pMIR-REPORT luciferase vector (Shanghai GeneChem Co., Ltd.) containing the CDK6 3'UTR or mutated forms. miR-320d mimics or mimic negative control (NC) (50 nmol/l) were co-transfected into the cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation for 48 h, luciferase activity was assessed using the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). The experiments were repeated three times.

Statistical analysis. The SPSS statistical software package (version 17.0) (IBM Corp., Armonk, NY, USA) was used for statistical analyses, and data were expressed as the mean \pm SD from at least three independent experiments. Kaplan-Meier was used to estimate the survival curves. The Pearson χ^2 test was used to analyze the expression of CDK6 between the test group (DLBCL) and negative control group (lymph node reactive hyperplasia). The comparison of the CDK6 expression between the blank control, mimic NC and miR-320d mimics was analyzed using one-way ANOVA, followed by least significant difference (LSD) test for differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CDK6 is upregulated in DLBCL patients with poor prognosis.

A total of 85 patients with primary DLBCL, including 51 men and 34 women (sex ratio, 1.5:1) with a median age of 57 years (range, 9-81 years) were included in the analysis. The median overall survival (OS) of patients was 39.8 months, and the OS rate was 43.5%. The positive rate of CDK6 expression in DLBCL was 76.47%, whereas it was negatively expressed in lymph node reactive hyperplasia (Fig. 1A). CDK6 expression was significantly higher in DLBCL than that in lymph node reactive hyperplasia tissues (P<0.01; Fig. 1B and Table I). Patients of DLBCL with high levels of CDK6 expression had a shorter median overall survival (39 months) than those with low levels of CDK6 expression (46.2 months) (Fig. 1C).

Overexpression of miR-320d or knockdown of CDK6 inhibits proliferation in GCB type of DLBCL cells. To investigate the

Table I. CDK6 expression in DLBCL and lymph node reactive hyperplasia tissues.

Samples	Total	CDK6 expression		OS rate (%)	P-value
		Positive (%)	Negative (%)		
DLBCL	85	65 (76.47)	20 (23.53)	43.5	<0.01
Lymph node reactive hyperplasia	19	0	19		

DLBCL, diffuse large B-cell lymphoma; OS, overall survival.

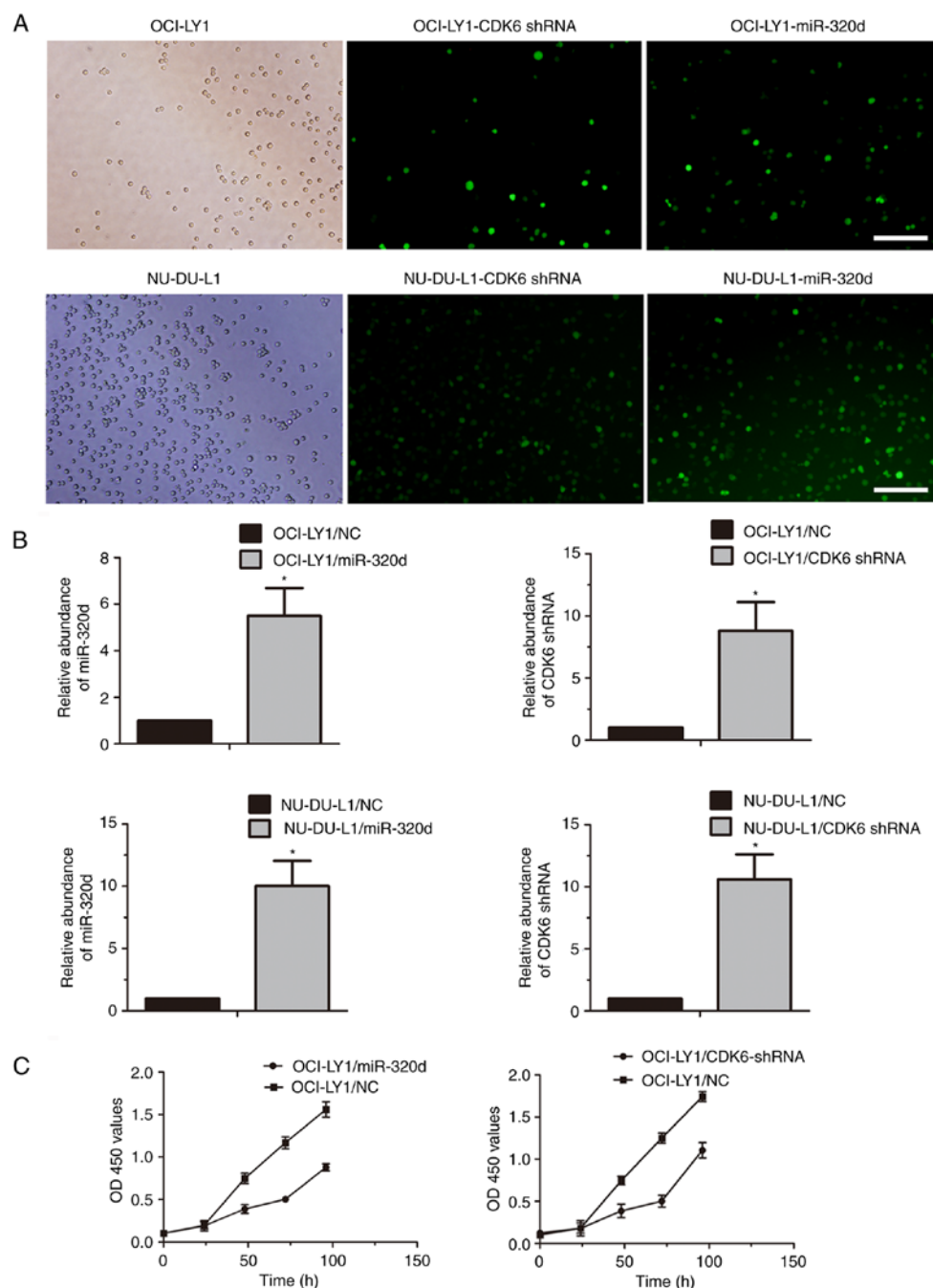


Figure 2. miR-320d and CDK6 shRNA inhibit DLBCL cell proliferation. (A) Laser scanning confocal microscope analysis of cells overexpressing miR-320d and suppressing CDK6 at 48 h after lentiviral transfection. OCI-LY1 (upper panel) and NU-DUL-1 (lower panel) cells were respectively transduced with CDK6 shRNA and miR-320d lentiviral vector. Scale bar, 100 μ m. (B) Overexpression of miR-320d (left panel) and suppression of CDK6 (right panel) in OCI-LY1 and NU-DUL-1 cells, respectively. The data shown were the mean \pm SE of three individual experiments (* P <0.05). (C) A CCK-8 assay revealing that CDK6 knockdown (lower panel) and miR-320d overexpression (upper panel) inhibited the proliferation of OCI-LY1 cells. Starting at 48 h after transfection, the OD450 value of the experimental group was obviously lower than that of the control group (P <0.05). This result was not detected in NU-DUL-1 cells. DLBCL, diffuse large B-cell lymphoma; CCK-8, Cell Counting Kit-8; OD, optical density.

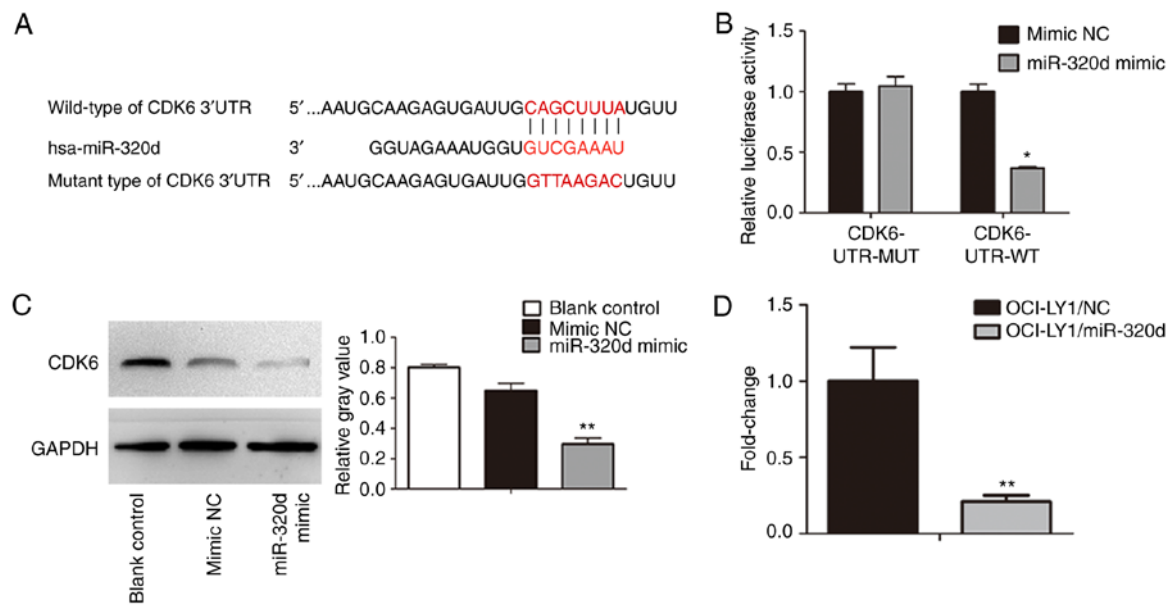


Figure 3. CDK6 is a target of miR-320d. (A) Predicted miR-320d binding to the 3'UTR of human CDK6. (B) Compared with the mutant type, overexpression of miR-320d suppressed luciferase activity of the wild-type 3'UTR of CDK6 reporter in DLBCL cells (* $P < 0.05$). Luciferase activity was not inhibited in the NC groups. (C) Western blot analysis confirmed the downregulation of the CDK6 protein in OCI-LY1 cells transfected with miR-320d lentiviral vector. CDK6 protein expression levels in miR-320d-overexpressing cells were significantly lower than those in cells transfected with the universal vector (NC) and untransfected cells, and the difference was statistically significant (** $P < 0.01$). (D) qRT-PCR results revealing that the relative expression levels of CDK6 in miR-320d-transfected cells was lower compared with the NC-transfected OCI-LY1 cells (** $P < 0.01$). 3'UTR, 3'untranslated region; mimic NC, mimic universal vector; DLBCL, diffuse large B-cell lymphoma.

potential role of miR-320d and CDK6 in DLBCL proliferation, exogenous miR-320d or CDK6-shRNA lentiviral vector was transfected into OCI-LY1 and NU-DUL-1 cells, and miR-320d or CDK6-shRNA stably-expressing cells were established. Both OCI-LY1 and NU-DUL-1 cells were infected with the lentivirus containing the miR-320d and CDK6-shRNA expression sequence (Fig. 2A). The levels of miR-320d and CDK6-shRNA in DLBCL cells transduced respectively with the miR-320d-expressing and CDK6-shRNA constructs were markedly higher than those transduced with the control constructs (Fig. 2B). The CCK-8 assay revealed that the cell proliferation of OCI-LY1 infected with lenti-miR-320d or lenti-CDK6-shRNA was decreased compared with those infected with lenti-NC (Fig. 2C). Although the CCK-8 assay was not detected in the NU-DUL-1 cells, the results indicated that miR-320d and CDK6 shRNA may participate in inhibiting at least GCB-DLBCL cell proliferation.

Overexpression of miR-320d decreases CDK6 expression, and CDK6 is a direct downstream target of miR-320d. To determine the direct downstream target of miR-320d, potential targets were predicted using TargetScan, miRanda and miRDB. By integrating the three strategies, CDK6 was found to be the potential target of miR-320d. Then, the 3'UTR of CDK6 with wild-type or mutant seed-sequence-recognizing sites was cloned into a dual-Luciferase reporter (Fig. 3A). miR-320d mimic or NC mimic with CDK6-UTR-WT or CDK6-UTR-MUT plasmid were co-transfected into OCI-LY1 cells, and then the luciferase activity was analyzed. The results indicated that compared with the plasmid carrying CDK6-UTR-MUT, the luciferase activity of the plasmid carrying CDK6-UTR-WT was suppressed in the miR-320d

mimic cells (Fig. 3B). To further analyze the expression of CDK6 in DLBCL cells, qRT-PCR and western blotting were used to detect CDK6 mRNA and protein expression in OCI-LY1 cells overexpressing miR-320d. The results revealed that overexpression of miR-320d could decrease CDK6 expression both at the protein level and at the mRNA level (Fig. 3C and D). Based on the results, it was indicated that miR-320d specifically bound to the CDK6 3'UTR and may directly target CDK6.

Discussion

The development and proliferation of tumor cells are closely associated with the cell cycle, which is regulated by CDKs and cell cyclins. CDK6, an important member of the CDK family, is involved in oncogenesis and tumor development in many malignancies. In the present study, CDK6 was highly expressed in DLBCL patients and associated with a shorter median overall survival, suggesting that CDK6 acts as an oncogene in DLBCL.

Increasing evidence supports the role of miRNAs in many neoplasms, and miRNAs are differentially expressed in different diseases. Notably, miRNAs not only contribute to tumorigenesis but can also inhibit cancer progression. Recent studies suggest that miR-155 (23), miR-21 (24) and miR-17-92 (25), may function as oncogenes and these miRNAs are overexpressed in DLBCL. Notably, miR-155 was revealed to be highly expressed in the ABC and primary mediastinal B cell lymphoma subtypes, but not in the GCB subtype (26). miR-1234 was upregulated in Egyptian patients with DLBCL compared with Swedish patients, and may play a role in the oncogenesis of ABC-DLBCL by targeting STAT3 (27).

Overexpression of miR-520c-3p resulted in the downregulation of the oncogene *eIF4GII*, which could account for its anti-tumor activity in DLBCL (28). miR-146b-5p upregulation in papillary thyroid carcinoma was significantly correlated with poor prognosis (29). Among miR-320 subtypes, miR-320d has attracted increased attention. Several studies have revealed that miR-320d was upregulated in the serum of AML patients (13). However, a previous study from our group indicated that low expression of miR-320d was related to poor prognosis in DLBCL patients treated with CHOP (21). These studies indicated that miR-320d plays diverse roles and this could be attributed to differences in target genes among different cells.

We previously revealed that miR-320d expression was downregulated in DLBCL cell lines, and the CCK-8 assay indicated that DLBCL cells transfected with a miR-320d overexpression vector (pri-miR-320d) exhibited decreased growth compared with control cells (21). This led us to hypothesize that miR-320d functions as a tumor suppressor. In the present study, we integrated three bioinformatics prediction software programs and identified the CDK6 gene as a target of miR-320d. We used qRT-PCR and western blot assays to test our hypothesis that miR-320d regulates its target gene, CDK6. The results indicated that miR-320d overexpression downregulated CDK6 at the mRNA and protein levels, suggesting that miR-320d negatively regulated the expression of CDK6. However, these results did not demonstrate the direct regulation of the gene. Considering that miRNAs bind to the 3'UTR of the target mRNA to regulate gene expression, we constructed luciferase reporter plasmids bearing the wild-type or a mutant CDK6 3'UTR downstream of the stop codon of the luciferase gene. Luciferase assays revealed that overexpression of miR-320d reduced luciferase activity in the wild-type CDK6 3'UTR, whereas it had no effect on cells transfected with the mutant construct. These results suggested that miR-320d can directly and negatively regulate CDK6 gene expression by binding to the 3'UTR of CDK6.

To further validate the effect of the relation between miRNAs and their target genes on cell proliferation, CDK6 was silenced by transfection with CDK6-shRNA, and the results indicated that knockdown of CDK6 reduced DLBCL cell growth. This result was consistent with that reported by van der Linden *et al* (30), who revealed that that CDK6 downregulation inhibits acute lymphoblastic leukemia (ALL) cell proliferation. By contrast, overexpression of CDK6 suppressed the proliferation of human breast tumor cell lines through a mechanism involving p130 and E2F4 (31). miR-29c can promote apoptosis by downregulating CDK6 in glioma cells (32). Similarly, let-7a promoted apoptosis by inhibiting CDK6 expression in Ewing's sarcoma (Ewing) cells (33). These differences among studies could be attributed to the different cell types and different diseases, and indicate the diverse effects of CDK6 on cell proliferation. Our previous results indicated that miR-320d had no significant effect on DLBCL cell apoptosis; however, Classon and Harlow revealed that CDK6 was not only associated with cell apoptosis but also involved in cell cycle progression. They indicated that knockdown of CDK6 can promote apoptosis and block the cell cycle at the G0/G1 stage. CDK6 cooperation with CDK4 promote the growth of cells through the early G1 phase of cell cycle by binding to cyclin Ds. Then, the CDK4/6-cyclin D complexes

phosphorylate members of the retinoblastoma (RB) protein family releasing E2F transcription factors from RB-mediated inhibition (34). Bustos *et al* indicated that overexpression of miR-200a in metastatic melanoma cells blocked the cell cycle by targeting CDK6, and reduced the levels of phosphorylated-Rb1 and E2F-downstream targets, decreasing cell proliferation (35). Therefore, we speculated that knockdown of CDK6 may regulate Rb and E2F, and then inhibit DLBCL cell proliferation.

In conclusion, we revealed that the CDK6 protein was highly expressed in DLBCL tissues and could serve as a predictor of poor outcome in DLBCL patients. Overexpression of miR-320d suppressed DLBCL cell proliferation by targeting CDK6, suggesting that miR-320d is a potential therapeutic target for the treatment of DLBCL with high CDK6 expression.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

HS, MX, JC and JW were responsible for the study design, original article drafting and editing, data acquisition, data analysis and the editing of the images. RS was responsible for the immunohistochemical evaluations and the critically manuscript revision. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The use of human tissues was approved by the Shanxi Provincial Cancer Hospital Ethics Committee (no. 201733), and patient consent was obtained.

Patient consent for publication

Publication of the clinical datasets in this study does not compromise anonymity, or confidentiality, or breach local data protection laws.

Competing interests

The authors declare that they have no competing interest.

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