**ORIGINAL PAPER**

**MiR-320a/b inhibits cell viability and cell cycle progression by targeting aryl hydrocarbon receptor nuclear translocator-like in acute promyelocyte leukaemia**

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Acute promyelocyte leukaemia (APL) is a subgroup of acute myeloid leukaemia. Dysregulation of clock genes has been revealed to be involved in APL progression. Herein, the mechanism of clock gene aryl hydrocarbon receptor nuclear translocator-like (ARNTL) in APL was explored. The expression of ARNTL, period circadian regulator 1 and 2 (PER1 and PER2) in APL tissue samples and normal samples was analysed by bioinformatic analysis. Gene expression in APL cells was detected by reverse transcription quantitative polymerase chain reaction. Acute promyelocyte leukaemia cell viability and cell cycle progression were assessed by cell counting kit 8 (CCK-8) assays and flow cytometry analyses, respectively. The protein levels of ARNTL and cell cycle markers were examined by western blotting. Interaction between ARNTL and miR-320a/b was confirmed by luciferase reporter assays. Aryl hydrocarbon receptor nuclear translocator-like was overexpressed in marrow tissues of patients with acute myeloid leukaemia and predicted poor outcome. Aryl hydrocarbon receptor nuclear translocator-like knockdown inhibited APL cell viability and arrested APL cells in the G1 phase. Mechanically, ARNTL was targeted by miR-320a/b. Moreover, miR-320a/b upregulation promoted cell cycle arrest in the G1 phase and suppressed the viability of APL cells, and the impacts were reversed by ARNTL overexpression. In conclusion, miR-320a/b suppresses cell viability and leads to cell cycle arrest by suppressing ARNTL in APL.

**Key words:** miR-320a/b, ARNTL, acute promyelocyte leukaemia, clock gene.

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**Introduction**

Acute myeloid leukaemia (AML or LAML) is a malignant blood disease [1]. As a subtype of AML, acute promyelocyte leukaemia (APL) was defined as type-M3 of AML [2]. Acute promyelocyte leukaemia ranks first among malignant diseases occurring in young people [3]. During the pathological process of APL, leukaemia cells uncontrollably proliferate in the bone marrow and other haematopoietic tissues and then enter the peripheral blood to inhibit the proliferation of normal cells [4, 5]. Multiple risk factors, such as viruses, genetic predisposition, chemical poisons, radiation, and drugs, can lead to APL [6]. Currently, therapies for APL include induced-differentiation therapy, arsenical therapy, and haematopoietic stem cell transplantation [7]. Despite a deeper understanding of the biological characteristics of APL and improvements in therapeutic approaches, the prognosis of APL patients is still poor [8]. Hence,
exploring the underlying mechanism of APL is of great necessity.

The circadian clock is an endogenous time maintenance system of the body, which is widely present in various organisms and is manifested in various aspects such as metabolism, cell proliferation, physiology, and behaviour [9]. Many epidemiological studies have confirmed that changes in physiological rhythms can cause a high risk of various malignant tumours [10]. In recent years, circadian clock genes have been reported to play an important role in the occurrence and progression of malignant tumours [11]. Clock genes mainly include CLOCK, Per1/2/3, BMAL1, DP, REV-ERB, and Cry1/2 [12]. Aryl hydrocarbon receptor nuclear translocator-like (ARNTL) is also known as BMAL1 [13]. Heterodimers formed by CLOCK and ARNTL can activate transcription of 3 Per (Per1, Per2, Per3) genes and 2 Cry (Cry1, Cry2) by binding to the E box at the promoter site of the Cry and Per genes, and when the corresponding formed proteins reach a certain concentration, feedback at the initiation site inhibits the continuation of transcription [14]. Circadian regulation is critical for normal haematopoiesis, and disruption of rhythm function is thought to potentially cause malignant diseases of the haematological system [15]. The promyelocytic leukaemia (PML) gene plays an important role in the pathogenesis of APL [16]. As a circadian clock regulator, PML can physiologically interact with PER2. PML enhances the transcription of ARNTL/Npas2 and ARNTL/CLOCK by regulating the nuclear distribution of PER2 [17]. Npas2, the abbreviation of neuronal PAS domain protein 2, is a mammalian transcription factor that binds DNA as an obligate dimeric partner of ARNTL and is involved in the regulation of circadian rhythm [18]. Although ARNTL has been reported to be associated with APL [19], its specific mechanism in APL is uncharacterized.

In this study, we aimed to explore the role and underlying mechanism of ARNTL in APL progression, which may provide a new effective therapeutic approach to patients with APL.

Material and methods

Bioinformatic analysis

The expression of ARNTL, period circadian regulator 1 and 2 (Per1 and Per2) in AML samples (n = 173) and normal samples (n = 70) and the expression correlation between Per1 and Per2 in AML marrow tissues were predicted using GEPIA (http://gepia2.cancer-pku.cn/). The potential upstream miRNAs of ARNTL 3'UTR were predicted using ENCORI (http://starbase.sysu.edu.cn/) with screening criteria of CLIP Data ≥ 2, Pan-cancer ≥ 6, and predicted program: microT, miRanda, and PITA. In addition, the relationship between survival of LAML patients and ARNTL expression, the correlation between ARNTL expression and miR-320a (or miR-320b, miR-421) expression in LAML samples (n = 83), and the binding site between miR-320a (or miR-320b) and ARNTL 3'UTR were all predicted with ENCORI.

Cell culture

Acute promyelocyte leukaemia cell lines HL-60 and NB4 (Mingzhou Biotechnology Co. Ltd, Ningbo, China) were cultured in RPMI-1640 medium (Gibco, MA, USA) with 10% foetal bovine serum (Gibco) and maintained in 5% CO₂ at 37°C.

Cell transfection

Short hairpin RNA (shRNA) targeting ARNTL (sh-ARNTL) was used to knock down ARNTL, and its corresponding negative control (NC) was named as sh-NC. Sense sequence of sh-ARNTL: GGTGTGTGTGTAGTTTTTA and antisense sequence of sh-ARNTL: TAAACCTAACAACACAACC. Sense sequence of sh-NC: TGTTGTGTGTAGTTTTTA and antisense sequence of sh-NC: AAACAATCCTACACAAACA. MiR-320a mimics (or miR-320b mimics) were used to overexpress miR-320a (or miR-320b) with NC mimics as a negative control. The full length of ARNTL was planted into the pcDNA3.1 vector to upregulate ARNTL with empty pcDNA3.1 vector as a negative control. Above plasmids were synthesized by GenePharma (Shanghai, China). Afterwards, cell transfection was conducted using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s recommendations, and the transfection efficiency was examined using reverse transcription quantitative polymerase chain reaction (RT-qPCR) after 48 hours.

Cell counting kit 8 assay

After transfection, HL-60 and NB4 cells were seeded in 96-well plates and cultured in RPMI-1640 containing 10% foetal bovine serum. Then, 10 µl of CCK-8 solution was added into each well for 4 hours of incubation at 37°C. Detection of cell viability was conducted at 0, 24, 48, 72, and 96 hours. Absorbance was determined using a microplate reader (BioTeck, CA, USA) at 450 nm.

Reverse transcription quantitative polymerase chain reaction

Total RNA from HL-60 and NB4 cells was extracted by TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Reverse transcription quantitative polymerase chain reaction analysis was carried out using 2X SYBR Green qPCR Master Mix (TaKaRa) on an ABI 7500 Real-Time PCR system (Applied Biosys-
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M A Y M I S A N A N D  A L L O T H E R S

...tems, USA). GAPDH acted as an endogenous control for ARNTL and U6 for miR-320a/b. Gene expression was measured by the 2-ΔΔCt method [20]. The primers used for real-time amplification were as follows: miR-320a: Forward, 5'-ACTATGGAAAGCTTG CGTTGAG-3'; Reverse, 5'-ATTCGTTGAGATCACAAGCGT-3'; miR-320b: Forward, 5'-GTCTGATCCAGTCCAGGG TCCGAGGTATTCGCACTGGATACGAC TTTCC-GAC-3'; Reverse, 5'-TCCGAAACGGGAGAGATGGTG-3'; ARNTL: Forward, 5'-AAATGCAAGGGAAGCT-CAC-3'; Reverse, 5'-GTTGGTACCAAAGAAGCCA-3'; GAPDH: Forward, 5'-CTCAAGATCATCAGCAATGCC-3'; Reverse, 5'-TCAAGATCATCAGCAATGCC-3'; U6: Forward, 5'-ATACAGAGAAAGTTAGCAC-GG-3'; Reverse, 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Western blotting

A bicinchoninic acid protein assay kit (Beyotime, China) was utilized to measure the concentration of proteins extracted from HL-60 and NB4 cells using RIPA lysis buffer. The proteins were loaded on 10% SDS-PAGE and subsequently transferred to PVDF membrane (Millipore, MA, USA). Next, the membranes were cocultured with primary antibodies of anti-Cyclin A1 (ab104617, 1 : 800, abcam, Cambridge, UK), anti-Cyclin B1 (ab32053, 1 : 800, abcam), anti-Cyclin D1 (ab16665, 1 : 800, abcam), anti-Cyclin E1 (ab33911, 1 : 800, abcam), anti-ARNTL (ab258314, 1 : 800, abcam), and anti-GAPDH (ab8245, 1 : 1000, abcam) at 4°C overnight. Then, the membranes were cocultured with secondary antibody horseradish peroxidase-labelled IgG (ab6721, 1 : 1000, abcam) at 4°C for 2 hours. The protein bands were visualized by chemiluminescence kit (PerkinElmer, Waltham) and analysed using ImageJ software (NIH; Bethesda, MA, USA).

Flow cytometry

After being cultured in 6-well plates for 48 hours, HL-60 and NB4 cells were collected, washed with phosphate buffered saline, and then resuspended. The cells were fixed with pre-chilled 70% ethanol, treated with olfactomized ethidium (PI) for 30 min at 37°C in the dark, and filtered once with a 400-mesh screen. This experiment was performed with a flow cytometer (Biosciences, CA, USA), and the percentage of cells at each stage was calculated.

Luciferase reporter assay

Aryl hydrocarbon receptor nuclear translocator-like 3'UTR containing binding sites of miR-320a/b was subcloned into pmirGLO vectors (Promega, Madison, WI, USA) to generate ARNTL 3'UTR-Wt/Mut reporters. All plasmids were synthesized by GenePharma (Shanghai, China). Aryl hydrocarbon receptor nuclear translocator-like 3'UTR Wt/Mut vectors were cotransfected along with NC mimics or miR-320a/b mimics into HL-60 and NB4 cells using Lipofectamine 2000 (Invitrogen). A luciferase reporter assay system (Promega) was applied to test luciferase activity levels after 48 hours of transfection.

Statistical analysis

All experiments were repeated in triplicate, and the results were summarized as the mean ± standard deviation. The difference between 2 groups was analysed by Student's t-test and that among multiple groups was compared using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. GraphPad (Prism 5.0) software was applied to perform statistical analyses. The value of p < 0.05 was considered as statistically significant.

The cell lines used in this study were purchased from (Mingzhou Biotechnology Co. Ltd, Ningbo, China). Ethical approval for the use of these cells is not required in accordance with local/national guidelines.

Results

High expression of aryl hydrocarbon receptor nuclear translocator-like is identified in marrow tissues and predicts poor outcome in patients with acute myeloid leukaemia

According to the results from GEPIA, ARNTL was overexpressed in 173 marrow tissues of LAML patients compared with that in 70 normal marrow tissues (Fig. 1A). Because ARNTL was reported to regulate the transcription of PER1 and PER2, the expression of PER1 and PER2 in LAML marrow tissues and normal marrow tissues was also analysed by GEPIA, which revealed that PER1 and PER2 were highly expressed in marrow tissues of LAML patients (Fig. 1B–C). In addition, high expression of ARNTL predicted poor prognosis of patients diagnosed with LAML (Fig. 1D). GEPIA also indicated that PER1 expression was positively correlated with PER2 expression in LAML marrow tissues (Fig. 1E). In summary, ARNTL is over expressed in marrow tissues of LAML patients, and its high expression predicts poor outcome. The finding suggests that ARNTL is associated with APL progression.

Aryl hydrocarbon receptor nuclear translocator-like knockdown inhibits acute promyelocyte leukaemia cell proliferation and arrests acute promyelocyte leukaemia cells in the G1 phase

Subsequently, the functions of ARNTL in APL cells were investigated. Reverse transcription quan-
Quantitative polymerase chain reaction and western blotting showed that the mRNA and protein levels of ARNTL were downregulated after transfection of sh-ARNTL in HL-60 and NB4 cells (Fig. 2A–C). According to CCK-8 assays, the viability of HL-60 and NB4 cells was suppressed by ARNTL knockdown (Fig. 2D). Flow cytometry analyses demonstrated that ARNTL silencing significantly increased the percentage of HL-60 and NB4 cells in the G1 phase (Fig. 2E–F). Furthermore, levels of cell cycle-associated proteins (Cyclin A1, Cyclin B1, Cyclin D1, and Cyclin E1) were decreased by ARNTL downregulation in HL-60 and NB4 cells, as shown by western blotting (Fig. 2G). All these findings suggest that ARNTL knockdown inhibits APL cell viability and arrests APL cells in the G1 phase, thus suppressing APL cell proliferation.

**Aryl hydrocarbon receptor nuclear translocator-like is targeted by miR-320a/b**

To explore the mechanism of ARNTL in APL, ENCORI was used to predict the potential upstream miRNAs of ARNTL, and 3 potential miRNAs (miR-320a, miR-320b, and miR-421) were identified (Supplementary Table 1). Based on the results from ENCORI, miR-320a/b expression was negatively correlated with ARNTL expression in LAML patients (n = 83), while there was no significant correlation between miR-421 expression and ARNTL expression in tissues of patients with LAML (Fig. 3A–C).
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**Fig. 2.** Aryl hydrocarbon receptor nuclear translocator-like (ARNTL) knockdown inhibits acute promyelocyte leukaemia (APL) cell proliferation and arrests APL cell cycle in the G1 phase. After knocking down ARNTL in HL-60 and NB4 cells. A–C) The mRNA and protein levels of ARNTL were tested by reverse transcription quantitative polymerase chain reaction and western blotting. D) The viability of HL-60 and NB4 cells was assessed by cell counting kit 8 assays. E) Cell cycle phase data in HL-60 and NB4 cells were analysed by flow cytometry.


* p < 0.05, *** p < 0.001
Thus, miR-320a and miR-320b were identified for further study. Next, miR-320a/b was overexpressed via transfection of miR-320a/b mimics. As suggested by RT-qPCR, the expression levels of miR-320a and miR-320b were markedly upregulated in the miR-320a/b mimics group compared with those in the NC mimics group (Fig. 3D–E). Furthermore, elevation of miR-320a/b decreased ARNTL expression in HL-60 and NB4 cells (Fig. 3F). Afterwards, luciferase reporter assays were conducted to explore the binding capacity between miR-320a (or miR-320b) and ARNTL 3’UTR. The binding site between miR-320a (or miR-320b) and ARNTL 3’UTR was predicted using ENCORI (Fig. 3G–H). Luciferase reporter assay revealed that miR-320a/b upregulation decreased the luciferase activity of ARNTL 3’UTR-Wt, while that of ARNTL 3’UTR-Mut was not significantly altered in APL cells transfected with miR-320a/b mimics (Fig. 3I–J). Therefore, miR-320a/b can bind with ARNTL. Moreover, the protein level of ARNTL was also decreased by miR-320a/b overexpression in HL-60 and NB4 cells according to the results of western blotting (Fig. 3K). Overall, ARNTL binds to miR-320a/b and is negatively regulated by miR-320a/b in APL cells.

miR-320a/b inhibits acute promyelocyte leukaemia cell viability and cell cycle progression by decreasing aryl hydrocarbon receptor nuclear translocator-like expression

Subsequent experiments were carried out to explore the impact of miR-320a/b on HL-60 cell viability and cell cycle progression in response to ARNTL overexpression or not. As shown by Figure 4A–C, the mRNA and protein levels of ARNTL were upregulated after transfection of pcDNA3.1/ARNTL vectors. Cell counting kit 8 assays showed that the inhibitory effect of miR-320a/b elevation on the viability of HL-60 cells was partially attenuated by ARNTL overexpression (Fig. 4D–E). Flow cytometry analyses indicated that miR-320a/b overexpression promoted APL cell cycle arrest in the G1 phase, and the promoting effect was reversed by overexpressing ARNTL (Fig. 4F–I). Additionally, elevated miR-320a/b expression reduced levels of cell cycle-associated proteins (Cyclin A1, Cyclin B1,
Fig. 3. Aryl hydrocarbon receptor nuclear translocator-like (ARNTL) is targeted by miR-320a/b. A–C Correlation between ARNTL expression and miR-421 (or miR-320a/b) expression in LAML samples (n = 83) was analysed by ENCORI. D–E Transfection efficiency of miR-320a/b mimics in acute promyelocyte leukaemia (APL) cells was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR).
**Fig. 3.** F) ARNTL expression in APL cells after overexpressing miR-320a/b was tested by RT-qPCR. G–H) The binding site of miR-320a (or miR-320b) for ARNTL 3’UTR was predicted using ENCORI. After overexpressing miR-320a/b in APL cells. I–J) The binding relationship between miR-320a/b and ARNTL was validated by luciferase reporter assays. K) Protein levels of ARNTL in APL cells were detected by western blotting

* **p < 0.01, *** p < 0.001*

**ARNTL** – aryl hydrocarbon receptor nuclear translocator-like, **LAML** – acute myeloid leukaemia, **NC** – negative control
miR-320a/b inhibits cell viability and cell cycle progression by targeting aryl hydrocarbon receptor nuclear translocator-like (ARNTL) expression. A–C) Transfection efficiency of pcDNA3.1/ARNTL was evaluated by reverse transcription quantitative polymerase chain reaction and western blotting. After overexpressing ARNTL or miR-320a/b. D–E) The viability of HL-60 cells was assessed by cell counting kit 8 assays. F–I) Cell cycle phase data in HL-60 cells was analysed by flow cytometry. J) Levels of cell cycle-associated proteins (Cyclin A1, Cyclin B1, Cyclin D1, and Cyclin E1) in HL-60 cells were quantified by western blotting.

* p < 0.05, *** p < 0.001
Cyclin D1, and Cyclin E1) in HL-60 cells, and the decrease in protein levels of cell cycle markers was offset by upregulation of ARNTL (Fig. 4J). In conclusion, ARNTL overexpression reverses the inhibitory impact on APL cell viability and the promoting impact on cell cycle arrest in the G1 phrase caused by miR-320a/b upregulation. The finding revealed that miR-320a/b inhibits APL cell viability and cell cycle progression by decreasing ARNTL expression.

**MiR-320a/b inhibits cell proliferation by suppressing aryl hydrocarbon receptor nuclear translocator-like in acute promyelocyte leukaemia**

A schematic diagram was used to describe the mechanism of miR-320a/b in APL cells. As Figure 5 indicates, miR-320a/b directly binds to ARNTL in APL cells, and ARNTL can promote cell proliferation via enhancement of cell cycle progression. Thus, miR-320a/b inhibits cell proliferation by inducing APL cell cycle arrest in the G1 phase via targeting of ARNTL.

**Discussion**

Acute promyelocyte leukaemia is a severe malignancy of the haematological system [21]. Because desynchrony of cell proliferation and metabolic rhythm often occurs in normal tissues and malignant tissues, disruption of circadian rhythms is closely related to tumourigenesis [22]. As a key gene in the circadian clock, ARNTL widely participates in various haematological malignancies [23]. Epigenetic inactivation of ARNTL leads to leukaemia progression by impairing the characteristic expression pattern of other clock genes [24]. In haematological malignancies, such as acute lymphocytic leukaemia and diffuse large B-cell lymphoma, ARNTL (BMAL1) is silenced by promoter CpG island hypermethylation at the transcriptional level [25]. Reintroduction of ARNTL inhibits proliferation of hypermethylated leukaemia/lymphoma cells, while ARNTL knockdown has the opposite effect on the cells [25]. In this study, we mainly investigated the functions and mechanism of ARNTL in APL development. First, we identified...
the dysregulation of ARNTL in AML or LAML by bioinformatics analysis, which showed that ARNTL expression was significantly higher in LAML samples than that in the normal samples. Because ARNTL can regulate the expression of Per genes, we also use GEPIA to analyse PER1/2 expression correlation in LAML tissues. Our results showed that PER1 and PER2 were highly expressed in marrow tissues, and PER1 expression was positively correlated with PER2 expression in marrow tissues of LAML patients. Notably, high expression of ARNTL was associated with poor prognosis of LAML patients, suggesting that ARNTL is involved in APL progression. Subsequently, the functional role of ARNTL in APL cell lines was explored. We found that ARNTL knockdown inhibited APL cell viability and arrested APL cell cycle in the G1 phase. Therefore, ARNTL promotes APL cell proliferation by promoting cell cycle progression.

MicroRNAs (MiRNAs) are small noncoding RNA molecules 19–25 nucleotides in length [26]. MiRNA interacts with the 3’UTR of targeted mRNA to negatively regulate gene expression, thus suppressing the degradation and translation of the target gene [27]. Accumulating studies have revealed the involvement of various miRNAs in the pathogenesis of APL [28–31]. In the current study, potential upstream miRNAs of ARNTL were predicted by bioinformatics analysis, and miR-320a and miR-320b were shown to bind to ARNTL in APL cells. MiR-320a and miR-320b are 2 clusters of the miR-320 family [32]. MiR-320 is markedly downregulated in patients with chronic myeloid leukaemia (CML) [33] and inhibits CML progression [34]. MiR-320 targets TfR-1 to suppress the proliferation of acute myelogenous leukaemia (AML) [35]. MiR-320a targets pre-B-cell leukaemia transcription factor 3 to induce multiple myeloma cell growth inhibition and apoptosis [36]. MiR-320b induces cell cycle arrest and apoptosis, and inhibits viability of AML cells [37]. As a tumour suppressor, miR-320a has an inhibitory effect on cell migration, invasion, proliferation, and epithelial-mesenchymal transition processes in CML development [38]. Herein, miR-320a expression and miR-320b expression were negatively correlated with ARNTL expression in LAML samples, suggesting that miR-320a/b expression was downregulated in marrow tissues of LAML patients. Moreover, miR-320a/b overexpression inhibited APL cell proliferation and induced cell cycle arrest in the G1 phase, and the effects were reversed by ARNTL overexpression.

Conclusions

MiR-320a/b suppresses APL cell proliferation by targeting ARNTL in APL. Specifically, miR-320a/b induced APL cell cycle arrest in the G1 phase by binding with ARNTL, subsequently inhibiting APL cell proliferation. Our study highlights that the miR-320a/b/ARNTL regulatory axis may be a novel therapeutic target for APL treatment. However, some limitations in this study cannot be ignored. First, normal samples and LAML samples used in bioinformatics analysis are relatively small. Second, in vivo studies focusing on the miR-320a/b-ARNTL network were necessary for further investigation, which will be conducted in our future studies.

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The authors declare no conflict of interest.
References

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