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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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