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Extracellular vesicles derived from T-cell acute lymphoblastic leukemia inhibit osteogenic differentiation of bone marrow mesenchymal stem cells via miR-34a-5p

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Abstract. Reduced bone formation in patients with T-cell acute lymphoblastic leukemia (T-ALL) may be related to the interaction between tumour cells and bone marrow stromal cells (BMSCs). The miRNAs in extracellular vesicles derived from leukemia cells play an essential role in regulating the function of BMSCs; however, the regulatory mechanisms remain unclear. The expression of miR-34a-5p in T-ALL patients and cells was measured by quantitative real-time PCR. BMSCs were co-cultured with extracellular vesicles isolated from T-ALL cells in mineralization medium. The osteogenic differentiation of BMSCs was evaluated by Alizarin Red S staining, alkaline phosphatase (ALP) staining, and detection of osteogenic differentiation markers. A dual-luciferase reporter assay was performed to confirm the targeting relationship between miR-34a-5p and Wnt family member 1 (WNT1). MiR-34a-5p expression was upregulated in T-ALL patients and Jurkat cells. After BMSCs were co-cultured with extracellular vesicles derived from T-ALL cells, the osteogenic differentiation of BMSCs was inhibited, and bone mineralization and ALP activity were decreased compared to those of control cells. MiR-34a-5p knockdown in T-ALL cells restored osteogenic differentiation of BMSCs co-cultured with extracellular vesicles. In addition, miR-34a-5p targets and negatively regulates WNT1 expression. In conclusion, our results demonstrated that knockdown of miR-34a-5p in extracellular vesicles derived from T-ALL cells promoted osteogenic differentiation of BMSCs by regulating WNT1.

Key words: T-cell acute lymphoblastic leukemia, Bone marrow stromal cells, Extracellular vesicles, MicroRNA-34a-5p, Osteogenic differentiation

T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) is an aggressive circulatory tumour formed by abnormal proliferation of precursor lymphoid T-cells [1], which transforms T-lymphocyte precursor cells into malignant T-ALL precursor cells [2, 3]. Abnormal immune cells in T-ALL destroy normal bone formation, that is, the osteogenic differentiation of bone marrow stromal cells (BMSCs) [4]. Osteoblasts are important participants in bone formation and play a significant role in maintaining haematopoietic function [5]; however, the mechanisms underlying how T-ALL cells disrupt the osteogenic differentiation of BMSCs remain unknown.

Extracellular vesicles are secreted by malignant tumour cells and serve as an important medium for tumour cells to communicate with the extracellular microenvironment and support their survival [6, 7]. There are special selection strategies for the secretion of extracellular vesicles from tumour cells and the incorporation of selected DNA, mRNA, miRNA and proteins into extracellular vesicles [8, 9]. T-ALL cells attach to stromal cells in the bone marrow environment so that tumour cells can survive and proliferate in the environment of normal cells [10, 11]. Leukemia-derived extracellular vesicles can transfer miRNA to BMSCs to change their characteristics, such as inhibiting the adhesion ability of BMSCs

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and activating the NF-κB signaling pathway [12]. Acute myeloid leukemia cells associate with BMSCs through extracellular vesicles to reduce the sensitivity of tumour cells to etoposide [13]. Histone deacetylase-mediated extracellular vesicle secretion contributes to the survival of myeloma cells in the bone marrow microenvironment [13]. BMSCs have the capacity to differentiate into different tissue lineages, such as fat, cartilage and bone [14]. Increasing bone mass by stimulating osteoblast differentiation of BMSCs is a common approach [15]. IncRUNX2-AS1 derived from the extracellular vesicles of myeloma cells has been shown to inhibit the osteogenesis of MSCs [16]. Another study indicated that hsa-miR-940 extracellular vesicles secreted by prostate cancer cell lines promoted the osteogenic differentiation of mesenchymal target cell cells [17]; however, the effects of extracellular vesicles derived from leukemia on the osteogenic differentiation of BMSCs have not been reported.

microRNAs (miRNAs) are non-coding RNAs with a length of 19–25 nucleotides [18]. MicroRNAs generally bind to the 3’-UTR of the target gene to negatively regulate its transcription [19]. The miR-34a-5p-Notch1 pathway plays a central role in osteoblast differentiation, which reduces the differentiation of bone marrow-derived monocytes in osteoclasts [20]. In addition, miR-34a-5p from extracellular vesicles from human amniotic epithelial cells stimulated osteoblast differentiation and promoted cell migration [21]. Through bioinformatics analysis, miR-34a-5p and WNT1 were predicted to have a targeted binding site. The Wnt/β-catenin pathway is essential for maintaining bone homeostasis [22]. Abnormal blockade of Wnt signaling in BMSCs will impair bone formation and femoral remodelling function [23]. Studies have also shown that osteogenic differentiation is promoted by Wnt pathway activation [24], suggesting the involvement of Wnt signaling in BMSC differentiation.

The current study aimed to explore the effects of miR-34a-5p in extracellular vesicles derived from T-ALL cells on the osteogenic differentiation of BMSCs. These findings provide a theoretical basis for a better understanding of the occurrence and development of T-ALL.

Materials and Methods

Clinical samples

Fasting peripheral blood samples were obtained in the morning from 30 T-ALL patients and 30 healthy subjects between May 2019 and March 2020. After centrifugation, the serum was separated and kept at –80°C for further use. None of the participants received treatment prior to blood collection. This study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University Hospital. Written informed consent was obtained from all subjects.

Cell culture

Five human T-ALL cell lines (Loucy, Jurkat, CCRF-CEM, KOPT-K1, and DND-41) were obtained from ATCC (Manassas, USA) and maintained in RPMI-1640 medium (Sigma, Saint-Louis, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin at 37°C with 5% CO₂. Human primary peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy volunteers from our hospital as previously described [25] and cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS. Human BMSCs (Shanghai Institute of Biological Sciences, Shanghai, China) were cultured in α-MEM (Sigma) containing 1% antibiotics and 10% FBS at 37°C with 5% CO₂. BMSCs subjected to osteogenic differentiation were cultured in mineralization medium containing β-glycerophosphate (10 mmol/L), dexamethasone (10−8 mol/L), and vitamin C (50 mg/L) for 14 days. The medium was changed every three days.

Plasmids and transfection

The WNT1 gene was inserted into the pcDNA3.1 vector (Invitrogen, Carlsbad, USA). The miR-34a-5p mimics and corresponding mimics control were purchased from GenePharma (Shanghai, China). Cells were transfected with vectors carrying the WNT1 gene (or empty vectors) or miR-34a-5p mimics (or mimic controls) using Lipofectamine™ 2000 Reagent (Invitrogen). Cells were collected 24 h post transfection, and transfection efficiency was confirmed by quantitative real-time PCR (qRT-PCR).

MTT assay

The proliferation of Jurkat cells was examined using the MTT assay (Roche, Mannheim, Germany). The cells were plated in 96-well plates (5 × 10³ cells/well) and transfected with designated plasmids. At 0 h, 24 h, 48 h, and 72 h post transfection, the cells were treated with 10 μL of 0.5 mg/mL MTT solution and cultured at 37°C for 3 h. Then, 150 μL of DMSO was added to determine the reaction. The absorbance was measured at 490 nm using a microplate reader (R&D Systems, Minneapolis, USA).

Isolation and identification of extracellular vesicles

T-ALL cells were cultured in 100-mm Petri dishes for 24 h. Then, serum-free culture medium was collected.
and centrifuged at 2,000 g for 20 min to remove cell debris. After ultracentrifugation at 20,000 g for 1.5 h and final centrifugation at 160,000 g for 3 h, extracellular vesicles were obtained. The isolated extracellular vesicles were then resuspended in filtered phosphate-buffered saline (PBS) and prepared for nanoparticle analysis. The extracellular vesicles were visualized by transmission electron microscopy (TEM; JOEL, Akishima, Japan). qNano (Izon Science, Medford, USA) was used to determine the size of extracellular vesicles. The expression levels of extracellular vesicles markers, including TSG-101, CD63, and GM130, were measured by Western blot.

**Alizarin Red S (ARS) staining**

To assess bone mineralization, BMSCs were incubated with ARS staining solution (Cyagen Bioscience, Santa Clara, USA) for 25 min and then observed under a microscope (Nikon, Tokyo, Japan). Subsequently, BMSCs were treated with 100 μM cetylpyridinium chloride (Sigma) for 60 min. The absorbance was measured at 570 nm using a microplate reader (Tecan Group, Zurich, Switzerland).

**Alkaline phosphatase (ALP) staining**

BMSCs were fixed with 95% ethanol and stained with ALP solution for 4 h at 37°C. Then, 2% cobalt nitrate (Tianjin Chemical Reagents, Tianjin, China) and ammonium sulfide (Tianli Chemical Reagents, Tianjin, China) were added to the cells which were then observed under a microscope (Nikon). The BMSCs were then incubated with 10 μM p-nitrophenyl phosphate (Meilunbio, Dalian, China) for 30 min. The absorbance was measured at 420 nm using a spectrophotometer.

**Dual-luciferase reporter detection**

A putative binding site between miR-34a-5p and WNT1 was identified by TargetScan (www.targetscan.org). The mutant (MUT) or wild-type (WT) binding site of miR-34a-5p for WNT1 was inserted into the pmirGLO luciferase vector (Promega, Madison, USA). BMSCs were co-transfected with vectors carrying WNT1-WT (or WNT1-MUT) and miR-34a-5p mimics (or mimicsNC). Luciferase activity was detected 48 h post transfection.

**Quantitative Real-time-PCR (qRT-PCR)**

Total RNA was extracted from the serum samples and cell lysates using TRIzoL™ Plus (Invitrogen). Reverse transcription was performed using a reverse transcription kit (Takara, Otsu, Japan). The ABI 7500HT fast RT-PCR system (Applied Biosystems, CA, USA) was used for qRT-PCR according to the following procedures: 95°C for 3 min, 95°C for 15 cycles of 15 s, 60°C for 30 s, and 72°C for 20 s. U6 and β-actin were used as internal references for miR-34a-5p or other proteins, respectively. The primers used in this experiment are shown in Table 1.

**Western blot analysis**

Total protein was isolated from cells using RIPA buffer (Sigma). Protein concentration was determined by bichinonic acid assay (Sigma). Equal amounts (50 μg) of protein samples were separated by 10% SDS-PAGE and then transferred to PVDF membranes. After a one-hour incubation with 5% skimmed milk powder in TBST, the membranes were stained with anti-mouse monoclonal antibodies (Abcam, Cambridge, UK): WNT1 (1:1000; ab15251), β-catenin (1:1000; ab6302), BMP2 (1:1000; ab14933), RUNX2 (1:1000; ab15251), TSG-101 (1 μg/mL; ab30871), CD63 (1:1000; ab59479), OCN (1:1000; ab93876), OPN (1:1000; ab8448), GM130 (1:1000; ab52649), and GAPDH (1:800; ab8245) at 4°C overnight and then incubated with a secondary antibody (1:10000; catalogue no. ASP00001; Agilent Technologies, Santa Clara, USA) at room temperature for 1 h. An ECL system (Pierce, Appleton, USA) was used to observe and analyze the immunoblots. Protein expression was quantified using Fusion Solo (version 4) software (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). GAPDH was used as an internal reference.

### Table 1 Primers used in qRT-PCR

| Gene   | Primers                                                                 |
|--------|-------------------------------------------------------------------------|
| miR-34a-5p | F 5'-AACAATTCAACTCCAGCTGTGACTGGTGTGCCGACCAGA-3'  
   | R 5'-CTCAACTGGTGTGCCGACCAGA-3'                                         |
| Wnt1   | F 5'-GCTTGCTTGCCTCGACTTGTGTCGTTCCATCGAATCCTGCAC-3'  
   | R 5'-GGTTCCATCGAATCCTGCAC-3'                                           |
| BMP2   | F 5'-TGTCACGACGAGGAGGCCAGAAGAGG-3'                                     |
|         | R 5'-TGTCACGACGAGGAGGCCAGAAGGG-3'                                      |
| RUNX2  | F 5'-GGTGCAGTGATTTGCTTTTG-3'                                           |
|         | R 5'-TGGTGCAGTGATTTGCTTTTG-3'                                          |
| OCN    | F 5'-GCCAGCGAGGTAGTGGAAGAG-3'                                          |
|         | R 5'-CTGGAGAGGAGGCCAGAAGGG-3'                                          |
| OPN    | F 5'-GGTGAGGAGGAGGCCAGAAGGG-3'                                         |
|         | R 5'-CTGGAGAGGAGGCCAGAAGGG-3'                                          |
| PPARy  | F 5'-GGACTTCTCGGAGGCAGAC-3'                                            |
|         | R 5'-GGACTTCTCGGAGGCAGAC-3'                                            |
| FABP4  | F 5'-GCGAAGCTCCATCGAATCGCTTCTGG-3'                                     |
|         | R 5'-GCGAAGCTCCATCGAATCGCTTCTGG-3'                                     |
| U6     | F 5'-GGTGCTATCCAGGCTGT-3'                                              |
|         | R 5'-GGTGCTATCCAGGCTGT-3'                                              |
| β-actin| F 5'-ACGAGAGGAGGCCAGAAGGG-3'                                           |
|         | R 5'-ACGAGAGGAGGCCAGAAGGG-3'                                           |
Statistical analysis

Data are presented as the mean ± SD from three independent experiments. Statistical analyses (Student’s t-test or one-way analysis of variance) were performed using SPSS (version 17.0) software (SPSS Inc., Chicago, USA). Chi-square test and Student’s t-test were used to compare the clinical characteristics (categorical variables and continuous variables, respectively) of patients with low or high miR-34a-5p expression. A p-value of <0.05 was considered statistically significant.

Results

MiR-34a-5p expression in T-ALL patients and T-ALL cell lines

To explore the regulatory effect of miR-34a-5p on human BMSCs, we first measured the expression of miR-34a-5p in the peripheral blood of T-ALL patients and T-ALL cell lines (DND-41, KOPT-K1, Loucy, Jurkat, and CCRF-CEM). The results showed that the serum level of miR-34a-5p in T-ALL patients was significantly higher than that in healthy subjects (Fig. 1A). Compared to primary human PBMCs, the expression of miR-34a-5p was significantly upregulated in all T-ALL cells, except the DND-41 cell line. Among all T-ALL cells tested, the highest miR-34a-5p expression was observed in Jurkat cells (Fig. 1B). Then, we analyzed the relationship between miR-34a-5p expression and patients’ clinical characteristics in Table 2 and Supplementary Table 1. High miR-34a-5p expression was positively associated with white blood cell count and risk classification, while no significant correlation was found between miR-34a-5p expression and patients’ age, gender, bone marrow blasts, platelet count, hemoglobin, or lactate dehydrogenase level. These findings suggest the involvement of miR-34a-5p in the progression of T-ALL.

Effect of T-ALL cell-derived extracellular vesicles on osteogenic differentiation of hBMSCs

To investigate whether extracellular vesicles derived from T-ALL cells would affect the biological behaviours

Table 2 Association between miR-34a-5p expression and clinical features of T-ALL patients

| Clinical features | miR-34a-5p | p value |
|-------------------|------------|---------|
| Gender            |            | 0.558   |
| Male              | 9          | 4       |
| Female            | 10         | 7       |
| Age (years)       |            | 0.510   |
| <5                | 11         | 5       |
| ≥5                | 8          | 6       |
| WBC count (×10⁹/L)|            | 0.020*  |
| <50               | 4          | 7       |
| ≥50               | 15         | 4       |
| Platelet count (×10⁹/L, Median) | 0.592 | |
| <100              | 12         | 8       |
| ≥100              | 7          | 3       |
| Hemoglobin (g/L)  |            | 0.389   |
| <110              | 10         | 4       |
| ≥110              | 9          | 7       |
| LDH – Median (U/L)|            | 0.216   |
| <250              | 6          | 6       |
| ≥250              | 13         | 5       |
| Risk Group        |            | 0.019*  |
| Low-risk          | 1          | 3       |
| Medium-risk       | 3          | 5       |
| High-risk         | 15         | 3       |

Note: * p < 0.05.
of BMSCs, we isolated extracellular vesicles from the culture medium of Jurkat cells. The extracellular vesicles were first visualized by TEM (Fig. 2A) and then analyzed for extracellular vesicle markers, including TSG-101, CD63, and GM130, by Western blot. The results showed that TSG-101 and CD63, but not GM130, were stably expressed in extracellular vesicles derived from T-ALL cells (Fig. 2B). The size of the isolated extracellular vesicles ranged from 30 nm to 120 nm (Fig. 2C). Then, BMSCs were cultured in mineralization medium to induce osteogenic differentiation with or without exposure to T-ALL cell-derived extracellular vesicles. The expression levels of osteogenic differentiation markers (i.e., BMP2, RUNX2, OCN, and OPN) in BMSCs co-cultured with extracellular vesicles were significantly lower than those in the control group (Fig. 2D–E). In addition, exposure to extracellular vesicles significantly reduced bone mineralization in BMSCs (Fig. 2F) and decreased ALP activity (Fig. 2G). These results indicate that T-ALL cell-derived extracellular vesicles suppress the osteogenic differentiation of BMSCs.

Knockdown of miR-34a-5p in extracellular vesicles derived from T-ALL cells promotes osteogenic differentiation of BMSCs

Extracellular vesicles have been shown to carry a large group of lipids, proteins, DNAs, and miRNAs that can be transferred from donor to recipient cells. Moreover, the
above results indicated that miR-34a-5p was implicated in the pathogenesis of T-ALL. To elucidate the regulatory mechanism underlying the effects of T-ALL cell-derived extracellular vesicles on BMSC osteogenic differentiation, we transfected Jurkat cells with a miR-34a-5p inhibitor, which significantly downregulated miR-34a-5p in both Jurkat cells and secreted extracellular vesicles (Fig. 3A). We further analyzed the expression of WNT1 and β-catenin, key components of Wnt signaling, inBMSCs. Co-culture with extracellular vesicles significantly downregulated the expression of both WNT1 and β-catenin in BMSCs, indicating the inhibition of Wnt signaling (Fig. 3A–B); however, the inhibition of miR-34a-5p significantly upregulated WNT1 mRNA and protein expression in BMSCs co-cultured with extracellular vesicles (Fig. 3A–B). The protein level of β-catenin in miR-34a-5p inhibitor-transfected Jurkat cells was also significantly increased (Fig. 3B), indicating the activation of Wnt signaling. When BMSCs were co-cultured with extracellular vesicles derived from Jurkat cells transfected with the miR-34a-5p inhibitor, the mRNA and protein expression of osteogenic differentiation markers was significantly elevated compared to the group exposed to extracellular vesicles with normal miR-34a-5p expression (Fig. 3C–D). ARS staining showed that extracellular vesicles secreted from cells with miR-34a-5p deficiency promoted mineralization in BMSCs (Fig. 3E). The ALP activity test revealed that miR-34a-5p knockdown contributed to increased enzyme activity in BMSCs (Fig. 3F). We further analyzed the mRNA expression of the adipogenic markers peroxisome proliferator-activated receptor gamma (PPARγ) and fatty acid binding protein 4 (FABP4). The results showed that co-culture with extracellular vesicles significantly upregulated PPARγ and FABP4 expression in BMSCs cells compared to the control group, while the inhibition of miR-34a-5p significantly downregulated the expression of both genes in BMSCs cells co-cultured with extracellular vesicles (Supplementary Fig. 1A). Moreover, we detected the effect of miR-34a-5p inhibition on Jurkat cell proliferation by the MTT assay. The results showed that the knockdown of miR-34a-5p significantly inhibited Jurkat cell proliferation (Supplementary Fig. 1B). Taken together, the results demonstrate that miR-34a-5p deficiency induces osteogenic differentiation of BMSCs.

**MiR-34a-5p negatively regulates WNT1 expression**

To further explore the regulatory mechanism of T-ALL cell-derived exosomal miR-34a-3p in osteogenic differentiation, we used TargetScan to predict the downstream target of miR-34a-3p. WNT1 was a target gene of miR-34a-3p with a putative binding site (Fig. 4A). To confirm the binding relationship between miR-34a-3p and WNT1, we performed a dual-luciferase reporter assay by co-transfecting BMSCs cells with luciferase plasmids carrying WNT1-WT (or WNT1-MUT) and miR-434a-3p mimics (or mimics control). Transfection with miR-34a-3p mimics significantly reduced the luciferase activity of the WNT1-WT reporter but had no effect on that of the WNT1-MUT reporter (Fig. 4B). Moreover, the inhibition of miR-34a-5p in T-ALL cells significantly upregulated WNT1 at both the mRNA (Fig. 4C) and protein levels (Fig. 4D). The above data indicate that miR-34a-5p targets and negatively regulates WNT1 expression.

**miR-34a-5p targets WNT1 to regulate osteogenic differentiation**

To determine whether miR-34a-5p regulated BMSC osteogenic differentiation by targeting WNT1, a rescue experiment was performed. We co-transfected BMSCs with miR-34a-5p mimics (or mimics control) and vectors overexpressing WNT1 (or empty vectors). miR-34a-5p upregulation significantly suppressed WNT1 expression in BMSCs, while WNT1 overexpression effectively restored the level of WNT1 without affecting miR-34a-5p mRNA expression (Fig. 5A). We also found that WNT1 overexpression partially but significantly restored the downregulation of osteogenic differentiation markers and β-catenin in BMSCs overexpressing miR-34a-5p (Fig. 5B). Moreover, miR-34a-5p overexpression significantly downregulated the level of β-catenin in BMSCs, while WNT1 upregulation effectively restored β-catenin expression in cells overexpressing miR-34a-5p, indicating the activation of Wnt/β-catenin signaling (Fig. 5C). Additionally, the upregulation of WNT1 also significantly reversed mineralization and ALP activity in cells overexpressing miR-34a-5p (Fig. 5D–E). These data support that miR-34a-5p inhibits BMSC osteogenic differentiation by regulating WNT1.

**Discussion**

Despite substantial progress made by chemotherapy in recent decades, the recurrence rate, metastasis rate and prognosis of T-ALL remain worse than those of other ALLs [26]. The inhibition of BMSC differentiation by abnormal immune cells is a key feature of T-ALL [27]. Therefore, new treatment strategies targeting the osteogenic differentiation of BMSCs are urgently needed for T-ALL patients. Our study showed that miR-34a-5p was highly upregulated in T-ALL patients and cell lines. Additionally, miR-34a-5p inhibited BMSC differentiation by targeting WNT1.

Extracellular vesicles are bridges between cells. The proteins and RNA within microvesicles are secreted out
Fig. 3  Knockdown of miR-34a-5p in extracellular vesicles derived from T-ALL cells promotes osteogenic differentiation of BMSCs.

T-ALL cells were transfected with miR-34a-5p inhibitor (or inhibitor control) followed by exosome isolation. BMSCs were co-cultured with T-ALL cell-derived extracellular vesicles for 14 days. (A) The expression levels of miR-34a-5p and WNT1 were examined by qRT-PCR. (B) The protein levels of WNT1 and β-catenin were examined by Western blot. The (C) mRNA and (D) protein levels of osteogenic differentiation markers (i.e., BMP2, RUNX2, OCN, OPN) were measured by qRT-PCR and Western blot. (E) ARS staining was used to evaluate mineralization in BMSCs. (F) ALP staining was performed to detect ALP activity in BMSCs. Data are shown as the mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.01.
of the cell through endocytosis [28]. Extracellular vesicles secreted by leukemia and other tumors play a critical role in promoting the survival, proliferation and metastasis of tumor cells by interfering with other normal cells in the microenvironment [29]. BMSCs are a type of bone marrow stem cells that mainly differentiate into osteoblasts to promote bone formation and maintain bone homeostasis [30]. The differentiation of BMSCs relies on intercellular communication with neighbouring cells or extracellular vesicle signaling between osteoblasts [31-33]. Here, we found that the coculture of extracellular vesicles isolated from T-ALL cells with BMSCs significantly reduced bone mineralization and the expression of osteogenic differentiation markers, suggesting the inhibitory effect of extracellular vesicles on the osteogenic differentiation of BMSCs.

MiR-34a, as a post-transcriptional regulator, is expressed in a variety of leukemia cells and other tumor cells [34, 35]. Additionally, miR-34a may be used as a predictive marker for the future development of Richter syndrome in patients with chronic lymphocytic leukemia [36]. In T cells infected with T gonorrhea virus 1, miR-34a isolation resulted in increased tumor cell death [37]. Similarly, we found highly expressed miR-34a-5p in the serum samples of T-ALL patients and all T-ALL cell lines. Mesenchymal stem cells are originally found in the bone marrow and can differentiate into stem cell populations of multiple lineages in vivo [38]. Here, we found that extracellular vesicles without miR-34a-5p had no inhibitory effect on BMSC differentiation, indicating that T-ALL-derived extracellular vesicles inhibited the osteogenic differentiation of BMSCs through miR-34a-5p. BMSCs not only have a high capacity for osteogenic differentiation but also possess the potential for adipogenic and neurogenic differentiation [39]. The levels of osteogenic and adipogenic markers in BMSCs are negatively correlated [40]. PPARγ is an adipogenic marker that has been shown to negatively regulate the differentiation of BMSCs into fibroblasts [41]. FABP4 is a carrier protein for fatty acids that has been identified as a marker associated with adipogenesis in BMSCs [42]. Here, we further analyzed the mRNA expression of PPARγ and FABP4. The data showed that co-culture with extracellular vesicles significantly upregulated PPARγ and FABP4 in BMSCs compared to the control group, while miR-34a-5p inhibition significantly decreased the expression of both PPARγ and FABP4 in BMSCs co-cultured with extracellular vesicles (Supplementary Fig. 1A). These results suggested a negative correlation between the osteogenic and adipogenic differentiation of BMSCs. Previous evidence suggests a link between osteogenesis and neurogenesis from an evolutionary perspective [43]. Porcine brain extract (PBE) promoted osteogenic differentiation of rBMSCs in a rat DO model, and there are multiple factors in PBE, particularly neuronal growth factors,

Fig. 4 MiR-34a-5p negatively regulates WNT1 expression.
(A) The WNT1 gene was predicted as a target of miR-34a-5p with a putative binding site. (B) A dual-luciferase reporter assay was performed to confirm the binding between miR-34a-5p and WNT1. The (C) mRNA and (D) protein expression levels of WNT1 in transfected T-ALL cells were measured by qRT-PCR and Western blot, respectively. Data are shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.01.
which are important for promoting bone regeneration [44]. Therefore, we speculated that a link exists between neurogenesis and osteogenesis; however, the relationship between neurogenesis and osteogenesis and which plays a key role during bone formation needs further investigation.  

MiR-34a has been shown to regulate WNT1, promote the proliferation and metastasis of tumour cells, and drive cancer development [45, 46]. The potential binding site between miR-34a-3p and WNT1 was predicted by TargetScan, indicating that miR-34a-5p may regulate...
WNT1 expression. Wnt/β-catenin signaling plays a key role in maintaining bone mass, promoting osteoblast maturation and bone mineralization, and negatively regulating osteoclast formation [47, 48]. Promoting BMSC differentiation by activating the Wnt/β-catenin pathway effectively prevents bone loss caused by surgery [49]. The downregulation of Wnt expression has been shown to inhibit BMSC differentiation and aggravate osteoporosis [23]. Moreover, WNT1 is a key component in the Wnt signaling pathway and has been identified as a major Wnt ligand implicated in osteoporosis [50]. The above studies suggested that WNT1 and Wnt signaling were involved in the regulation of BMSC differentiation.

In the current study, we found that miR-34a-5p targeted WNT1 and inhibited its expression. Moreover, WNT1 overexpression diminished the inhibitory effect of miR-34a-5p on BMSC differentiation. To ascertain the involvement of the canonical Wnt/β-catenin signaling pathway in osteogenic differentiation, we detected the expression of WNT1 and β-catenin. The inhibition of miR-34a-5p significantly elevated the protein level of WNT1 and β-catenin in BMSCs cells co-cultured with extracellular vesicles, while the overexpression of WNT1 significantly restored the downregulation of WNT1 and β-catenin in BMSCs overexpressing miR-34a-5p. Hence, the overexpression of miR-34a-5p inhibited osteogenic differentiation of BMSCs by inhibiting the WNT1 expression.

Taken together, our results demonstrated that miR-34a-5p in T-ALL cells may have clinical benefit in reducing T-ALL-inhibited bone formation.

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Not applicable.

Disclosure

The authors declare that there is no conflict of interest.

Declarations

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Availability of data and material

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution

Conception and study design: TY;
Data acquisition: CS;
Data analysis: WX, HLY;
Manuscript drafting: BX;
Manuscript revising: CT.

All authors have read and approved the final version of this manuscript to be published.

| Clinical features | miR-34a-5p High | miR-34a-5p Low | p value |
|-------------------|-----------------|----------------|---------|
| Age (years)       | 5.26 ± 1.12     | 5.73 ± 1.35    | 0.313   |
| WBC count (×10⁹/L)| 65.42 ± 8.27    | 57.45 ± 6.34   | 0.010*  |
| Platelet count (×10⁹/L) | 98.11 ± 17.88 | 99.27 ± 21.40  | 0.875   |
| Hemoglobin (g/L)  | 149.74 ± 33.51  | 142.23 ± 37.78 | 0.576   |
| LDH (U/L)         | 288.84 ± 30.45  | 272.73 ± 33.10 | 0.187   |

Note: WBC, white blood cell; LDH, lactate dehydrogenase. * p < 0.05.

Supplementary Table 1 Association between miR-34a-5p expression and clinical features of T-ALL patients
Supplementary Fig. 1 (A) The expression levels of PPARγ and FBP4 were examined by qRT-PCR. (B) The effect of miR-34a-5p inhibition on Jurkat cell proliferation was examined by MTT assay.

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