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MicroRNA-206 serves as a tumor suppressor in pediatric acute myeloid leukemia by targeting Cyclin D1

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ABSTRACT

Pediatric acute myeloid leukemia (AML) is a complex and heterogeneous disease. Several studies have shown the regulatory functions of microRNAs (miRNAs) in pediatric AML progression, and in this study, we aimed to evaluate the biological role of miR-206 in pediatric AML. The results demonstrated that miR-206 expression levels in the bone marrow and serum of pediatric AML patients were remarkably decreased than those in normal controls, and low serum miR-206 expression was closely associated with the unfavorable clinicopathological characteristics and prognosis of pediatric AML patients. In addition, in vitro functional experiments revealed that overexpression of miR-206 significantly inhibited AML cell proliferation partly through induction of cell cycle arrest. Further studies showed that Cyclin D1 might be a direct target of miR-206 in AML cells, and the impaired proliferation ability of miR-206-overexpressing AML cells was notably rescued by Cyclin D1 restoration. Accordingly, the findings of our study suggested that miR-206 might serve as a promising prognostic marker and a potential therapy target for patients with pediatric AML.

1. Introduction

Acute myeloid leukemia (AML), a type of cancer that arises from myeloid cells, is the second most common type of leukemia in children, and its incidence is increasing year by year [1]. In spite of the rapid development of diagnostic and therapeutic methods, the prognosis of patients with pediatric AML remains largely dismal, with long-term survival rates of 50% to 65% [2]. Accordingly, it is of critical importance to elucidate the underlying mechanisms involved in pediatric AML progression and to identify novel therapeutic strategies.

MicroRNAs (miRNAs) are a class of highly conserved, non-protein-coding, single-stranded small RNAs (˜22 nucleotides in length) that post-transcriptionally regulate gene expression [3]. miRNAs show aberrant expression patterns and functional abnormalities in many human cancers, and miRNA profiling can be used as a tool for cancer diagnosis and prognosis [4]. miR-206, transcribed from the genomic region chromosome 6p12.2, is a miRNA commonly found to be downregulated in a variety of human cancers, including prostate cancer [5] and epithelial ovarian cancer [6]. But its regulatory role in leukemia has not been reported. Therefore, in the present study, we sought to evaluate the biological role of miR-206 in pediatric AML.

2. Materials and methods

2.1. Patients and clinical samples

Bone marrow (BM) and serum samples were collected from 73 patients with pediatric AML (46 boys and 27 girls; median age 6 years; range 3–10 years) before any interventional treatments at Daqing Longnan Hospital (Daqing, China). These patients were classified and treated according to the protocol for Chinese AML children by Subspecialty Group of Hematology Diseases, Society of Pediatrics and Chinese Medical Association [7], and their clinicopathological characteristics were summarized in Table 1. As normal controls, BM samples were collected from 20 pediatric patients with normal bone marrow morphology (14 boys and 6 girls; median age 8 years; range 4–14 years), and serum samples were collected from 20 healthy children (13 boys and 7 girls; median age 8 years; range 4–15 years). The study was approved by the Ethics Committee of Daqing Longnan Hospital, and written informed consent for clinical studies was obtained.

2.2. Cell culture and transfection

Two AML cell lines, THP-1 and HL-60, and normal HS-5 cells from

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Table 1
Association between serum miR-206 expression and clinicopathological characteristics of pediatric AML patients.

Characteristics	Total number (n = 73)	miR-206 expression		*P* value
		Low (n = 41)	High (n = 32)	
Age (years)				0.448
> 6	31	19	12	
≤6	42	22	20	
Gender				0.936
Male	46	26	20	
Female	27	15	12	
Leukocyte counts (/μl)				0.725
> 10,000	45	26	19	
≤10,000	28	15	13	
French–American–British classification				0.017
M0-M5	61	30	31	
M7	12	11	1	
Cytogenetics				0.002
Favorable	23	7	16	
Intermediate	38	23	15	
Unfavorable	12	11	1	
Extramedullary disease				0.112
Present	28	19	9	
Absent	45	22	23	

marrow stroma were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

miR-206 mimics (miR-206) and mimics negative control (miR-NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The Cyclin D1 overexpression plasmid was established by inserting the full-length human Cyclin D1 cDNA into the pcDNA3.1 vector (Invitrogen). The empty vector was considered as the control. Cells were grown in 24-well plates at a density of 1×10^5 cells/well overnight, and then transfected with the oligonucleotides or vectors using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). After 48 h, cells were used for further experiments.

2.3. RNA extraction and RT-qPCR analysis

Total RNA was extracted using the miRNeasy extraction kit (Qiagen, Valencia, CA, USA) and reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Invitrogen). qPCR analysis was then carried out on a 7500 Fast Real-Time Sequence detection system (Applied Biosystems, Foster City, CA, USA) using the miScript SYBR Green PCR Kit (Qiagen). The sequences of primers were shown as follows: miR-206, RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACCACACC-3′, forward primer: 5′-CGATGGAATGTAA GGAAGT-3′ and reverse primer: 5′-GTGCAGGGTCCGAGGT-3′; U6, RT: 5′-AACGCTTCACGAATTTGCGT-3′, forward primer: 5′-CTCGCTTCGGC AGCACA-3′ and reverse primer: 5′-AACGCTTCACGAATTTGCGT-3′. The relative gene expression was detected by $2^{-\Delta\Delta Ct}$ method [8], with U6 as an internal control.

2.4. MTT assay

Cell proliferation was measured using 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were cultured in 96-well plates at 2×10^3 cells/well, and at indicated time points, 20 μl of MTT solution (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubation for additional 4 h, the supernatant was removed and 100 μl dimethylsulfoxide (DMSO; Sigma-Aldrich) was then added to each well. The absorbance of each well was read at 570 nm on a microplate reader (Bio-Tek Company, Winooski, VT, USA).

2.5. Cell cycle analysis

The CycleTEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) was used to detect cell cycle progression. Cells were harvested, and fixed in 70% ethanol overnight at 4 °C. RNA was removed from the cells by incubation with RNase A (Sigma-Aldrich) at 37 °C for 30 min. Cells were then stained with propidium iodide solution at room temperature for 30 min. Cell cycle distribution was detected and analyzed using the FACSCalibur flow cytometry (BD Biosciences).

2.6. Western blot analysis

Total protein was extracted using radioimmunoprecipitation-assay buffer (Beyotime, Shanghai, China). The protein concentration was detected using a BCA Protein Assay Kit (Solarbio, Beijing, China). Equal amount of proteins was separated by SDS-polyacrylamide gel electrophoresis, and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% (wt/vol) skimmed milk, and then incubated with specific primary antibodies at 4 °C overnight. After three washes with TBST, the membranes were further incubated with HRP-conjugated secondary antibody. The protein bands were visualized using an enhanced chemiluminescent detection kit (Pierce, Rockford, IL, USA), and GAPDH was used as an internal control.

2.7. Dual-luciferase reporter assay

The fragment of Cyclin D1 3′-UTR that contains specific miR-206 binding site was amplified by PCR and cloned into the psiCHECK2 vector (Promega, Madison, WI, USA). The putative binding site was mutated using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen). HEK293 T cells were seeded into 24-well plates and then co-transfected with the WT or MUT luciferase constructs and miR-206 mimics or mimics negative control. Cells were harvested at 48 h after transfection, and the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA) and SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). For comparisons between groups, Student's *t*-test or $\chi 2$-test was used. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. A P-value < 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. miR-206 is downregulated in pediatric AML

We initially investigated miR-206 expression in the BM and serum samples from 73 pediatric AML patients and 20 normal controls using RT-qPCR analysis, and the results demonstrated that, compared with normal controls, miR-206 expression levels in the BM and serum samples of pediatric AML patients were remarkably decreased (Fig. 1A-B). Besides, as indicated by Pearson correlation analysis, in pediatric AML patients, serum miR-206 expression is closely correlated with miR-206 expression in the BM samples ($r = 0.245$, $P = 0.036$; Fig. 1C).

Collection of serum samples is more convenient. To further investigate the association between serum miR-206 expression and clinicopathological characteristics of pediatric AML patients, these patients were then classified into two groups, including high expression group

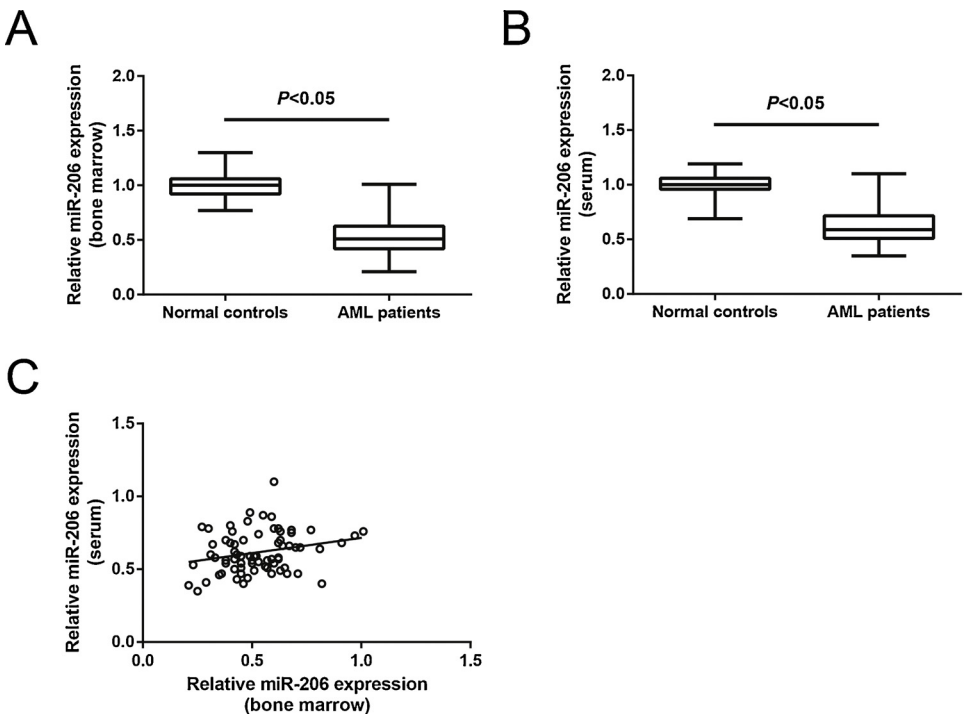

Fig. 1. miR-206 is downregulated in pediatric AML. (A) RT-qPCR analysis of miR-206 expression levels in the BM samples of pediatric AML patients and normal controls. (B) RT-qPCR analysis of miR-206 expression levels in the serum samples of pediatric AML patients and normal controls. (C) The correlation between serum miR-206 expression and BM miR-206 expression in pediatric AML patients.

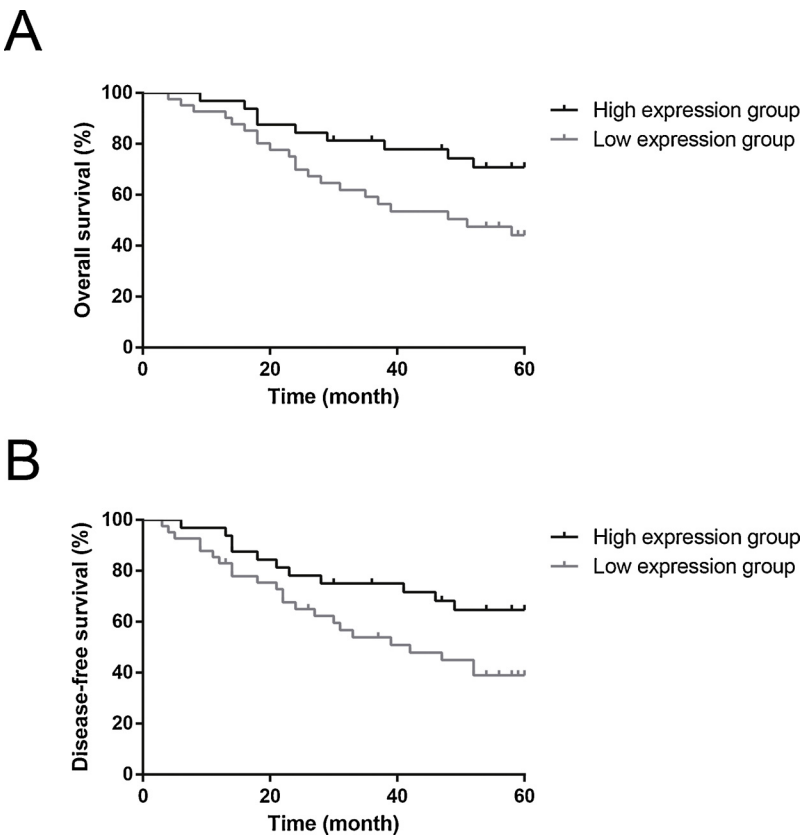

Fig. 2. Kaplan-Meier analysis of (A) overall survival and (B) disease-free survival in 73 pediatric AML patients according to serum miR-206 expression.

(n = 32; > mean) and low expression group (n = 41; ≤ mean). As listed in Table 1, serum miR-206 expression was closely associated with French–American–British classification ($P = 0.017$) and cytogenetic abnormality ($P = 0.002$) of pediatric AML patients. In addition, pediatric AML patients with higher expression of miR-206 predicted better overall survival ($P = 0.028$; Fig. 2A) and disease-free survival ($P = 0.041$; Fig. 2B) than those with lower expression.

3.2. miR-206 inhibits AML cell proliferation and cell cycle progression

Moreover, we found that the expression of miR-206 in the AML cell lines, THP-1 and HL-60, was significantly lower compared with that in

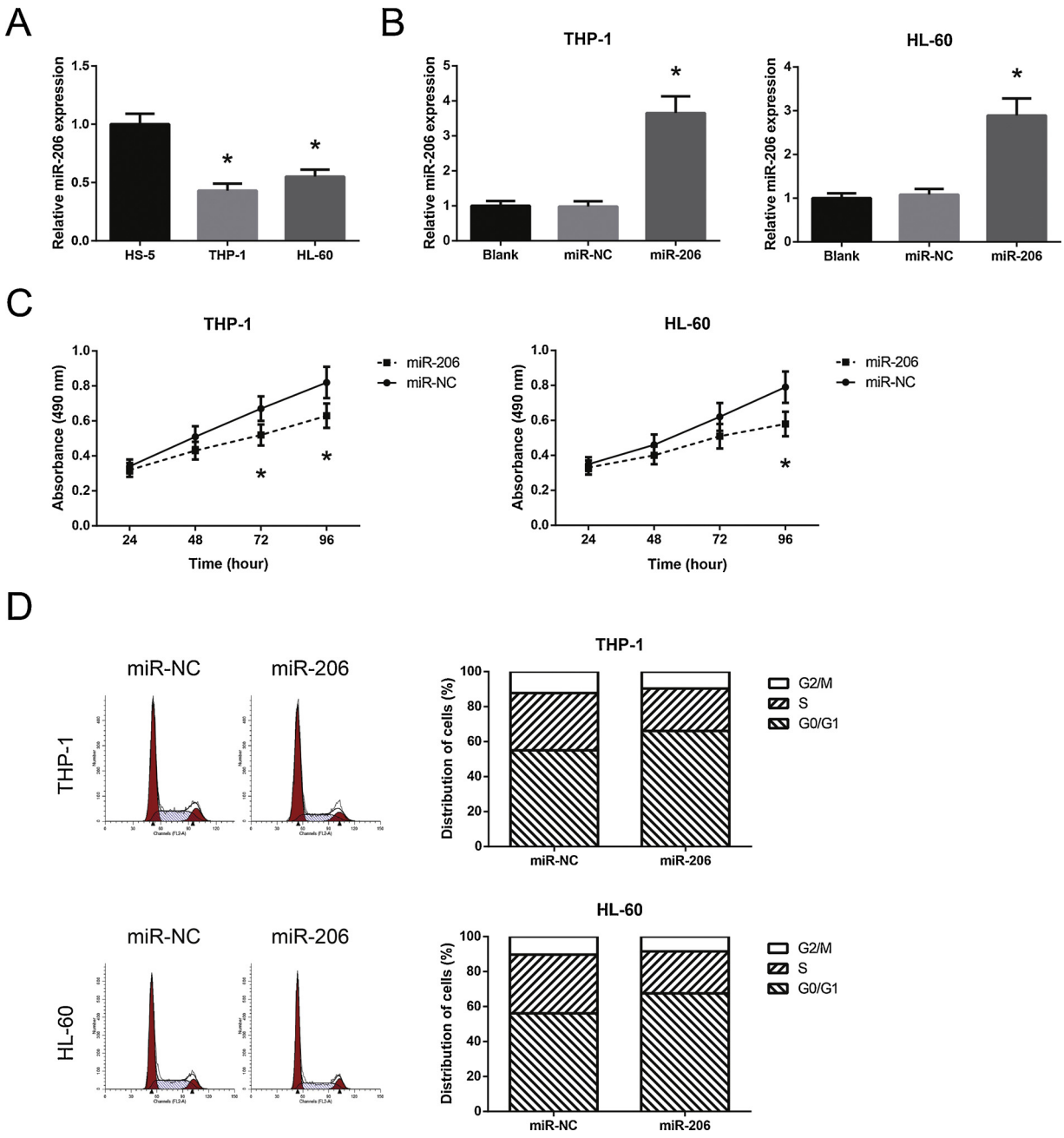

Fig. 3. miR-206 inhibits AML cell proliferation and cell cycle progression. (A) RT-qPCR analysis of miR-206 expression levels in two AML cell lines (THP-1 and HL-60) and normal HS-5 cells. *$P < 0.05$ vs. HS-5 cells. (B) RT-qPCR analysis of miR-206 expression levels in THP-1 and HL-60 cells after transfection. (C) MTT assay was performed to detect the proliferation of THP-1 and HL-60 cells after transfection. (D) Flow cytometric analysis was performed to detect the cell cycle progression of THP-1 and HL-60 cells after transfection. *$P < 0.05$ vs. mimics control-transfected cells.

normal HS-5 cells (Fig. 3A). To further explore the biological role of miR-206 in human AML cell lines in vitro, we overexpressed miR-206 in THP-1 and HL-60 cells through transfection with miR-206 mimics, and the transfection efficacy was confirmed by RT-qPCR analysis (Fig. 3B). By MTT assay, we noticed that the proliferation rates were significantly inhibited when miR-206 was overexpressed in THP-1 and HL-60 cells (Fig. 3C). Besides, as shown in Fig. 3D, miR-206 overexpression in THP-1 and HL-60 cells induced a significant accumulation of cells in the G0/G1 phase and a remarkable reduction of cells in the S phase and G2/M phase.

3.3. miR-206 targets Cyclin D1 in AML cells

To further elucidate the mechanisms underlying the tumor suppressive role of miR-206 in pediatric AML, we then screened for potential targets of miR-206. Through TargetScan database (http://www.targetscan.org/vert_71/) [9], a conserved domain within the 3′-UTR of Cyclin D1 with a potential miR-206 binding site was identified (Fig. 4A). To confirm this assumption, dual-luciferase reporter assay was then performed, and the results indicated that the luciferase activity was notably reduced in HEK293 T cells co-transfected with miR-206 mimics and Cyclin D1-WT (Fig. 4B). In addition, as shown in Fig. 4C, overexpression of miR-206 significantly decreased the levels of Cyclin D1 protein in THP-1 and HL-60 cells. Rescue experiments showed that the impaired proliferation abilities of miR-206-overexpressing THP-1 and HL-60 cells were notably rescued by Cyclin D1 restoration (Fig. 4D).

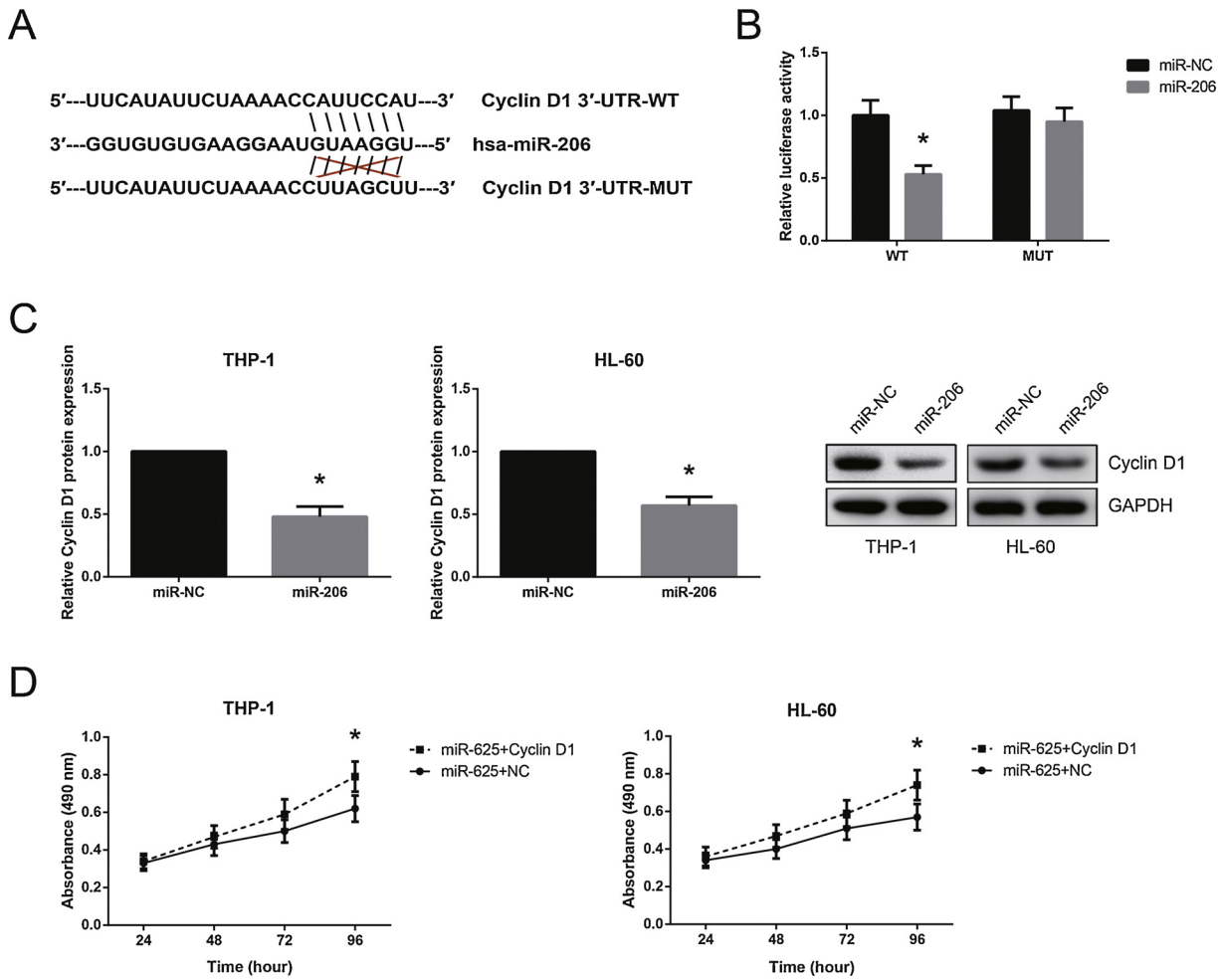

Fig. 4. miR-206 targets Cyclin D1 in AML cells. (A) Putative binding region between miR-206 and the 3′-UTR of Cyclin D1 was predicted. (B) Dual-luciferase reporter assay was performed to validate the combination between miR-206 and the 3′-UTR of Cyclin D1. (C) Western blot analysis of Cyclin D1 protein expression levels in THP-1 and HL-60 cells after transfection. (D) MTT assay was performed to detect the proliferation of THP-1 and HL-60 cells after transfection. $^{*}P < 0.05$ vs. mimics control-transfected cells, $^{\#}P < 0.05$ vs. empty vector-transfected cells.

4. Discussion

Pediatric AML is a complex and heterogeneous disease featured by various molecular and cytogenetic abnormalities. Recently, emerging studies have identified the diagnostic and prognostic roles of miRNAs in pediatric AML progression. For example, Lin et al. reported that serum miR-370 level could efficiently screen pediatric AML patients from healthy controls [10], whereas Hong et al. showed that serum miR-195 may serve as an independent prognostic factor for both relapse-free and overall survivals of pediatric AML patients [11].

In this study, we observed the decreased expression levels of miR-206 in the BM and serum samples from pediatric AML patients. Then, the low serum miR-206 level was found to be closely correlated with the unfavorable clinicopathological characteristics and prognosis of pediatric AML patients. Loss of cell cycle control often leads to abnormal cell proliferation and tumorigenesis [12], and our experimental data further showed that overexpression of miR-206 inhibited cell proliferation partly through induction of cell cycle arrest in AML cells. Hence, these findings suggest that miR-206 may act as a tumor suppressor in pediatric AML and can be considered as a potential prognostic indicator for pediatric AML patients.

The regulatory functions of miRNAs primarily rely on their target gene(s) [13]. The potential target of miR-206 that could be involved in cell cycle progression prompted us to focus on Cyclin D1, a recognized oncogene involved in the promotion of cell cycle transition [14]. The direct binding relation between miR-206 and Cyclin D1 was previously reported [15], and this study verified that miR-206 can directly bind to the 3′-UTR of Cyclin D1 and decrease its expression in AML cells. More importantly, restoration of Cyclin D1 expression partially blocked the tumor suppressive role of miR-206 in AML cells. These results provided adequate evidence to suggest that Cyclin D1 downregulation is essential for the tumor suppressive role of miR-206 in pediatric AML.

In conclusion, this study provided the convincing evidence that miR-206 serves as a tumor suppressor in pediatric AML, inhibiting cell proliferation and cell cycle progression by downregulating Cyclin D1 expression. Therefore, the miR-206/Cyclin D1 axis may be a useful therapeutic target for pediatric AML treatment.

Declaration of Competing Interest

None.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2015, CA Cancer J. Clin. 65 (1) (2015) 5–29 Epub 2015/01/07.
[2] U. Creutzig, T. Buchner, M.C. Sauerland, M. Zimmermann, D. Reinhardt, H. Dohner, et al., Significance of age in acute myeloid leukemia patients younger than 30 years: a common analysis of the pediatric trials AML-BFM 93/98 and the adult trials AMLCG 92/99 and AMLSG HD93/98A, Cancer 112 (3) (2008) 562–571 Epub 2007/12/14.
[3] L. He, G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation, Nat.

Rev. Genet. 5 (7) (2004) 522–531 Epub 2004/06/24.
[4] Y.S. Lee, A. Dutta, MicroRNAs in cancer, Annu. Rev. Pathol. 4 (2009) 199–227 Epub 2008/09/27.
[5] Y. Wang, H. Xu, L. Si, Q. Li, X. Zhu, T. Yu, et al., MiR-206 inhibits proliferation and migration of prostate cancer cells by targeting CXCL11, Prostate 78 (7) (2018) 479–490 Epub 2018/03/16.
[6] C. Dai, Y. Xie, X. Zhuang, Z. Yuan, MiR-206 inhibits epithelial ovarian cancer cells growth and invasion via blocking c-Met/AKT/mTOR signaling pathway, Biomed. Pharmacother. 104 (2018) 763–770 Epub 2018/05/29.
[7] Suggestion of diagnosis and treatment of acute myelocytic leukemia in childhood, Zhonghua er ke za zhi = Chin. J. Pediatr. 44 (11) (2006) 877–878 Epub 2007/02/06.
[8] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (4) (2001) 402–408 Epub 2002/02/16.
[9] B.P. Lewis, I.H. Shih, M.W. Jones-Rhoades, D.P. Bartel, C.B. Burge, Prediction of mammalian microRNA targets, Cell 115 (7) (2003) 787–798 Epub 2003/12/31.
[10] X. Lin, Z. Wang, Y. Wang, W. Feng, Serum MicroRNA-370 as a potential diagnostic and prognostic biomarker for pediatric acute myeloid leukemia, Int. J. Clin. Exp. Pathol. 8 (11) (2015) 14658–14666 Epub 2016/01/30.
[11] Z. Hong, R. Zhang, H. Qi, Diagnostic and prognostic relevance of serum miR-195 in pediatric acute myeloid leukemia, Cancer Biomark. 21 (2) (2018) 269–275 Epub 2017/12/12.
[12] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (1) (2000) 57–70 Epub 2000/01/27.
[13] V. Ambros, The functions of animal microRNAs, Nature 431 (7006) (2004) 350–355 Epub 2004/09/17.
[14] V. Baldin, J. Lukas, M.J. Marcote, M. Pagano, G. Draetta, Cyclin D1 is a nuclear protein required for cell cycle progression in G1, Genes Dev. 7 (5) (1993) 812–821 Epub 1993/05/01.
[15] S.J. Elliman, B.V. Howley, D.S. Mehta, H.O. Fearnhead, D.M. Kemp, L.R. Barkley, Selective repression of the oncogene cyclin D1 by the tumor suppressor miR-206 in cancers, Oncogenesis 3 (2014) e113 Epub 2014/08/12.