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Original Article

MiR-140 Targets lncRNA DNAJC3-AS1 to Suppress Cell Proliferation in Acute Myeloid Leukemia

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Competing interests: The authors declare no conflict of Interest.

Abstract. ***Objectives:*** **MiR-140 and DNAJC3-AS1 have been demonstrated to play critical roles in cancer biology, while their participation in acute myeloid leukemia (AML) is unclear. This study aimed to explore the role of miR-140 and DNAJC3-AS1 in AML.**
Methods: **The expression of DNAJC3-AS1 and miR-140 were detected by RT-qPCR. Then, the role of DNAJC3-AS1 and miR-140 in regulating each other was explored by overexpression assay. Next, the direct interaction between DNAJC3-AS1 and miR-140 was analyzed using an RNA pull-down assay. Next, the subcellular location of DNAJC3-AS1 was explored using cellular, subcellular fractionation assay. Finally, cell proliferation analysis was evaluated with BrdU assay.**
Results: **Increased expression levels of DNAJC3-AS1 and decreased expression levels of miR-140 were observed in AML patients. DNAJC3-AS1 was detected in nuclear and cytoplasm samples and direct interaction between DNAJC3-AS1 and miR-140 was observed.**
Discussion: **Reduced expression levels of DNAJC3-AS1 were observed after overexpression of miR-140 in AML cells. DNAJC3-AS1 increased cell proliferation and inhibited the role of miR-140 in suppressing cell proliferation.**
Conclusion: **In conclusion, miR-140 may target DNAJC3-AS1 to suppress cell proliferation in AML.**

Keywords: DNAJC3-AS1; miR-140; Acute myeloid leukemia; Proliferation.

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Introduction. As the most common type of myeloid leukemia, acute myeloid leukemia (AML) develops from bone marrow, where blood cells are produced.[1] With disease development, AML cells will move into the blood quickly. They may even move to lymph nodes and many important organs of the human body, such as the spleen, liver, testicles, and even the central nervous system, leading to poor prognosis.[2,3] AML is estimated to affect about 4.3 out of 100,000 people each year.[3] Moreover, only less than 28% of these patients could survive more than five years after the initial diagnosis.[4,5] AML patients are frequently treated with chemotherapy,[6,7] while AML mainly affects patients older than 65 years and usually cannot tolerate the adverse effects of chemotherapy.[6,7] Therefore, treatment strategies of AML should be improved.

Targeted therapies have been proven to be potential novel targets for the treatment of AML because of the

high specificity and fewer adverse effects.[8,9] Some signal pathways, such as the hedgehog pathway and the PI3K/AKT pathway, have shown promising potentials to treat AML.[10,11] However, more effective targets with higher safety are needed to improve targeted therapies for AML further. Noncoding RNA (ncRNAs), such as microRNAs (miRNAs) and long ncRNAs (lncRNAs), lack protein-coding capacity but affect protein synthesis to regulate cancer progression.[12,13] Therefore, ncRNAs are potential candidates for AML treatment. MiR-140 and DNAJC3-AS1 have been proven to play critical roles in cancer biology, while their participation in AML is unclear.[14-17] Therefore, we predicted that miR-140 might target DNAJC3-AS1. Therefore, this study was conducted to explore the interaction between DNAJC3-AS1 and miR-140 in AML.

Materials and Methods

Patients and Samples of Peripheral Blood Mononuclear Cells (PBMCs). Blood samples were donated by a total of 49 AML patients (30 males and 19 females, 66.7 +/- 5.9 years old) and 49 healthy controls (30 males and 19 females, 66.8 +/- 5.6 years old) at the First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital from May 2018 to May 2020. Blood was extracted from patients after overnight fasting. FicolHistopaque gradient centrifugation method was used to separate PBMCs. All healthy controls exhibited normal physiological parameters during a systemic physiological examination. All 49 AML patients were classified following the French-American-British (FAB) classification, and 12, 11, 10, and 16 participants were classified into M1/M2, M3, M4/5, and M6/7, respectively. No anti-AML therapy was performed on these patients before admission. This study was approved by the Ethics Committee of the hospital mentioned above. All patients and controls signed the informed consent.

AML Cells and Cell Culture. Two AML cell lines HL60 and U937, were purchased from BeNa Culture Collection (Suzhou, China). DMEM medium (Gibco) was supplemented with 10% FBS (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) to serve as the culture medium of both cell lines. Cells were cultivated at 37 °C with 5% CO_2 and 95% humidity.

Cell Transfections. DNAJC3-AS1 and miR-140 were overexpressed in HL60, and U937 cells by transfecting DNAJC3-AS1 vector (pcDNA3.1 backbone) and/or miR-140 mimic (RiboBio, China). All transfections were mediated by Neon Electroporation Transfection device (Thermo Fisher Scientific). Negative control (NC) experiments were performed, transfecting cells with empty vector or NC miRNA. Untransfected cells were cultivated until the following assays to serve as the control. The overexpression was checked every 24 h until 96 h.

Preparation of RNA Samples. EZ RNA Miniprep Kit (EZBioResearch) was used to prepare RNA samples from PBMCs and HL60 and U937 cells. RNA samples were digested with DNase I (Invitrogen, USA) to remove genomic DNA. The integrity and RNA concentrations were evaluated by Bioanalyzer. A RIN value higher than 8.0 was reached in all cases, indicating high RNA integrity.

RT-qPCRs. With 3,000 ng total RNAs as the template, cDNA samples were prepared through reverse transcriptions (RTs). With cDNA samples as the template, the expression levels of DNAJC3-AS1 and miR-140 were determined by performing qPCRs. The endogenous control for DNAJC3-AS1 and miR-140 was 18S rRNA and U6, respectively. Ct values of DNAJC3-AS1 and miR-140 were normalized to their endogenous control using the $2^{-\Delta\Delta Ct}$ method.

Subcellular Fractionation Assay. Cytoplasmic and nuclear samples were separated from both HL60 and U937 cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific). The two fractions were separated by centrifuging cell lysate at 1,200 g for 10 min. Then, the cytoplasmic sample was added into an RNase-free tube, while a nuclear sample was incubated with nuclear lysis buffer on ice for another 60 min. RNA isolation from these two samples was performed, and RT-PCRs were followed to determine the expression levels of DNAJC3-AS1. PCR products were separated by 2% agarose gel. After staining with EB, images were taken with a MyECL imager.

RNA Pull-Down Assay. DNAJC3-AS1 or NC RNA expression vector was linearized using T7 RNA polymerase (Thermo Fisher Scientific) to prepare a biotinylated *in vitro* transcript of both DNAJC3-AS1 and NC RNA. The biotinylated RNAs were named Bio-DNAJC3-AS1 or Bio-NC. These two RNAs were transfected into HL60 and U937 cells, followed by cell lysis for 48 h. Streptavidin-agarose beads were then used to pull down the complex, followed by RNA isolation and RT-qPCRs to determine the expression levels of miR-140.

BrdU Cell Proliferation Assay. In this assay, the incorporation of 5-Bromo-2-deoxyUridine (BrdU), which reflects DNA synthesis, was determined to quantify cell proliferation. Transfected cells were incubated in a medium containing 10 μM BrdU (BD Pharmingen) for 48 h, then incubated with anti-BrdU-antibody (peroxidase-coupled, Sigma–Aldrich) at room temperature for 60 min. After three washing times with

PBS, the cells were incubated with peroxidase substrate for 30 minutes. Finally, OD values at 450 nm were determined to reflect cell proliferation.

Statistical Analysis. The unpaired t-test and ANOVA Tukey's test were used to compare two or multiple independent groups. Correlation analysis was performed with Pearson's correlation coefficient. $P < 0.05$ was statistically significant.

Results.

The Expression of DNAJC3-AS1 and miR-140 in AML Patients and Controls. Samples of PBMCs were subjected to RT-qPCRs to detect the expression of DNAJC3-AS1 and miR-140 in AML patients and controls. The results showed increased expression levels of DNAJC3-AS1 (**Figure 1A**, $p < 0.01$) and decreased expression levels of miR-140 (**Figure 1B**, $p < 0.01$) in AML patients. Our data illustrated the potential participation of these two ncRNAs in AML. Correlation analysis performed with Pearson's correlation coefficient revealed that DNAJC3-AS1 and miR-140 were inversely correlated across both AML (**Figure 1C**) and control (**Figure 1D**) samples, suggesting the potential interaction between them.

To detect the expression of DNAJC3-AS1 (A) and miR-140 (B) in AML patients and controls, samples of PBMCs were subjected to RNA isolations, followed by RT-qPCRs. Pearson's correlation coefficient was applied to explore the correlations between DNAJC3-AS1 and miR-140 across AML and control samples. **, $p < 0.01$.

The Direct Interaction Between DNAJC3-AS1 and miR-140 and the Subcellular Location of DNAJC3-AS1. The online program IntaRNA 2.0 was applied to explore the potential interaction between DNAJC3-AS1 and miR-140. It was observed that DNAJC3-AS1 and miR-140 could form potential base pairing (**Figure 2A**). RNA-pull down assay was performed to validate the direct interaction between DNAJC3-AS1 and miR-140 in both HL60 and U937 cells. Compared to the Bio-DNAJC3-AS1 pull-down group, the Bio-NC pull-down group exhibited significantly lower expression levels of miR-140, confirming the direct interaction between DNAJC3-AS1 and miR-140 (**Figure 2B**, $p < 0.01$). Subcellular location of DNAJC3-AS1 in both cell lines was explored with a subcellular fractionation assay. Unlike GAPDH, the expression of DNAJC3-AS1 was detected in both nuclear and cytoplasm samples (**Figure 2C**).

The online program IntaRNA 2.0 was applied to explore the potential interaction between DNAJC3-AS1 and miR-140 (A). RNA-pull down assay was performed to validate the direct interaction between DNAJC3-AS1 and miR-140 in both HL60 and U937 cells (B). Subcellular location of DNAJC3-AS1 in both cell lines was explored with subcellular fractionation assay (C). **, $p < 0.01$.

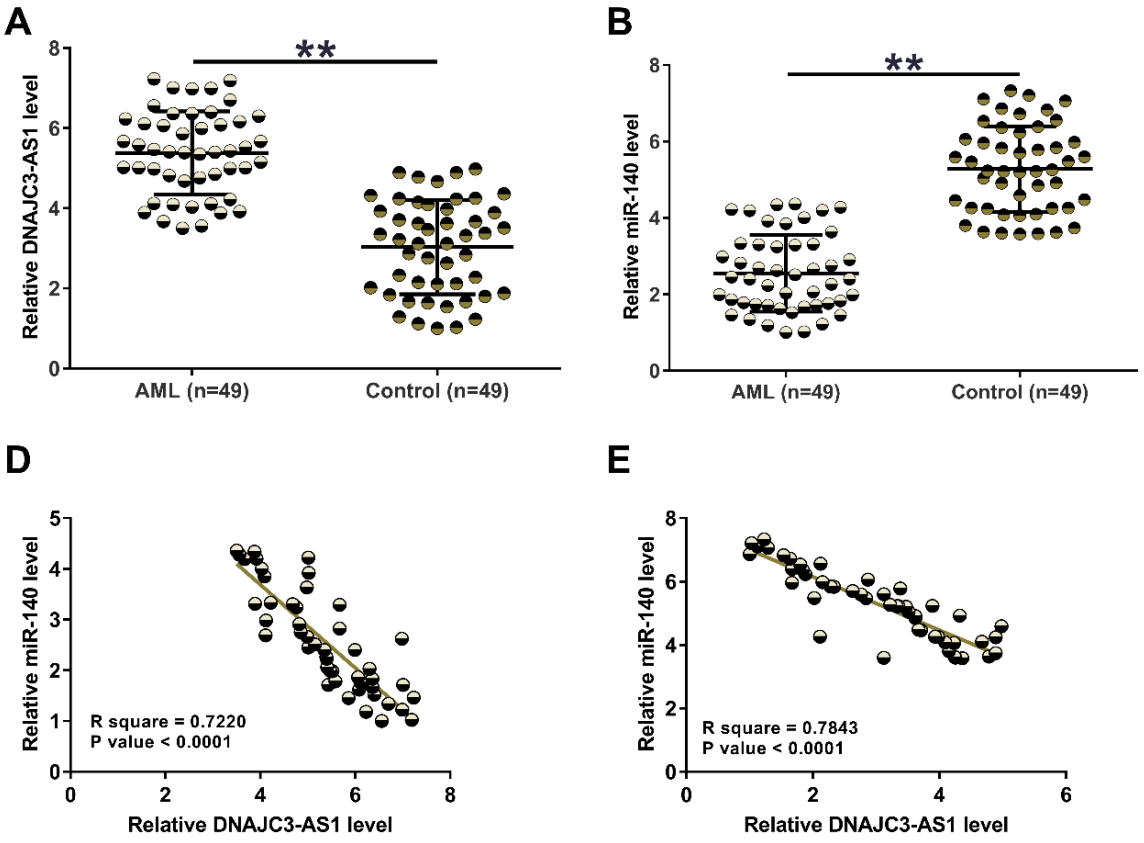

Figure 1. Analysis of the expression of DNAJC3-AS1 and miR-140 in AML patients and controls.

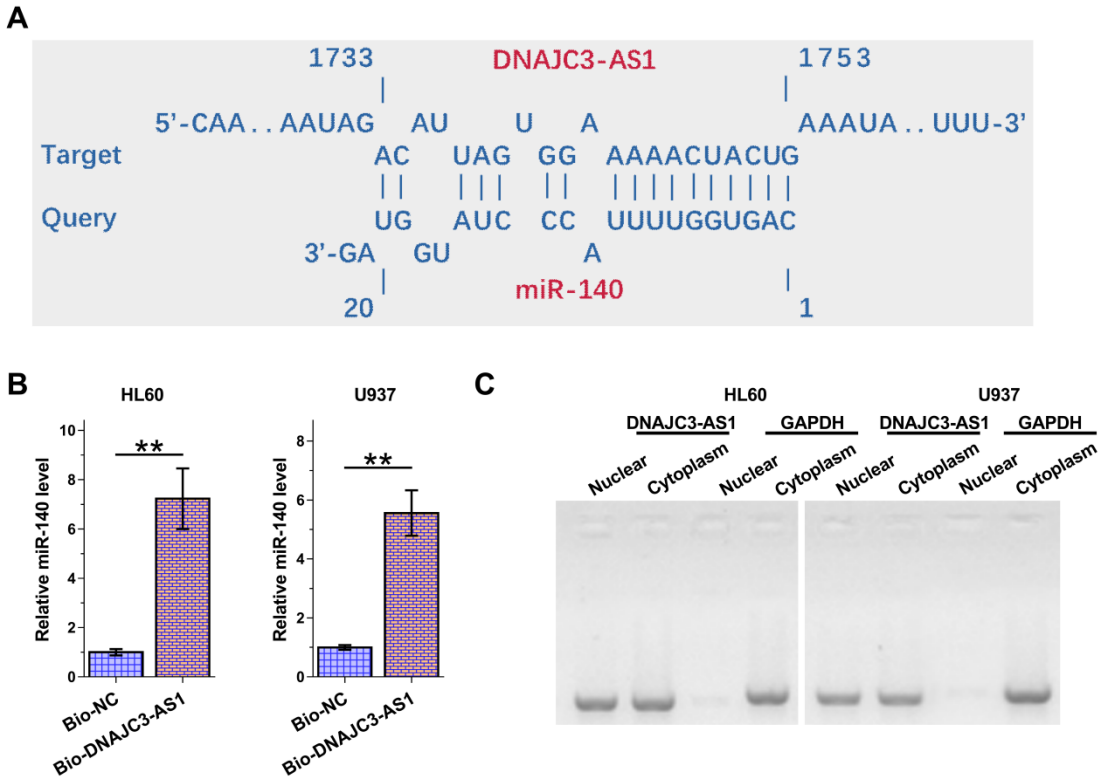

Figure 2. Analysis of the direct interaction between DNAJC3-AS1 and miR-140, and the subcellular location of DNAJC3-AS1.

The Role of miR-140 in the Expression of DNAJC3-AS1. DNAJC3-AS1 or miR-140 was overexpressed in HL60 and U937 cells, followed by confirmation of the expression of DNAJC3-AS1 and miR-140 every 24 h until 96 h (**Figure 3A**, $p < 0.05$). Reduced expression levels of DNAJC3-AS1 were observed after the overexpression of miR-140 in both cells (**Figure 3B**, $p < 0.05$). In contrast, no altered expression of miR-140 was observed after the overexpression of DNAJC3-AS1 (**Figure 3C**). Therefore, DNAJC3-AS1 could be targeted by miR-140 in AML cells.

HL60 and U937 cells were overexpressed with DNAJC3-AS1 or miR-140, followed by confirming the expression of DNAJC3-AS1 and miR-140 every 24 h until 96 h (A). The role of miR-140 in the expression of DNAJC3-AS1 (B) and the role of DNAJC3-AS1 in the expression of miR-140 (C) were explored by overexpression assays, followed by RT-qPCRs. *, $p < 0.01$.

The Role of DNAJC3-AS1 and miR-140 in the Proliferation of HL60 and U937 Cells. BrdU assay was performed to explore the role of DNAJC3-AS1 and miR-140 in the proliferation of HL60 and U937 cells.

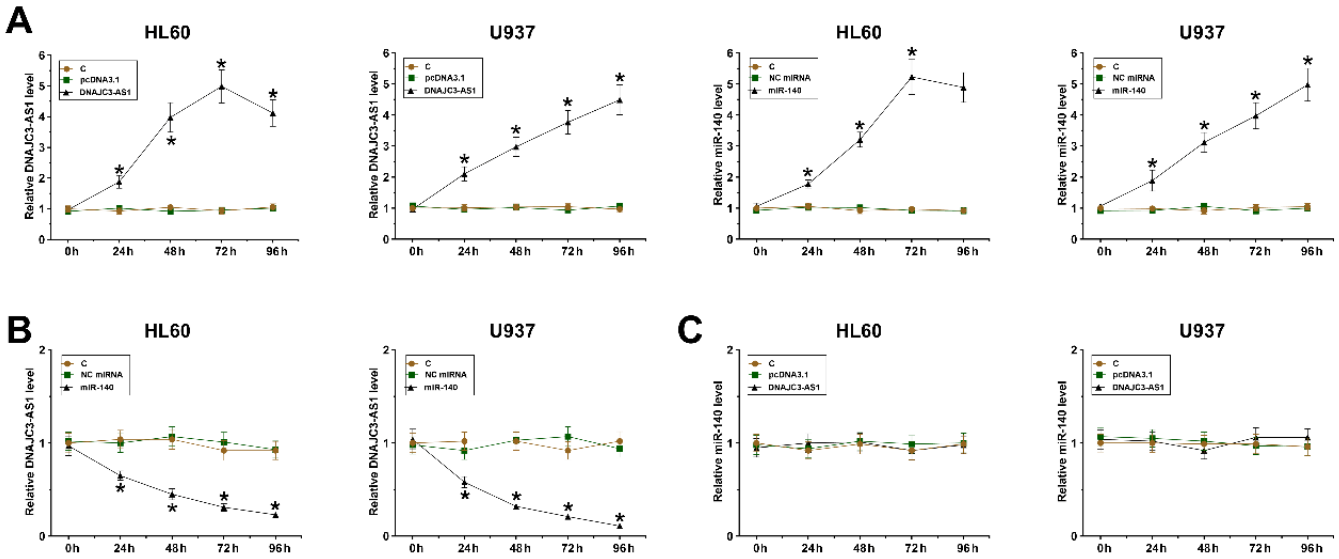

Figure 3. Analysis of the role of miR-140 in the expression of DNAJC3-AS1.

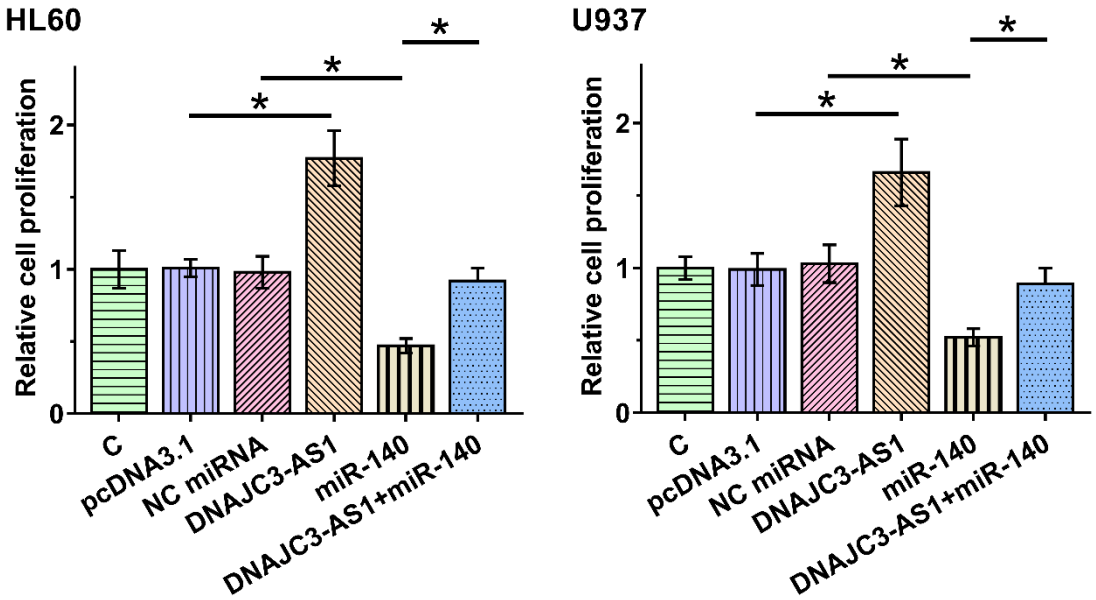

Figure 4. Analysis of the role of DNAJC3-AS1 and miR-140 in the proliferation of HL60 and U937 cells.

DNAJC3-AS1 increased cell proliferation, and miR-140 decreased cell proliferation. Moreover, DNAJC3-AS1 inhibited the role of miR-140 in suppressing cell proliferation (**Figure 4**, $p < 0.01$). Therefore, DNAJC3-AS1 is likely downstream of miR-140 in regulating AML cell proliferation.

BrdU assay was performed to explore the role of DNAJC3-AS1 and miR-140 in the proliferation of HL60 and U937 cells. Experiments were repeated in three biological replicates, and the mean +/- SD values of these three were presented. *, $p < 0.01$.

Discussion. This study explored the crosstalk between two ncRNAs DNAJC3-AS1 and miR-140 in AML. We showed that DNAJC3-AS1 could be targeted by miR-140 in AML cells.

Previous studies have reported the oncogenic role of DNAJC3-AS1 in several types of cancer, including colorectal cancer, renal cell carcinoma, and osteosarcoma.[14-16] DNAJC3-AS1 is upregulated in colorectal cancer to interact with the EGFR pathway, thereby regulating fatty acid synthase to promote cancer progression.[14] In another study, DNAJC3-AS1 was found to regulate the miR-27a-3p/PRDM14 axis to promote the development of tumors.[15] In osteosarcoma, DNAJC3-AS1 interacts with its sense-cognate gene DNAJC3 to accelerate tumor progression.[16] Therefore, DNAJC3-AS1 could interact with different tumor suppressive and oncogenic factors in different cancers to play an oncogenic role. In this study, we first reported the upregulation of DNAJC3-AS1 in AML. Overexpression of DNAJC3-AS1 led to the increased proliferation of AML cells. Therefore, DNAJC3-AS1 also plays an oncogenic role in AML. However, the mechanism that mediates the increased expression levels of DNAJC3-AS1 in AML is unknown. Future studies on this topic are still needed.

MiR-140 has been characterized as a tumor suppressor in many types of cancer, and it targets different genes in different cancers to inhibit cancer growth and/metastasis.[17] For instance, decreased expression levels of miR-140 were observed in esophageal cancer, and it regulates the wnt/β-catenin pathway by targeting ZEB2, leading to inhibited cancer development.[17] Furthermore, MiR-140 is also downregulated in lung cancer, and it inhibits the growth and metastasis of tumors by downregulating insulin-like growth factor 1 receptors through direct targeting.[18] In addition, the expression of miR-140 is also inhibited in gastric cancer, where it targets TES1 to suppress tumor metastasis and growth. However, the involvement of miR-140 in AML has not been reported in previous studies. In this study, we observed the downregulation of miR-140 in AML and the inhibitory effects of miR-140 on AML cell proliferation. Therefore, miR-140 is a tumor suppressor in AML.

Interestingly, we detected DNAJC3-AS1 in both nuclear and cytoplasm of AML cells. Moreover, miR-140 directly interacted with DNAJC3-AS1, and overexpression of miR-140 decreased the expression levels of DNAJC3-AS1. Because mature miRNAs are only in the cytoplasm and DNAJC3-AS1 can be detected in the cytoplasm, we concluded that DNAJC3-AS1 could be targeted by miR-140. Therefore, besides protein-coding genes, miR-140 can also target oncogenic lncRNAs to suppress cancers.

Conclusions. miR-140 is downregulated in AML, and DNAJC3-AS1 is upregulated in AML. Therefore, MiR-140 may target DNAJC3-AS1 to suppress AML cell proliferation.

Ethics Approval and Consent to Participate. This study was approved by the Ethics Committee of the First

Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital and all the patients or parents/guardians of patients provided written informed consent.

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