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miR-137 downregulates *c-kit* expression in acute myeloid leukemia

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ABSTRACT

The oncogene *c-kit* plays a vital role in the pathogenesis of acute myeloid leukemia (AML). However, the mechanism of microRNAs targeting *c-kit* in AML has not been determined in detail. Moreover, the role *miR-137 in tumor cell proliferation remains controversial. The aim of this work was to verify whether miR-137* targets *c-kit* and to research the biological effects of restoring *miR-137* expression in leukemia cells. We found that *miR-137* binds specifically to the 3′-UTR of *c-kit* and suppresses the expression and activities of *c-kit*. There is a negative correlation between *miR-137* and *c-kit* expression in both patients and cell lines determined by screening large clinical samples. We found that *miR-137* can inhibit proliferation, promote apoptosis, and induce differentiation of *c-kit*+ AML cells. We determined that *miR-137* can participate in the leukemogenesis by regulating *c-kit*, which could be used as a therapeutic target for acute myeloid leukemia.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the inhibition of cell differentiation and the accumulation of immature cells at various different stages, leading to an overall decrease of the hematopoietic system [1]. There are 2.76 leukemia patients per 10 million in China; China ranks third in the incidence of AML worldwide and has the highest levels of mortality in young patients with AML [2]. Therefore, it is most urgent that the detailed pathogenesis of AML is understood so that the most effective treatment of AML can be determined.

c-kit is an 80Kb proto-oncogene that encodes a 145 KD transmembrane tyrosine kinase receptor named C-KIT, also known as CD117 [3]. C-KIT expresses in a minority (<5%) of bone marrow myeloid progenitor cells [4] and is found in the tumor cells of over 70% AML patients [5]. Recent large sample studies of patients with core-binding factor AML (CBF AML) indicate that approximately 37% of adult patients and 19% of pediatric patients have *c-kit* mutations [6], leading to increased C-KIT activity. Tyrosine kinase inhibitors (TKI), such as imatinib, dasatinib, and PKC412, have been shown to suppress tumor growth by decreasing aberrant activities of C-KIT. However, patients respond variously to TKI depending on the specific mutation in *c-kit*, and often display drug resistance and inefficiency of clinical treatment [7]. Therefore, the pathway underlying regulation of C-KIT needs to be further investigated. Thus far, it has been proven that several microRNA (miRNA) regulated C-KIT and play a vital role in the development of tumors including miR-221/222, 193a, 193b, 29b, and 34 [8–12]. miRNAs target C-KIT function by influencing three prominent signaling pathways that regulate cellular proliferation and self-renewal: Sp1/NF-κB, STAT5A/B, and PI3K/AKT [8,10,11].

We predicted several miRNAs, *miR-128, miR-218, miR-137*, and *miR-296,* might target the 3′-UTR of *c-kit* according to our bioinformatic analyses: targetscan and miRbase. Luciferase activity of the *c-kit* 3′-UTR reporter was reduced about 3.62–42.56% by ectopic expression of the miRNAs; among them, *miR*-137 was the most efficient inhibitor. The KASUMI-1 cell line contains a mutation on N822 of *c-kit* [13], meanwhile the K562 cell line expresses high levels of wild-type *c-kit*; therefore, we chose these two cell lines to research *c-kit*-driven AML. In the current study, we hypothesized that *miR-137* may modulate tumor growth by targeting *c-kit*. We analyzed the correlation between *c-kit* and *miR-137 in* large samples of AML patients as well as cell lines and explored whether restoration of *miR-137* can affect biological characteristics of AML cells.

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Table 1
Primers and inhibitors.

Name	Sequence(5′-3′)
miR137 RT1	GTTGGCTCTGGTGCAGGGTCCGAGGTATT-CGCACCAGAGCCAACCTACGCGT
miR137 RT2-F	CGGCGGTTATTGCTTAAGAATAC
miR137 RT2-stemloop	GTGCAGGGTCCGAGGT
Ghkit-F	GGCGACGAGATTAGGCTGTT
Ghkit-R	CATTCGTTTCATCCAGGATCTCA
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGATTCACGAATTTGCGT
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG
wtUTR-F	TCTAGAACACCATAAGGTTTCGTTTCTG
wtUTR-R	TCTAGAGACTCATGGGCTTGGGAAT
mutUTR-F	TGAAATGTAGAATGTCTTTTGAATATTCCC
mutUTR-R	ATTCAAAAGACATTCTACATTTCAATATTTACAA
pmiR137-F	GAATTCGCAGCAAGAGTTCTGGTGGC
pmiR137-R	TCTAGACTACCTTGGCAACCACGGGCG
iNC	CAGUACUUUUGUGUAGUACAA
inh137	CUACGCGUAUUCUUAAGCAAUAA

2. Materials and methods

2.1. Patients

Bone marrow (BM) from 77 patients and 57 healthy controls were collected at Shengjing Hospital of China Medical University between December 2013 and March 2016. Healthy BM was obtained from healthy transplantation donors. Clinical samples were obtained from 63 male and 71 female patients whose median age was 50.00 (41.076–51.567). The samples were divided into five groups: healthy controls (n = 57), primary AML (n = 49), complete remission AML (CR AML) (n = 28), $CD117^{-}$ cell lines (n = 2) and $CD117^{+}$ cell lines (n = 3) respectively (Table S1). All diagnostic methods were performed independently from each other including morphology and immunophenotyped as well as cytogenetic and molecular tests. The comprehensive diagnosis was confirmed according to World Health Organization (WHO) classification criteria [14].

2.2. Real-Time PCR

Mononuclear cells from BM samples were prepared using Ficoll-Hypaque (Sigma-Aldrich, USA) gradient centrifugation. RNA was extracted from BM samples and cell lines manually according to the standard protocols. The cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using an RNA PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Co, USA). *c-kit* was reversely transcribed using random primers and *miR-137* was amplified using a miRNA-specific stem-loop primer. Primer sequences were listed in Table 1. Real-time PCR was performed using the SYBR Premix Ex Taq II (TaKaRa Co, USA) on the Applied Biosystems 7900 Fast Real-time PCR System. The relative expression of mRNA copy number was calculated by $2^{-\triangle\triangle CT}$, with U6 small nuclear RNA used for normalization and the average CT value of healthy people as a control. The CT value was analyzed using SDS 2.4 software.

2.3. Plasmid construction

The human *miR-137* precursor (*pmiR-137*) was amplified by PCR using the primers listed in Table 1 and cloned into pcDNA3.1(+) (Life Technologies, USA) to generate the microRNA expression vector (pmiR). The wild type 3′-UTR of *c-kit* (named *wtUTR*) (55640984–55642244) was inserted downstream of the firefly luciferase-coding region of the pGL3-promotor vector (Promega, USA). The mutation type of *c-kit* 3′-UTR was also constructed (named *mutUTR*) using PrimeSTAR® Max DNA Polymerase (TAKARA, USA). Primer sequences were listed in Table 1. The authenticity and orientation of all the constructs were confirmed by DNA sequencing (Genscript, China).

2.4. Cell culture, and transfection

The human hepatoma cells (Bel7402), human megakaryoblast (DAMI), human promyeloblast (HL60), human macrophage promyeloblast (Kg1a), human myeloblast (kasumi-1), and human chronic myeloid leukemia cell lines (K562) were cultured in RPMI 1640 while the human embryo kidney cells (HEK293) were grown in DMEM, supplemented with 10–20% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.5. Luciferase reporter assay

HEK293 and Bel7402 cells were co-transfected with 125 ng *PGL3/wtUTR/mutUTR*, 375 ng *PCDNA3.1/pmiR137/miR-137 inhibitor* (*inh137*)/*inhibitor NC* (*iNC*) (Ambion, USA), and 10 ng pTK. Sequences of *inh137* and *iNC* were listed in Table 1. Firefly and Renilla luciferase activities were measured consecutively after 24 h via a Lumat LB9507 luminometer (Berthold Technologies, Germany) using a Dual-Glo Luciferase Assay (Promega, USA).

2.6. Western blot analysis

2.5 μg *PCDNA3.1 vacant/pmiR137* and 75 pmol *inh137/iNC* were transfected into cell lines. Cells were harvested 96 h after transfection. Total protein was extracted and transferred to a PVDF membrane; then, the membrane was blocked for 2 h and incubated with antibodies against C-KIT (Abcam, Cambridge, MA) or GAPDH antibody (Proteintech Group Inc, Chicago, IL) overnight at 4 °C. Immunoreactive bands were visualized using ECL plus chemiluminescence reagents (Thermo, Rockford, IL) and analyzed with an ECL chemiluminescence detection system (Bio-Rad). The gray value was analyzed using ImageJ software (Version 1.49 V).

2.7. Proliferation, apoptosis, and differentiation assays

2.5 μg *PCDNA3.1/pmiR137* and 75 pmol *inh137/iNC* were transfected into cell lines. Cell proliferation ability was assessed via a Cell Counting Kit-8 (CCK8) (Dojindo, JP) and detected with a MB580 microplate reader (Heales Technology, China). Cell apoptosis ability was assessed using an Annexin V FITC apoptosis detection kit (Dojindo, JP), and detected with a Beckman FC500 with 10,000 events acquired. For the differentiation assay, the cells were stained with anti-CD15-FITC (Beckman, USA), and detected by Beckman FC500 and Navios; 10,000 events were acquired. IgG-FITC isotype control was used simultaneously. Flow cytometric data was analyzed using CXP analysis software (Version 2.1).

2.8. Statistics analysis

Data were subjected to statistical analysis using the SPSS statistical software (version 17.0). All data are expressed as the mean ± SD from three or more experiments. Comparison of measurement data was performed using the following statistical tests: *t*-test, one-way ANOVA, repeated-measure ANOVA; comparison of the count data was performed using a Pearson Chi-square test; and the correlation analysis used the Spearman r test. All P values were two-tailed and $P < 0.05$ was considered statistically significant.