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IRF3 is involved in human acute myeloid leukemia through regulating the expression of miR-155

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ABSTRACT

Acute myeloid leukemia (AML) is a serious disease of the hematopoietic system characterized by de-differentiation and uncontrolled proliferation of immature hematopoietic precursor cells in the bone marrow. However, the underlying mechanism of AML development remains largely unknown. Here in this study, we report the function of IRF3, a member of the interferon-regulatory factor (IRF) family, in human AML. We first show that IRF3 mRNA and protein levels are significantly up-regulated in human AML compared with healthy donors. IRF3 knockdown inhibits cellular proliferation and colony formation in OCI/AML-2 and OCI/AML-3 cells. In addition, IRF3 knockdown induces apoptosis of OCI/AML-2 and OCI/AML-3 cells, whereas IRF3 overexpression promotes cell survival. Further mechanism study shows that IRF3 is positively correlated with miR-155, which is considered as an oncogenic microRNA in AML. We show that IRF3 binds to the promoter of miR-155 and promotes the expression of miR-155 in OCI/AML-2 and OCI/AML-3 cells. In conclusion, our evidence show that IRF3 overexpression in AML promotes cell growth and survival, and miR-155 is involved, indicating that IRF3 may be a potential new biomarker and therapeutic target for AML.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease that is associated with a very poor prognosis. Multiple cytogenetic and molecular abnormalities that characterize different forms of AML have been used to better prognosticate patients and inform treatment decisions [1]. Indeed, risk status in patients with this disease has classically been based on cytogenetic findings. However, additional molecular characteristics have been shown to inform risk assessment, including FLT3, NPM1, KIT, and CEBPA mutation status [2]. Advances in AML biology and its genetic landscape should ultimately lead to more subset-specific AML therapies, ideally tailored to each patient's disease [1,3]. Recent advances in sequencing technology have led to the discovery of novel somatic mutations in tissue samples from patients with AML, providing deeper insight into the mutational landscape of the disease [2,4]. However, the mechanism underlying AML is still largely unknown.

The interferon-regulatory factor (IRF) family, originally identified as transcriptional regulators of the type I interferon system, consists of nine members (IRF1-IRF9) in mammals [5]. The members of the IRF family have gained much attention for their regulation of the development and responses by immune cells because of their markedly diverse roles in regulating gene-expression networks within the immune system [5]. Recently, other biological functions of IRF family have been reported, including metabolism, cardiovascular diseases, and cancer [6,7]. Lack of IRF1 expression in acute promyelocytic leukemia and in a subset of acute myeloid leukemias with del(5) (q31) [8]. Constitutive IRF8 expression inhibits AML by activation of repressed immune response signaling [9]. The functions of other IRF members in AML remains unknown.

MicroRNAs (miRNAs) are a newly recognized class of regulatory genes which repress the expression of protein-coding genes. In the immune system, miR-155 is unique in its ability to shape the transcriptome of activated myeloid and lymphoid cells controlling diverse biological functions ranging from inflammation to immunological memory [10]. Earlier publications reveal that AML patients bearing an FLT3-ITD mutation have an increased expression of miR-155 [11–13]. A recent report reveals a novel network in which FLT3-ITD signaling induces oncogenic miR-155 by p65 and

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STAT5 in AML [14]. However, the mechanism by which miR-155 is regulated in human AML is not fully understood.

In the present work, we report that IRF3 promotes AML partly by regulating the expression of miR-155. IRF3 is overexpressed in human AML and IRF3 overexpression promotes AML cell growth and survival. Mechanism study shows that IRF3 binds to the promoter of miR-155 and promotes the expression of miR-155 in AML cells.

2. Materials and methods

2.1. Patients

AML patient samples were obtained from the *Department of Hematology, the First Affiliated Hospital of Zhengzhou University* between June 2006 and August 2014. The diagnosis of AML was made by expert clinical hematopathologists according to the morphological and immunological criteria of the National Cancer Institute expert panel. The study protocols used for AML patient sample collection were approved by the ethics committees of the participating centers of *Zhengzhou University*. All patients provided written informed consent in accordance with the Declaration of Helsinki. All samples were analyzed by cytogenetic and molecular genetic analyses.

2.2. Cell lines and cell culture

OCI/AML-2 and OCI/AML-3 cell lines were cultured in alpha-minimal essential medium (Corning, 10-022-CV), 293T cells were cultured in Rosewell Park Memorial Institute 1640 (Thermo, 11875-085), all with 10% fetal bovine serum (Hyclone, 16000-044), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2. For growth curves, cells were seeded at 1×10^4 or 1×10^3 cells/ml in 10 cm dishes and counted daily.

2.3. Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Thermo, 15596026). cDNA was synthesized using 1 μg RNA with the Advantage RT-for-PCR kit (Clontech, PCR5914). We quantified PCR amplifications using SYBR Green PCR Master Mix (TAKARA, RR820A) and normalized results against GAPDH gene expression. The miRNA quantification was performed as previously described by using snoRNA-135 expression for normalization. Taqman reverse transcription (RT) and PCR primers, snoRNA-135, hsa-miR-155 were obtained from Applied Biosystems (Darmstadt, Germany). Primers for IRF3 and GAPDH are as follows:

IRF3 forward: 5′-AGAGGCTCGTGATGGTCAAG-3′
IRF3 reverse: 5′-AGGTCCACAGTATTCTCCAGG-3′
GAPDH forward: 5′-TGTGGGCATCAATGGATTTGG-3′
GAPDH reverse: 5′-ACACCATGTATTCCGGGTCAAT-3′

2.4. Western blot

Cells were lysed in RIPA lysis buffer (Beyotime, P0013) with a mixture of protease inhibitors (Roche, 11697498001). 20 μg cell lysis were applied to 12% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, which were then blocked in 5% fat-free milk for 1 h. The membranes were then probed with primary antibody for IRF3 (Abcam, ab25950), or GAPDH (Abcam, ab37168) at 4 °C overnight. Then the membranes were washed for 3 times with TBST and incubated with the HRP-conjugated secondary antibody (in 5% fat-free milk) for 2 h. Finally, the membranes were washed with TBST for 4 times and visualized using Chemiluminescent ECL reagent (Beyotime, P0018).

2.5. Lentivirus packaging and transduction

shIRF3 and ctrl shRNA retroviral particles were purchased from Invitrogen. The shRNA sequences targeting IRF3 is as follow: 5′-GATGAGCTACGTGAGGCATGT-3′. The shRNAs were cloned into pSMAL-GFP/Puro. For retroviral packaging, 293T cells were co-transfected with the retroviral particles with psPAX2 and VSVG as described by the manufacturer (Life Technologies). For transduction, cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 h, infected cells were selected for 72 h with puromycin (2 mg/ml) or hygromycin (200 mg/ml).

2.6. Methylcellulose colony-forming cell assay

The methylcellulose colony-forming cell assay was performed as described previously [15]. In all, 0.9 ml of 1×10^3 cells/ml were combined with 1.2 ml of 2.1% (w/v) methylcellulose and 0.9 ml fetal bovine serum; 3 ml was plated in triplicate on 35 mm plates with gridlines. Plates were imaged and counted after 9 days at 37 °C in 5% CO_2 with the EVOS XL Core Imaging System (Life Technologies).

2.7. Apoptotic analysis

Cellular apoptosis analysis was conducted with an Annexin V-FITC Apoptosis Detection Kit (Sigma, APOAF) according to the manufacturer's protocol. An FACSCalibur flow cytometer was used for data analysis.

2.8. Luciferase assay

For promoter luciferase assays, we co-transfected 293T cells with 0.7 μg miR-155 promoter construct (pGL3-1783) or pGL3-control and 0.2 μg pcDNA4.1, IRF3 (pcDNA4.1-IRF3) expression constructs. Firefly luciferase and Renilla luciferase activities were determined 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, E1910). Values were normalized by using firefly luciferase or Renilla luciferase, respectively.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described by the Upstate protocol with some modifications. AML cells were harvested and cross-linked with 1% formaldehyde at room temperature for 10 min. After washing four times with 20 ml PBS in 50 ml conical tubes, cells were scraped and swelled in hypotonic swelling buffer (25 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, protease inhibitor cocktail from Roche) and incubated on ice for 10 min. Following centrifugation at 2000 rpm for 10 min, the nuclei were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris (pH 8.1)) and sonicated with Branson 150 sonicator. Antibodies against IRF3 (Abcam, ab25950) and IgG antibody (Santa Cruz, sc-2027) were used for IP. q-PCR was carried out with specific primers to amplify the IRF3-binding region of the miR-155 promoter (forward, 5′-CAGCCTGGAGGAGGATCGA-3′; reverse, 5′-TCCCAAAGCCCC-CAATCT-3′).

2.10. Statistical analysis

All values are expressed as the mean ± SEM. Statistical differences among groups were determined using either Student's *t*-test (for two groups) or one-way ANOVA (for more than two groups)

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using Graph-Pad Prism 6 Software. Linear regression analysis was performed to analyze the relationship between IRF3 and miR-155.

3. Results

3.1. IRF3 expression in human acute myeloid leukemia

To explore the potential function of IRF3 in human acute myeloid leukemia (AML), we tested the expression of IRF3 in human AML and non-AML controls. We first compared the mRNA levels of IRF3 in twenty AML patients and seven normal donors. The results showed that IRF3 mRNA was significantly enriched in human AML compared with non-AML normal controls (Fig. 1A). We further performed western blot to study the protein level of IRF3 in AML cells from patients or cells from non-AML healthy donors. As shown in Fig. 1B and C, we found that the protein level of IRF3 was also markedly up-regulated in AML patients compared with normal healthy controls. These findings demonstrate that IRF3 mRNA and protein levels are increased in AML patients, indicating that IRF3 may participate in the development of human AML.

3.2. IRF3 promotes AML cells growth

We next investigated whether IRF3 influence AML cells growth by using two cells lines, OCI/AML-2 and OCI/AML-3. We knocked down the expression of IRF3 in these two cell lines, as evidenced by western blot (Fig. 2A and B). We performed cell proliferation assay in OCI/AML2 cells and found that IRF3 knockdown reduced the proliferation rate of OCI/AML-2 cells since day 4 (Fig. 2C). In addition, similar results were observed in OCI/AML-3 cells (Fig. 2D). Furthermore, we measured clonogenic potential using methylcellulose colony-forming cell assays. Colony forming cell assays demonstrated that IRF3 knockdown resulted in a decline in colony formation potential of OCI/AML2 and OCI/AML3 cells (Fig. 2E and F). Finally, we also overexpressed IRF3 in OCI/AML2 with lentivirus (Fig. 2G) and performed colony formation assay. The results indicated that IRF3 overexpression conferred a significant increase in colony formation potential of OCI/AML2 cells (Fig. 2H). Taken together, these results demonstrate that IRF3 promotes AML cells proliferation and colony formation.

3.3. IRF3 regulates AML cell survival

To explain the effects of IRF3 on proliferation and colony formation, we examined the basal viability of OCI/AML-2 and OCI/AML-3 cell lines with/without IRF3 knockdown or overexpression. Annexin-V staining revealed a higher level of apoptosis in OCI/AML-2 and OCI/AML-3 with IRF3 knockdown compared with those without IRF3 knockdown (Fig. 3A and B). Furthermore, we found that IRF3 overexpression markedly reduced the basal level of apoptosis in both OCI/AML-2 and OCI/AML-3 cells (Fig. 3C and D). Altogether, these findings demonstrate that IRF3 overexpression provides AML cells with a growth advantage as observed in colony-forming cell assays and suspension culture, a phenotype that may be fostered, in part, by decreased basal apoptotic activity.

3.4. IRF3 promotes the expression of miR-155

Earlier publications reveal that AML patients have an increased expression of miR-155 [14], however, the underlying mechanism is not fully understood. Indeed, our findings also revealed that miR-155 was overexpressed in human AML (Fig. 4A). Therefore, we conjectured that IRF3 may regulate the expression of miR-155 and we analyzed the relationship of IRF3 and miR-155. Interestingly, our linear regression analysis showed that the expression of miR-155 was positively correlated with IRF3 mRNA level (Fig. 4B), indicating that IRF3 may regulate the expression of miR-155. Next, we cloned the promoter of miR-155 and performed luciferase assay to determine the effect of IRF3 on miR-155 promoter. Our results showed that IRF3 overexpression could significantly promote the promoter activity of miR-155 (Fig. 4C). We next analyzed the enrichment of IRF3 on miR-155 promoter in OCI/AML-2 cells and found that IRF3 could selectively bind to the promoter of miR-155 (Fig. 4D). Therefore, these findings indicate that IRF3 binds to the promoter of miR-155 and facilitates the promoter activity of miR-155. We also analyzed whether IRF3 regulates the level of miR-155 in OCI/AML-2 and OCI/AML-3 cells. As shown in Fig. 4E and F, IRF3 knockdown reduced the level of miR-155 in OCI/AML-2 and OCI/AML-3 cells. In contrast, IRF3 overexpression up-regulated the level of miR-155 in OCI/AML-2 and OCI/AML-3 cells (Fig. 4G and H). Taken together, these findings support that IRF3 binds to the promoter of miR-155 and activates the expression of miR-155, which may conduct the effects of IRF3 on cell growth and survival in AML cells.

4. Discussion

The roles of the IRF family in human AML remain largely unknown. Here in this work, using clinical samples and AML cells, we demonstrate that IRF3 promotes AML development. We first found that IRF3 mRNA and protein levels were significantly up-regulated in human AML compared with healthy donors. Using loss-of-function and gain-of-function strategies, we showed that IRF3 promoted OCI/AML-2 and OCI/AML-3 cell growth and survival. Finally, we provided evidence that IRF3 promoted the expression of miR-155 by binding to its promoter.

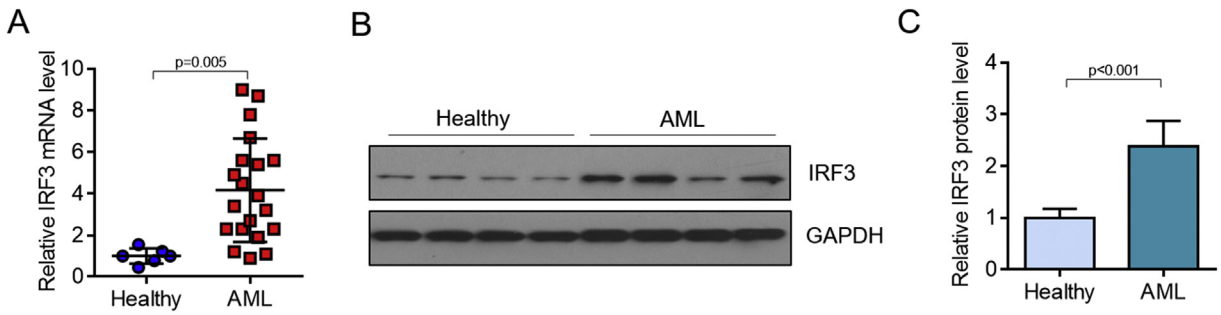

Fig. 1. IRF3 is up-regulated in human acute myeloid leukemia. (A) IRF3 mRNA level is up-regulated in human acute myeloid leukemia (AML, n = 20) compared with healthy control (n = 7). Data was analyzed by two-tailed unpaired Student's *t*-test. (B) Representative western blot showing IRF3 protein level is up-regulated in human AML compared with healthy control. (C) Quantitative results of IRF3 protein levels in (B). N = 4 in each group. Data was analyzed by two-tailed unpaired Student's *t*-test.

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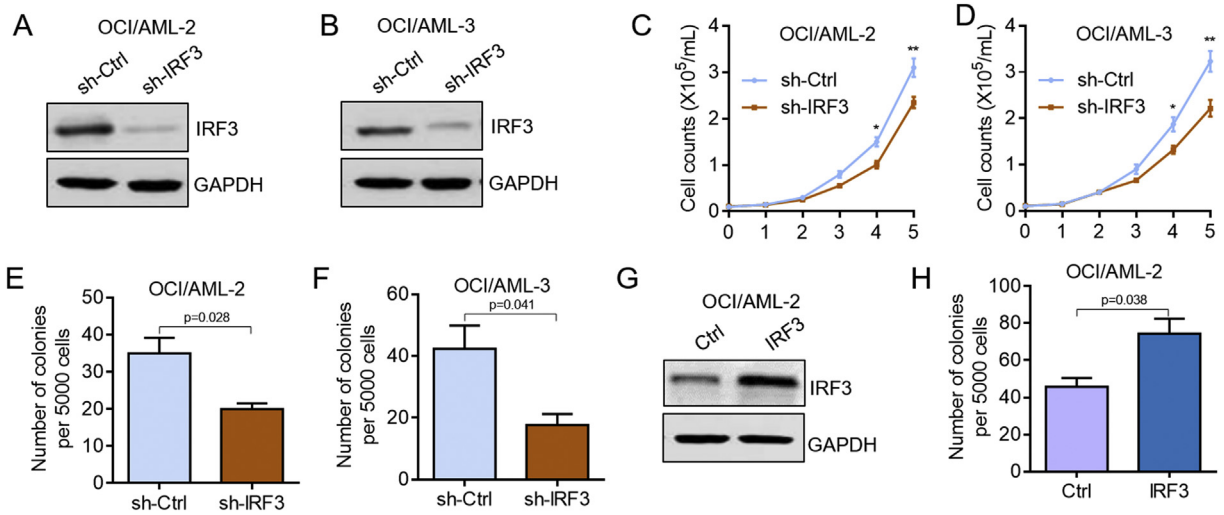

Fig. 2. IRF3 promotes proliferation and colony formation of acute myeloid leukemia cells. (A) Representative western blot showing IRF3 knockdown in OCI/AML-2 cells. OCI/AML-2 cells were transduced with lentivirus expressing sh-Ctrl or sh-IRF3. (B) Representative western blot showing IRF3 knockdown in OCI/AML-3 cells. OCI/AML-3 cells were transduced with lentivirus expressing sh-Ctrl or sh-IRF3. (C) IRF3 knockdown inhibits OCI/AML-2 cell proliferation. Sorted OCI/AML-2 cells with/without IRF3 knockdown were grown in normal serum condition and Trypan blue cell counts were performed daily. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* sh-Ctrl. Data was analyzed by one-way ANOVA. (D) IRF3 knockdown represses OCI/AML-3 cell proliferation. Sorted OCI/AML-3 cells with/without IRF3 knockdown were grown in normal serum condition and Trypan blue cell counts were performed daily. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* sh-Ctrl. Data was analyzed by one-way ANOVA. (E) IRF3 knockdown inhibits colony formation of OCI/AML-2 cells. One thousand sorted OCI/AML-2 cells with/without IRF3 knockdown were plated in methylcellulose for colony-forming assay, quantitative results of colonies are presented. Data was analyzed by two-tailed unpaired Student's *t*-test. (F) IRF3 knockdown represses colony formation of OCI/AML-3 cells. One thousand sorted OCI/AML-3 cells with/without IRF3 knockdown were plated in methylcellulose for colony-forming assay, quantitative results of colonies are presented. Data was analyzed by two-tailed unpaired Student's *t*-test. (G) Representative western blot showing IRF3 overexpression in OCI/AML-2 cells. OCI/AML-2 cells were transduced with Ctrl or IRF3 overexpressing lentivirus. (H) IRF3 overexpression facilitates colony formation of OCI/AML-2 cells. One thousand sorted OCI/AML-2 cells with/without IRF3 overexpression were plated in methylcellulose for colony-forming assay, quantitative results of colonies are presented. Data was analyzed by two-tailed unpaired Student's *t*-test.

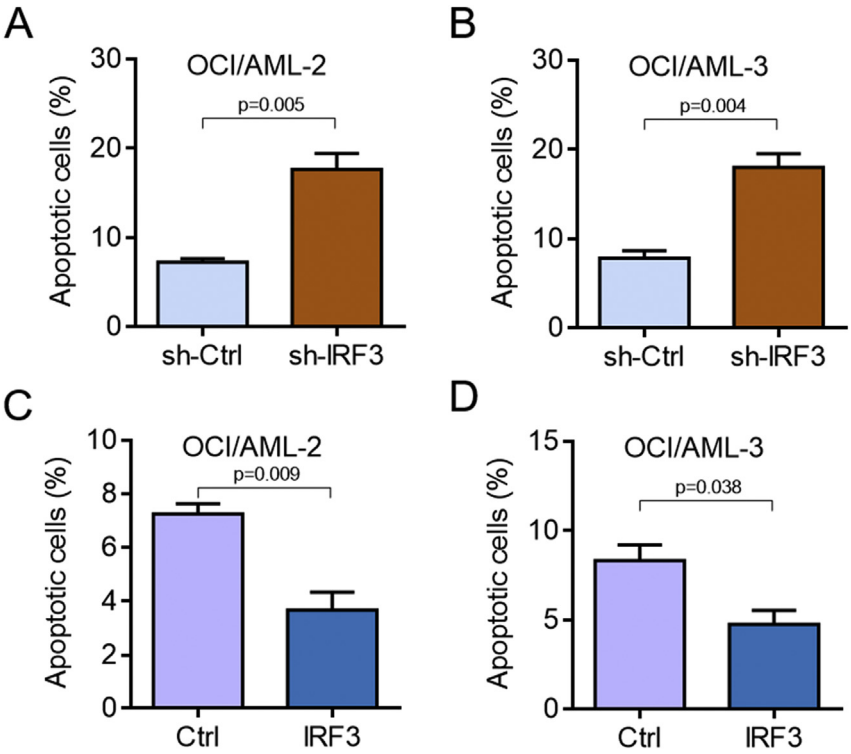

Fig. 3. IRF3 maintains survival of acute myeloid leukemia cells. (A) IRF3 knockdown induces apoptosis in OCI/AML-2 cells. OCI/AML-2 cells were infected with lentivirus expressing control or IRF3 shRNA. Annexin V/DAPI staining was used to analyze cell apoptosis. Data was analyzed by two-tailed unpaired Student's *t*-test. (B) IRF3 knockdown induces apoptosis in OCI/AML-3 cells. OCI/AML-3 cells were infected with lentivirus expressing control or IRF3 shRNA. Annexin V/DAPI staining was used to analyze cell apoptosis. Data was analyzed by two-tailed unpaired Student's *t*-test. (C) IRF3 overexpression reduces apoptosis in OCI/AML-2 cells. OCI/AML-2 cells were infected with lentivirus expressing control or IRF3 constructs. Annexin V/DAPI staining was used to analyze cell apoptosis. Data was analyzed by two-tailed unpaired Student's *t*-test. (D) IRF3 overexpression represses apoptosis in OCI/AML3 cells. OCI/AML-3 cells were infected with lentivirus expressing control and IRF3 constructs. Annexin V/DAPI staining was used to analyze cell apoptosis. Data was analyzed by two-tailed unpaired Student's *t*-test.

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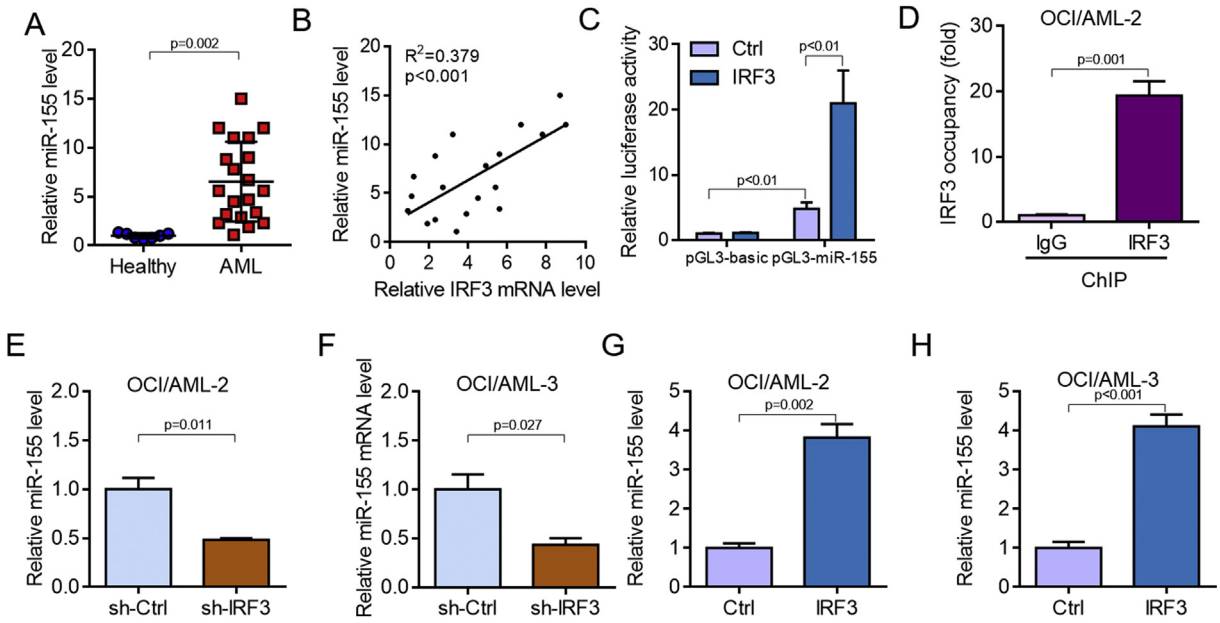

Fig. 4. IRF3 regulates the expression of miR-155. (A) MiR-155 is overexpressed in human AML (N = 20) compared with healthy controls (N = 7). (B) Linear regression analysis showing that miR-155 level is positively correlated with IRF3 mRNA level in human AML. N = 20, $R^2 = 0.379$, $p < 0.001$. (C) Luciferase assay showing that IRF3 promotes the promoter activity of miR-155. The promoter of miR-155 (−1500~ + 70 bp) was cloned into pGL3 plasmid and luciferase assay was performed in 293T cells with indicated plasmids. Data was analyzed by one-way ANOVA. (D) IRF3 binds to the promoter of miR-155. Chromatin immunoprecipitation was performed with IgG or IRF3 antibody and followed by quantitative real-time PCR with primers amplifying promoter of miR-155 (−455~−300 bp). Data was analyzed by two-tailed unpaired Student's *t*-test. (E) IRF3 knockdown reduces miR-155 level in OCI/AML-2 cells. OCI/AML-2 cells were infected with sh-Ctrl or sh-IRF3 lentivirus, and the level of miR-155 was analyzed. Data was analyzed by two-tailed unpaired Student's *t*-test. (F) IRF3 knockdown reduces miR-155 level in OCI/AML-3 cells. OCI/AML-3 cells were infected with sh-Ctrl or sh-IRF3 lentivirus, and the level of miR-155 was analyzed. Data was analyzed by two-tailed unpaired Student's *t*-test. (G) IRF3 overexpression up-regulates miR-155 level in OCI/AML-2 cells. OCI/AML-2 cells were infected with lentivirus overexpressing Ctrl or IRF3 constructs, and the level of miR-155 was analyzed. Data was analyzed by two-tailed unpaired Student's *t*-test. (H) IRF3 overexpression promotes the expression of miR-155 in OCI/AML-3 cells. OCI/AML-3 cells were infected with lentivirus overexpressing Ctrl or IRF3 constructs, and the level of miR-155 was analyzed. Data was analyzed by two-tailed unpaired Student's *t*-test.

Studies over the past two decades have implicated the critical functions performed by the IRF family. Many IRF members play central roles in the cellular differentiation of hematopoietic cells and in the regulation of gene expression in response to pathogen-derived danger signals. Moreover, the role of several IRF family members in the regulation of the cell cycle and apoptosis has important implications for understanding susceptibility to and progression of several cancers, including AML [16]. Lack of IRF1 expression in acute promyelocytic leukemia and in a subset of acute myeloid leukemias with del(5) (q31). In these patients, accelerated exon skipping results in a loss of IRF1 expression and function that cannot be overcome by exposure to inducing agents [8]. Constitutive IRF8 expression inhibits AML by activation of repressed immune response signaling [9]. The prognostic significance of IRF8 transcripts in adult patients with acute myeloid leukemia [17].

IRF3 plays a key role in innate responses against viruses. Indeed, activation of this transcription factor triggers the expression of type I interferon and downstream interferon-stimulated genes in infected cells [18]. The TEL-AML1 fusion protein of acute lymphoblastic leukemia modulates IRF3 activity during early B-cell differentiation [19]. We found that IRF3 was overexpressed in human AML compared with healthy donors (Fig. 1). We knocked down the expression of IRF3 in AML cell lines OCI/AML-2 and OCI/AML-3 and found that IRF3 knockdown reduced cell growth and induce apoptosis. IRF3 overexpression obtained oppose results (Figs. 2 and 3). These findings indicate that IRF3 facilitate the development of human AML and may serve as a potential prognostic factor. Further work is needed to elucidate the association between IRF3 and patients survival and evaluate its prognostic value.

MicroRNAs (miRNAs) are small RNAs of 19–25 nucleotides that are regulators of gene expression. miRNA expression in AML is closely associated with cytogenetics and FLT3-ITD mutations. A small subset of miRNAs is correlated with survival [20]. One of such miRNA is miR-155. MiR-155 expression is upregulated in both adult and pediatric patients with cytogenetically normal AML (CN-AML) and correlates with adverse clinical outcomes. KDM7A is targeted by miR-155 in acute myeloid leukemia and impacts differentiation [21]. NF-κB/STAT5/miR-155 network targets PU. 1 in FLT3-ITD-driven acute myeloid leukemia [14]. A recent preclinical study developed an LNA Antimir-155 (MRG-106) for the treatment of acute myeloid leukemia and validated miR-155 as a therapeutic target in AML and support the testing of MRG-106 in AML patients in the context of phase 1 clinical trial [22]. However, the mechanism by which miR-155 is regulated is still not fully understood. Indeed, we found that miR-155 was overexpressed in human AML and its expression was positively correlated with IRF3 mRNA level. We further demonstrated that IRF3 bound to miR-155 promoter and promoted its promoter activity. In OCI/AML-2 and OCI/AML-3 cells, we also validated that IRF3 regulates the transcription of miR-155 (Fig. 4). Therefore, IRF3 regulates the expression of miR-155, which may be one of the mechanisms underlying IRF3 function in AML. However, further work should be carried out to explore whether miR-155 is the major target for the effects of IRF3 on AML cells growth.

In conclusion, we identify IRF3 as a potential oncogenic gene in human AML. IRF3 promotes the growth and survival of AML cells partly through regulating the expression of miR-155. Therefore, IRF3 may serve as a promising target for AML treatment.

Conflict of interest

None.

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