Original Article

CircRNA CircEHBP1 Regulates the Maturation of MiR-129 to Increase the Chemoresistance of Cancer Cells to Adriamycin in Acute Myeloid Leukaemia

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Abstract. Background: MicroRNA-129 (miR-129) is known to promote chemosensitivity of many types of cancer cells. However, the role of miR-129 in acute myeloid leukaemia (AML) is unclear. We predicted that premature miR-129 might interact with circEHBP1, a well-characterized oncogene in bladder cancer, and analyzed the interaction between circEHBP1 and miR-129 in AML.

Methods: Expression of circEHBP1 and miR-129 in AML patients before and after adriamycin (ADR) treatment was determined by RT-qPCR. CircEHBP1 distribution in nuclear and cytoplasm fractions of AML cells was determined using a cellular fractionation assay. The direct interaction of circEHBP1 with premature miR-129 was explored with an RNA-RNA pull-down assay. Finally, the role of circEHBP1 in regulating miR-129 maturation was analyzed in overexpression cells by RT-qPCRs.

Results: Compared to the controls, AML patients exhibited increased circEHBP1 and premature miR-129 levels but decreased mature miR-129 levels. Altered gene expression was more obvious in ADR resistant group than in ADR sensitive group. CircEHBP1 was detected in both nuclear and cytoplasm fractions of AML cells and directly interacted with premature miR-129. CircEHBP1 overexpression increased premature miR-129 level but decreased mature miR-129 level. In AML cells, circEHBP1 suppressed ADR-induced cell apoptosis and attenuated the enhancing effects of miR-129 on cell apoptosis. More importantly, the role of circEHBP1 in regulating cell apoptosis was more obvious in ADR resistance cells.

Conclusion: CircEHBP1 may suppress miR-129 maturation to increase the chemoresistance of cancer cells to ADR in AML.

Keywords: acute myeloid leukaemia; circEHBP1; miR-129; Adriamycin.

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Introduction. As a type of blood malignancy developing in bone marrow, acute myeloid leukaemia (AML) originates from blood-forming cells and mainly affects people older than 45 years old. Even compared to other types of leukaemia, AML deteriorates quickly without proper treatment. Even with appropriate treatment, fewer than 30% of AML patients can survive more than 5 years. Although some novel therapeutic approaches,
such as targeted therapy and stem cell transplantation, have been developed to treat AML, chemotherapies, including adriamycin (ADR), remains the most widely used AML treatment modality.\textsuperscript{6,7} However, chemoresistance will inevitably develop in many cases after treatment, leading to a poor prognosis.

Circular RNAs (circRNAs), a novel type of endogenous non-coding RNA (ncRNA), play vital roles after treatment, leading to a poor prognosis.\textsuperscript{10} endogenous non-coding RNA (ncRNA), play vital roles after treatment, leading to a poor prognosis.

A possible mechanism is that circRNAs can act as a sponge to bind miRNAs, thereby regulating their target genes.\textsuperscript{11,12} However, the role of circRNAs in AML is still unclear. Therefore, we predicted that premature miR-129 might interact with circEHBP1, a characterized oncogene in bladder cancer,\textsuperscript{13} and hypothesized that circEHBP1 might also participate in chemosensitivity via interaction with miR-129 in AML cells.

MicroRNAs (miRNAs) are a class of ncRNAs with about 22 nucleotides in length.\textsuperscript{14} The expression of miRNAs is frequently altered in AML, and changes in miRNA expression may trigger AML deterioration or remission.\textsuperscript{15} MiR-129 is known to promote chemosensitivity of cancer cells in many types of cancers and plays a critical player in developing chemosensitivity.\textsuperscript{16-18} However, its role in AML is still unclear. Therefore, we predicted that premature miR-129 might interact with circEHBP1, a characterized oncogene in bladder cancer,\textsuperscript{13} and hypothesized that circEHBP1 might also participate in chemosensitivity via interaction with miR-129 in AML cells.

Materials and Methods. Clinical samples. The present study included bone marrow (BM) specimens donated by a total of 60 AML patients (38 males and 22 females, 63.4 ± 6.9 years old) and 48 bone marrow transplantation donors (29 males and 19 females, 63.7 ± 7.1 years old). The 60 patients were admitted to The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital between May 2019 and May 2021. This study was carried out after obtaining approval from the Ethics Committee of The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital in compliance with the principles of the most recent version of the Declaration of Helsinki. AML patients were diagnosed through multiple approaches, including immune phenotyping and bone marrow routine. Only AML patients who received ADR treatment were enrolled. The 60 patients were grouped into ADR-sensitive (n=40) and resistant (n=20) groups based on treatment outcomes. Patients in the sensitive group were remitted completely or partially, and patients in the resistant group were at stable status or underwent deterioration. Bone marrow mononuclear cells (BMMCs) were isolated using Ficoll-Isopaque Plus (Sigma-Aldrich) through density gradient centrifugation and stored in a liquid nitrogen tank prior to the subsequent assays. All participants signed the written informed consent.

AML cells and cell culture. ADR-sensitive cell line (HL60) and ADR-resistant cell line (HL60/ADR) were purchased from BeNa Culture Collection (Suzhou) and cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Sigma-Aldrich, USA) and 1% antibiotic-antimycotic mixture (Thermo Fisher Scientific) at 37°C in an incubator with 5% CO\textsubscript{2} and 95% humidity.

Cell transfections. Both HL60 and HL60/ADR cells were overexpressed with circEHBP1 or miR-129 through the transfections of circEHBP1 expression vector or mimic of miR-129 using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) following the manufacturer's protocol. In each transfection, 2x10\textsuperscript{4} cells were transfected with 10 nM vector or 50 nM miRNA mimicking Lipofectamine 2000 use. After incubation with a transfection mixture for 6h, cells were further cultured in a fresh medium for 48h, and overexpression was confirmed by RT-qPCRs.

MTT (3-(4,5-Dimethylthiazol-2-yl) assay. ADR sensitivity was determined using the IC\textsubscript{50} value (half maximal inhibitory concentration) of ADR. By calculating the cell inhibition rate of ADR treatment with different concentrations (0, 2, 4, 8, 16, 32, 64, and 128 μM), the representative curve of AML cell growth inhibition was plotted. ADR concentration corresponding to 50% cell viability in the inhibitory rate curve was IC\textsubscript{50} value.

RNA preparations. Total RNA was isolated from both BMMCs and in vitro cultured HL60 cells using EasyPure® RNA Purification Kit (TransGen Biotech Co., LTD) and eluted in RNase-free water. After removal of contaminated genomic DNA using DNease I digestion, RNA samples were subjected to Bioanalyzer analysis to determine their integrity and determination. RNA samples were re-isolated if their quality was unsatisfactory (RIN value < 8.0 and/or RNA concentration < 500 ng/μl).

RT-qPCR. About 1000ng total RNA from each sample was reverse transcribed into cDNA using LunaScript® RT SuperMix Kit (NEB). qPCRs were performed to determine the expression of circEHBP1 and premature miR-129 using 18S rRNA as the internal control, and mature miR-129 expression was analyzed using All-in-One™ miRNA qRT-PCR Reagent Kits (Genecopoeia) with U6 as the internal control. Ct values were processed using the method of 2\textsuperscript{ΔΔCt}. Primer sequences were 5'-
TGGGATTTACCCTGTGAAACAG-3' (forward) and 5'-GACATACATGCAAAGTTCCTT-3' (reverse) for circEHBP1, 5'-AAACGGCTACCACATCCAAG-3' (forward) and 5'-TCGCGGAAGGATTTAAAGTG-3' (reverse) for 18S rRNA, 5'-GGAUCTTTTGCGGTCTGG-3' (forward) and 5'-AGATACTTTTGGGTAAGGGC-3' (reverse) for premature miR-129, and 5'-CTTTTGGCGGTCTGGGCTTG-3' (forward) for mature miR-129. Universal reverse primer and U6 primers were included in the kit.

**RNA-RNA pull-down assay.** In vitro transcripts of both circEHBP1 and negative control (NC), RNA were prepared through reverse transcriptions using HiScribe™ T7 High Yield RNA Synthesis Kit (NEB). To perform RNA-RNA pull-down, we labeled the 3’ end of both transcripts using Pierce Biotin 3' End DNA Labeling Kit (Thermo Scientific) and re-named Bio-circEHBP1 and Bio-NC, respectively. Bio-circEHBP1 and Bio-NC were transfected both into HL60 and HL60/ADR cells. At 48h post-transfection, cells were lysed on ice for 10 min and incubated with magnetic beads to pull-down RNA complex. After that, the RNA complex was purified and subject to RT-PCR to determine the expression of circEHBP1.

**Subcellular fractionation assay.** The nuclear and cytoplasm fractions of both HL60 and HL60/ADR cells were prepared using a cell fractionation kit (ab109719, Abcam). In brief, cells were lysed on ice for 10 min and centrifuged for 10 min at 600g. The supernatants were collected as the cytoplasm fraction. The pellets (nuclear fraction) were further incubated with cell lysis buffer for 10 min on ice. After that, RNAs were isolated from both cellular fractions and subjected to RT-qPCR to determine the expression of miR-129.

**Flow cytometry analysis.** HL60 and HL60/ADR cells were harvested after the conformation of cell transfections and incubated in a medium containing 5 μM ADR for 48h. After that, cells were stained with Annexin V-FITC and PI and subjected to flow cytometer S3™ Cell Sorter (Bio-Rad) to analyze cell apoptosis.

Statistical analysis. Two groups were compared by unpaired t-test. Two-time points of the same group were compared by paired t-test. Multiple independent groups were compared by ANOVA Tukey's test. The 60 AML patients were grouped into high and low circEHBP1/mature miR-129 level groups (cutoff = median). Chi-squared test was applied to study the associations between circEHBP1/mature miR-129 expression and patients’ clinical factors. P<0.05 was statistically significant.

**Figure 1. Analysis of the expression of circEHBP1 and miR-129 in AML patients.** The 60 patients were grouped into ADR-sensitive (n=40) and –resistant (n=20) groups based on treatment outcomes. Samples of BMMCs from ADR-sensitive group (n=40), ADR–resistant (n=20) and control group (n=48) were subjected to the determination of circEHBP1 (A), premature miR-129 (B) and mature miR-129 (C) through RT-qPCRs. **, p<0.01.
Table 1. Associations between circEHBP1 and miR-129 expression and AMP patients’ clinical factors.

| Factors                  | CircEHBP1 |         | MiR-129 |         |
|--------------------------|-----------|---------|---------|---------|
|                          | n         | High (n=30) | Low (n=30) | p        | High (n=30) | Low (n=30) | p        |
| Age (year)               |           |         |         |         |
| >=60                     | 34        | 15      | 19      | >0.05   | 16         | 18         | >0.05   |
| <60                      | 26        | 15      | 11      |         | 14         | 12         |         |
| Gender                   |           |         |         |         |
| Female                   | 38        | 20      | 18      | >0.05   | 21         | 17         | >0.05   |
| Male                     | 22        | 10      | 12      |         | 19         | 13         |         |
| WBC (x10^9/L)            |           |         |         |         |
| >=15                     | 32        | 17      | 15      | >0.05   | 18         | 14         | >0.05   |
| <15                      | 28        | 13      | 15      |         | 12         | 16         |         |
| Stages                   |           |         |         |         |
| M1-M3                    | 19        | 3       | 16      | 0.000   | 15         | 4          | 0.002   |
| M4-M6                    | 41        | 27      | 14      |         | 15         | 26         |         |
| Monosomal karyotype      |           |         |         |         |
| Yes                      | 8         | 3       | 5       | >0.05   | 2          | 6          | >0.05   |
| No                       | 52        | 27      | 25      |         | 28         | 24         |         |
| FLT3-ITD mutation        |           |         |         |         |
| Yes                      | 12        | 7       | 5       | >0.05   | 8          | 4          | >0.05   |
| No                       | 48        | 23      | 25      |         | 22         | 26         |         |
| NPMI mutation            |           |         |         |         |
| Yes                      | 23        | 12      | 11      | >0.05   | 13         | 10         | >0.05   |
| No                       | 37        | 18      | 19      |         | 17         | 20         |         |

Results.

Expression of circEHBP1 and miR-129 in AML patients. The 60 patients were grouped into ADR-sensitive (n=40) and resistant (n=20) groups based on treatment outcomes. BMNCs from ADR-sensitive (n=40), ADR-resistant (n=20), and control (n=48) groups were subjected to RT-qPCR to determine circEHBP1, premature miR-129 and mature miR-129 levels. Compared to the controls, AML patients exhibited increased expression of circEHBP1 (Figure 1A, p<0.01) and premature miR-129 (Figure 1B, p<0.01) but decreased mature miR-129 (Figure 1C, p<0.01). Altered gene expression was more obvious in the ADR-resistant group than in the ADR-sensitive group. Association analysis showed that circEHBP1 and mature miR-129 expression was closely associated with patients’ stages but not other factors (Table 1).

Expression of circEHBP1 and miR-129 in ADR-sensitive and resistant HL60 cells. The cell viability was determined by MTT assay in HL60/ADR cells and its parental cells HL60 (A). ADR-sensitive and resistant HL60 cells were also subjected to RT-qPCR to determine the expression of circEHBP1 (B), premature miR-129 (C) and mature miR-129 (D) through RT-qPCRs. **, p<0.01.
Figure 3. Detection of circEHBP1 in nucleus and cytoplasm and its direct interaction with premature miR-129. Cellular fractionation assay was carried out to detect circEHBP1 in the nucleus and cytoplasm in both types of HL60 cells (A). IntaRNA 2.0 was applied to predict the direct interaction between circEHBP1 and premature miR-129 (B). RNA-RNA pulldown assay was applied to further confirm the direct interaction between circEHBP1 and miR-129 (C). **, p<0.01.

Figure 4. Analysis of the role of circEHBP1 in the maturation of miR-129. Both types of HL60 cells were overexpressed with circEHBP1 or miR-129, and their overexpression was confirmed by RT-qPCRs (A). Premature miR-129 (B) and mature miR-129 (C) were detected in ADR-resistant and ADR-sensitive HL-60 cells transfected with circEHBP1 by RT-qPCRs. **, p<0.01.
Figure 5. The role of circEHBP1 and miR-129 in ADR-induced cell apoptosis. Flow cytometry analysis was used to detect cell apoptosis of miR-129 and circEHBP1+miR-129. Data from three biological replicates were used as average values to compare between groups. *, p<0.05; **, p<0.01.

Detection of circEHBP1 in nucleus and cytoplasm and its direct interaction with premature miR-129. A cellular fractionation assay was carried out to detect circEHBP1 in the nucleus and cytoplasm in both HL60 and HL60/ADR cells. It was observed that circEHBP1 could be found in both the nucleus and cytoplasm (Figure 3A). IntaRNA 2.0 was applied to predict the direct interaction between circEHBP1 and premature miR-129. The prediction revealed a strong base pairing between them (Figure 3B). RNA-RNA pull-down assay was applied to confirm their direct interaction further. Compared to the Bio-NC group, significantly higher levels of miR-129 were observed in Bio-circEHBP1, establishing the direct interaction between them (Figure 3C, p<0.01).

Analysis of the role of circEHBP1 in the maturation of miR-129. HL60 and HL60/ADR cells were overexpressed with circEHBP1 or miR-129, and RT-qPCR confirmed their overexpression. Furthermore, it was observed that circEHBP1 and miR-129 were significantly overexpressed; the overexpression of circEHBP1 was more obvious in ADR-sensitive cells than in ADR-resistant cells, while overexpression of miR-129 was less obvious in ADR-sensitive cells than in ADR-resistant cells (Figure 4A). In both HL60 and HL60/ADR cells, circEHBP1 increased the level of premature miR-129 (Figure 4B, p<0.01), but decreased the level of mature miR-129 (Figure 4C, p<0.01).

The role of circEHBP1 and miR-129 in ADR-induced cell apoptosis. Cells with transfections were subjected to the analysis of ADR-induced cell apoptosis. In each transfection group, cell apoptotic rates were higher in ADR-sensitive cells than in ADR-resistant cells. Moreover, circEHBP1 suppressed ADR-induced cell apoptosis and attenuated the enhancing effects of miR-129 on cell apoptosis. More importantly, the impact of circEHBP1 and miR-129 on regulating cell apoptosis was more evident in ADR-resistance cells (Figure 5, p<0.01).

Discussion. The present study analyzed the expression pattern and functionality of miR-129 and circEHBP1 in AML. We showed that circEHBP1 expression was increased in AML patients, and the maturation of miR-129 was suppressed in AML. Interestingly, the maturation of miR-129 was found to be regulated by circEHBP1.

Previous studies have characterized miR-129 as a critical player in the development of chemoresistance of cancer cells to multiple chemical drugs. For instance, miR-129 was downregulated in colorectal cancer, and its overexpression increased the sensitivity of cancer cells to 5-fluorouracil. In addition, miR-129 suppressed neuroblastoma growth, and overexpression of miR-129 increased the sensitivity of cancer cells to Cytoxan via MYO10. In gastric cancer, miR-129 targeted P-gp to suppress cisplatin resistance. Based on our knowledge, the role of miR-129 in AML is unclear. In this study, we showed the upregulation of premature miR-129 and downregulation of mature miR-129, and the alteration was more obvious in ADR-resistant patients than in ADR-sensitive patients. Therefore, inhibition of miR-129 maturation is likely involved in AML. Moreover, overexpression of miR-129 increased the sensitivity of AML cells to ADR. Thus, miR-129 may be targeted to raise the chemosensitivity of AML cells to ADR.

A recent study reported that circEHBP1 promoted bladder cancer progression by increasing lymphatic metastasis and lymphangiogenesis through its interaction with miR-130a-3p/TGFβ1R1/VEGF-D. The present study characterized the expression pattern of circEHBP1 and observed its upregulation in AML. Moreover, circEHBP1 overexpression suppressed ADR-induced cell apoptosis. Therefore, circEHBP1 is likely an oncogenic circRNA in AML. Most importantly, we detected circEHBP1 in both nuclear and cytoplasm fractions of AML cells, and circEHBP1 directly interacted with premature miR-129.
Moreover, circEHBP1 overexpression suppressed miR-129 maturation. Therefore, premature miR-129 may be sponged by circEHBP1 in the nucleus, reducing its maturation. It is worth noting that circEHBP1 was predicted to interact with multiple premature miRNAs (data not shown), while our functional assays only validated its interaction with premature miR-129. Interestingly, although the effects of circEHBP1 and miR-129 are stronger in ADR-resistant cells than in ADR-sensitive cells, they regulated ADR-induced apoptosis in both cell types. Therefore, circEHBP1 and miR-129 can control the sensitivity of cancer cells in both ADR-resistant and sensitive patients.

**Conclusions.** CircEHBP1 was overexpressed in AML, and the maturation of miR-129 was suppressed in AML. In addition, circEHBP1 may suppress miR-129 maturation to decrease ADR-induced cell apoptosis.

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