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Knockdown of IARS2 Inhibited Proliferation of Acute Myeloid Leukemia Cells by Regulating p53/p21/PCNA/eIF4E Pathway

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IARS2 encodes mitochondrial isoleucine-tRNA synthetase, which mutation may cause multiple diseases. However, the biological function of IARS2 on acute myeloid leukemia (AML) has not yet been identified. In the present study, qRT-PCR was used to determine the expression of IARS2 in K562, THP1, and HL-60 leukemia cells. Additionally the mRNA levels of IARS2 in CD34 cells and AML cells obtained from patients were detected by qRT-PCR. IARS2-shRNA lentiviral vector was established and used to infect acute myeloid leukemia HL-60 cells. qRT-PCR and Western blot analysis were employed to assess the knockdown effect of IARS2. The proliferation rate and cell cycle phase of HL-60 cells after IARS2 knockdown were evaluated by CCK-8 assay and flow cytometry. The PathScan Antibody Array was used to determine the expression of cell cycle-related proteins in HL-60 cells after IARS2 knockdown. The expression of proliferation-related proteins in HL-60 cells after IARS2 knockdown was determined by Western blot analysis. Results showed that IARS2 expression was stable and much higher in HL-60, THP-1, and K562 leukemia cells and AML cells obtained from patients than that of human CD34 cells. Compared with cells of the shCtrl group, IARS2 was markedly knocked down in cells that were transfected with lentivirus encoding shRNA of IARS2 in HL-60 cells ($p < 0.05$). IARS2 knockdown significantly inhibited the proliferation and induced cycle arrest at the G_1 phase in HL-60 cells. Additionally IARS2 knockdown significantly increased the expression of p53 and p21, and decreased the expression of PCNA and eIF4E in HL-60 cells. In conclusion, IARS2 knockdown can inhibit acute myeloid leukemia HL-60 cell proliferation and cause cell cycle arrest at the G_1 phase by regulating the p53/p21/PCNA/eIF4E pathways.

Key words: Acute myeloid leukemia (AML); IARS2; Proliferation

INTRODUCTION

Acute myeloid leukemia (AML) is the most common type of leukemia in adults, and its incidence has been increasing[1]. AML is a clonal, malignant disease of hematopoietic tissues caused by accumulation of abnormal blast cells in the bone marrow, ultimately leading to impaired normal hematopoiesis[2]. Presently the precise underlying mechanism of action of AML remains unclear, and several studies have suggested a significant correlation between the onset of the disease and several genes[3–5]. Therefore, exploring novel genes related to AML development and understanding the underlying molecular mechanisms is of significant importance for the development of therapeutic strategies and applicable diagnostics for the treatment of AML.

Mitochondrial isoleucine-tRNA synthetase is coded by the IARS2 gene in the zone 4 band 1 of chromosome 1. It is synthesized in the cytoplasm and then transported to the mitochondrion to catalyze binding of isoleucine to specific tRNAs for completion of mtDNA translation[6,7]. The IARS2 gene is a nuclear gene that encodes mitochondrial isoleucine-tRNA synthetase[8]. In previous studies, it has been indicated that a mutation in the gene encoding mitochondrial isoleucine-tRNA synthetase may cause skeletal dysplasia[9], CAGSSS syndrome[7], growth hormone deficiency[10], and hypertrophic cardiomyopathy[11].

It has been reported that IARS2 is a carcinogenic gene, silencing of IARS2 inhibited the proliferation of tumor cells, and that it is closely related to cell apoptosis[12,13]. Increased expression of mitochondrial IARS has also

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been reported in hereditary nonpolyposis colorectal cancer[14]. Moreover, it is indicated that IARS2 may be a novel target for the treatment of non-small cell lung cancer (NSCLC) because silencing of IARS2 induced cell cycle arrest, caused inhibition of NSCLC growth, and promoted cell apoptosis[15]. Knockdown of IARS2 suppressed the growth of gastric cancer cells by regulating the phosphorylation of cell cycle-related proteins[16]. Taken together, the above results suggested that IARS2 may be involved in the development of cancer. However, the potential role of IARS2 in AML has not yet been identified.

The purpose of this study was to investigate the expression of the IARS2 gene in several leukemia cell types and to identify the biological effect of IARS2 knockdown on proliferation, apoptosis, and the cell cycle in vitro, which would be helpful for exploring potential therapeutic strategies for AML therapy.

MATERIALS AND METHODS

Ethics Statement

The study was conducted with permission from the medical ethics committee of the Second Affiliated Hospital of Shaanxi University of Chinese Medicine. Written informed consent was acquired from all subjects or guardians prior to using their specimens.

Materials

RPMI-1640 media and fetal bovine serum (FBS) were purchased from Gibco Co., Ltd (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich China, Inc. (Shanghai, P.R. China). Antibodies against p21, p53, elF4E, proliferating cell nuclear antigen (PCNA), and GAPDH were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

This study included 12 newly diagnosed adult primary acute myeloid leukemia (M3) patients (age 20–55 years) who were treated in the Second Affiliated Hospital of Shaanxi University of Chinese Medicine (Xianyang, Shaanxi, P.R. China) between April 2015 and October 2017. AML cells were isolated to analyze the mRNA levels of IARS2.

Cell Culture

HL-60, THP-1, HL-60, and CD34 cells were acquired from the Institute of Hematology of China Academy of Chinese Medical Sciences (Beijing, P.R. China). All cell lines were cultured as monolayer with RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Lentivirus Packaging and Infection

Lentivirus expressing short hairpin RNA (shRNA) targeting the IARS2 sequence was constructed by Genechem Co. Ltd (Shanghai, P.R. China). Lentivirus-expressing scrambled shRNA was used as a negative control (Genechem Co. Ltd.). shRNA sequences were obtained by chemical synthesis of a single-strand DNA oligo and were annealed, digested, and inserted between *Age*I and *Eco*RI restriction sites of the Gv115 plasmid vector, which contained green fluorescent protein (GFP)-encoding sequences. The shRNA sequence is 5′-ACGTACTGGCGGCAGATAA-3′. Each nucleotide sequence was inserted into the IARS2 shRNA-expressing vector. Recombinant plasmids containing the IARS2-siRNA sequence were prepared by transfecting *Escherichia coli* to amplify the plasmid. Positive clones were screened by PCR identification, and sequence comparison was performed.

Lentiviruses were generated by triple transfection of 80% confluent 293T cells with the GV115 plasmid vector, pHelper 1.0 and pHelper 2.0 helper plasmids according to the manufacturer's guidelines. Subsequently, 293T cells were harvested by centrifugation at 4,000×*g* for 10 min at 4°C and filtered through a 0.45-mm filter. Then supernatant containing lentiviral particles was centrifuged at 25,000×*g* for 2 h at 4°C, and the lentivirus harvested.

A total of 200 μl of transfection reagent was added dropwise to the HL-60 cell suspension in six-well plates, and the cells were gently mixed. The cells were maintained for 12 h at 37°C in a humidified incubator with 5% CO_2. After 96 h, the infection efficiency, indicated by GFP expression, was determined by a fluorescence microscope.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from HL-60 cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA). Cells were collected for TRIzol treatment by centrifuging for 5 min at 2,000 rpm at 4°C and by the addition of 1 ml of TRIzol to the cell supernatant. After mixing for 5 min at room temperature, samples were precipitated and transferred to a new 1.5-ml tube.

Next, cDNA was obtained by reverse transcription using the Promega M-MLV kit (Promega, Madison, WI, USA). Quantitative real-time (qRT)-PCR was performed using a Real-Time PCR Detection System (Agilent, Santa Clara, CA, USA). SYBR Master Mixture Kit (Takara, Japan) and RNA reverse transcription were performed to determine expression. The primers used were as follows: IARS2 5′-TGGACCTCCTTATGCAAACGG-3′ (forward) and 5′-GGCAACCCATGACAATCCCA-3′ (reverse); GAPDH 5′-AGCCACATCGCTCAGACAC-3′ (forward) and 5′-GCCCAATACGACCAAATCC-3′ (reverse).

Western Blot Analysis

After lentivirus infection, HL-60 cells were lysed and centrifuged at 12,000 × *g* for 15 min at 4°C. Total protein was then extracted from the resulting supernatant and the concentration quantified by bicinchoninic acid (BCA) assay. Equal amounts of protein (30 μg) were separated on 10% SDS-polyacrylamide gels and were transferred onto PVDF membranes. After blocking with TBST containing 5% skimmed milk for 1 h, membranes were incubated overnight at 4°C with Flag-conjugated rabbit monoclonal anti-human GAPDH primary antibodies (1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA). Next, membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies, and proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Millipore, Boston, MA, USA).

CCK-8 Assay

The Cell Counting Kit-8 (CCK-8) assay was used to determine the cell proliferation after IARS2 knockdown. The CCK-8 assay was performed using 2×10^3 cells/well in 96-well plates. HL-60 cells were infected with lentivirus, and on days 1, 2, 3, 4, and 5, CCK-8 (10 μl) was added to the cells, respectively. Cells were cultured for another 4 h after treatment with CCK-8. Cell suspensions were then prepared and vortexed for 5 min, after which the absorbance was read at 450 nm, and cell proliferation rate was determined.

Cell Cycle Detection

IARS2 knockdown HL-60 cells at a density of 1×10^6/well in six-well plates were incubated in a humidified incubator at 37°C with 30% humidity and 5% CO_2 for 24 h. Cells were fixed with 70% ethanol overnight and incubated for 30 min with 1 ml of PI solution (20 μg/ml in PBS, containing 1% Triton X-100) supplemented with RNase A at 37°C in the dark. Cell cycle phases were assessed by flow cytometry (BD Bioscience, San Jose, CA, USA) with the CellQuest software (BDIS).

Detection of Relative Caspase 3 Activity

Caspase-Glo®3/7 Assay (Invitrogen) was used to determine apoptosis by measuring the activity of caspase 3 in HL-60 cells. Cells (1×10^4/well) in six-well plates were incubated in a humidified incubator at 37°C with 30% humidity for 72 h. Then 100 μl of Caspase-Glo3/7 was added, and cells were vortexed for 30 min at 500 rpm at 25°C. Cell suspensions were incubated at 25°C for 2 h, and fluorescence was recorded at 1 h on a GloMax® Multi+ System using the Blue (490 nm Ex, 510-70 nm Em) optical kit. Relative caspase 3 activity was determined using the formula: Relative caspase 3 activity = activity of caspase 3 of shIARS2 group/activity of caspase 3 of shCtrl group.

PathScan Intracellular Signaling Array

Cell lysates were prepared as described above, and total proteins were isolated. Intracellular signaling molecules were determined using the PathScan Intracellular Signaling Array Kit (Cell Signaling Technology) according to the manufacturer's guidelines.

Statistical Analysis

Data were presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed by one-way ANOVA. Software SPSS 17.0 was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Expression of IARS2 Gene in Leukemia Cells

The expression of IARS2 was determined in four types of human myeloid leukemia cells, CD34 cells, and AML cells obtained from patients by qRT-PCR. The data showed mRNA expression of IARS2 in HL-60, THP-1, k562 cell lines, and AML cells obtained from patients were much higher than that of CD34 cells, and their ΔCt were 5.55 ± 0.139, 5.70 ± 0.234, 9.05 ± 0.135, and 6.32 ± 0.232, respectively (Fig. 1). The CD34 cell line is 13.57 ± 0.342.

Expression of IARS2 Is Inhibited by Lv-shIARS2 in HL-60 Cells

HL-60 cells were infected with lentivirus vector Lv-shIARS2, and green fluorescence signal indicated infection efficacy. Infection efficacy was evaluated using a fluorescent microscope after infection for 72 h. The results indicated that more than 80.2% of HL-60

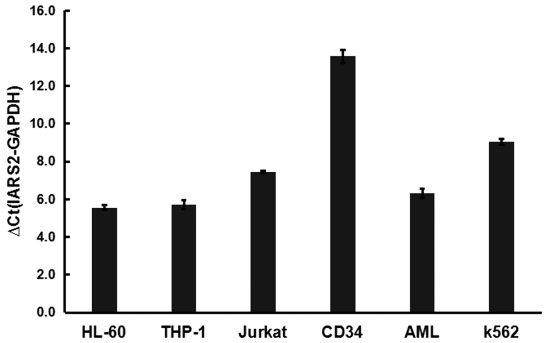

Figure 1. Expression of the IARS2 gene in different leukemia cell lines. Different human myeloid leukemia cells and expression of IARS2 mRNA was determined by quantitative real-time (qRT)-PCR.

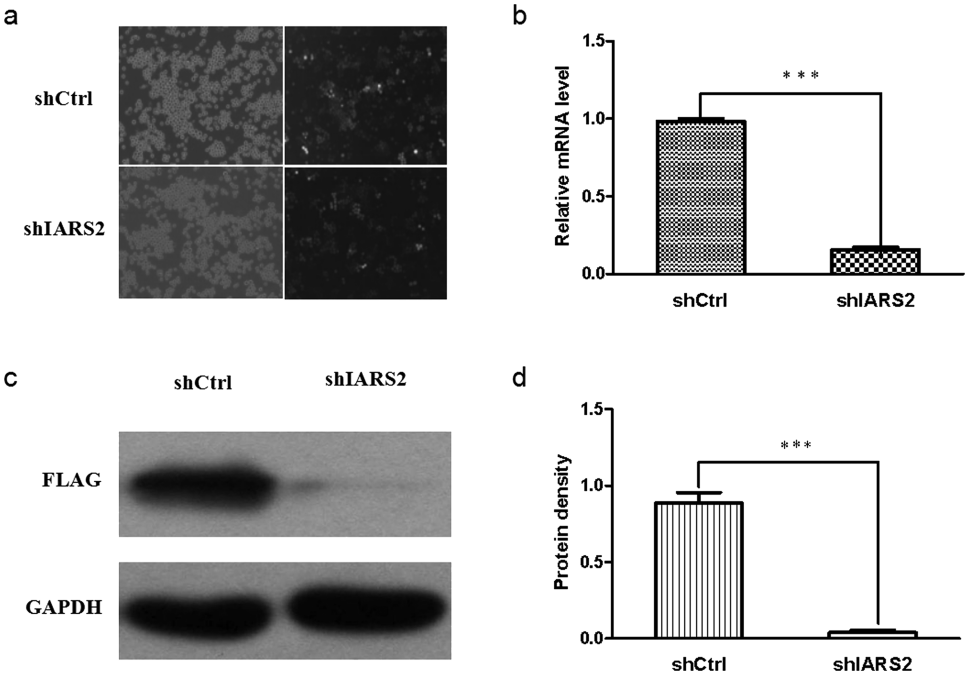

Figure 2. IARS2 knockdown effect in HL-60 cells using lentivirus-mediated RNAi. (a) After lentivirus (Lv) infection, HL-60 cells exhibited a green fluorescence signal. (b) In HL-60 cells, Lv-shIARS2 significantly decreased mRNA expression of IARS2 when compared to the Lv-Ctrl group. (c) In HL-60 cells, Lv-shIARS2 significantly decreased protein expression of IARS2 when compared to the Lv-Ctrl group. (d) Histograms of IARS2 protein expression in IARS2 knockdown and shCtrl cells. Lv-shIARS2 was established and used for transfection in HL-60 cells, and green fluorescence signal was observed using a microscope after 24 h. qRT-PCR and Western blot analysis were performed to determine IARS2 mRNA and protein expression in IARS2 knockdown and control HL-60 cells. ***$p<0.001$ versus shIARS2.

cells exhibited green fluorescence signal after shRNA lentivirus infection, indicating high infection efficiency ($p<0.05$) (Fig. 2a).

To determine the effect of IARS2 knockdown, both IARS2 mRNA and protein levels in HL-60 cells that were infected with Lv-shDGKZ and Lv-shCtrl were determined by qRT-PCR and Western blot analysis. The results showed that the mRNA expression level of the IARS2 gene was significantly suppressed (Fig. 2b), and the knockdown efficiency was 80.6% ($p<0.05$). Additionally, IARS2 expression in HL-60 cells by Western blot analysis showed that the gene knockdown at the protein level was successful (Fig. 2c and d). Together, these data indicated that the lentivirus-mediated RNAi could efficiently suppress the expression of endogenous IARS2 in HL-60 cells.

IARS2 Knockdown Inhibits the Proliferation of HL-60 Cells

After infection with shRNA-lentivirus, 2×10^3 HL-60 cells were plated in 96-well plates and cultured in a humidified incubator at 37°C with 30% humidity and 5% CO_2 for 5 days. The proliferation rates of shIARS2 HL-60 and control cells (shCtrl) were compared daily for 5 days. As shown in Figure 3, a significant decrease in proliferation rate was observed in cells of the shIARS2 group compared to that of the shIARS2 group cells since the fourth day, indicating that IARS2 knockdown inhibited proliferation of HL-60 cells (Fig. 3).

IARS2 Knockdown Alteration in Cell Cycling

To investigate the mechanism of the proliferation suppression effect of IARS2 knockdown, cell cycle distribution of HL-60 cells was evaluated using a flow

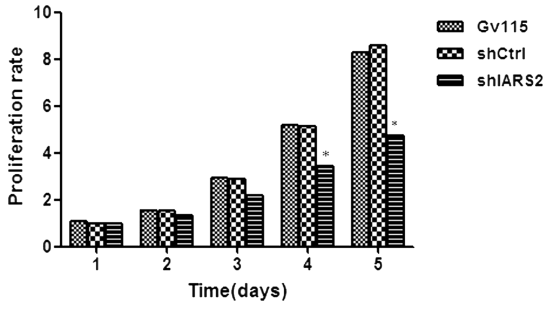

Figure 3. Effects of IARS2 on the proliferation rate of HL-60 cells after knockdown of IARS2. The proliferation rate was evaluated using a Cell Counting Kit-8 (CCK-8) assay on a daily basis. Data represent the mean ± SD from three independent experiments. *$p<0.05$ versus shIARS2.

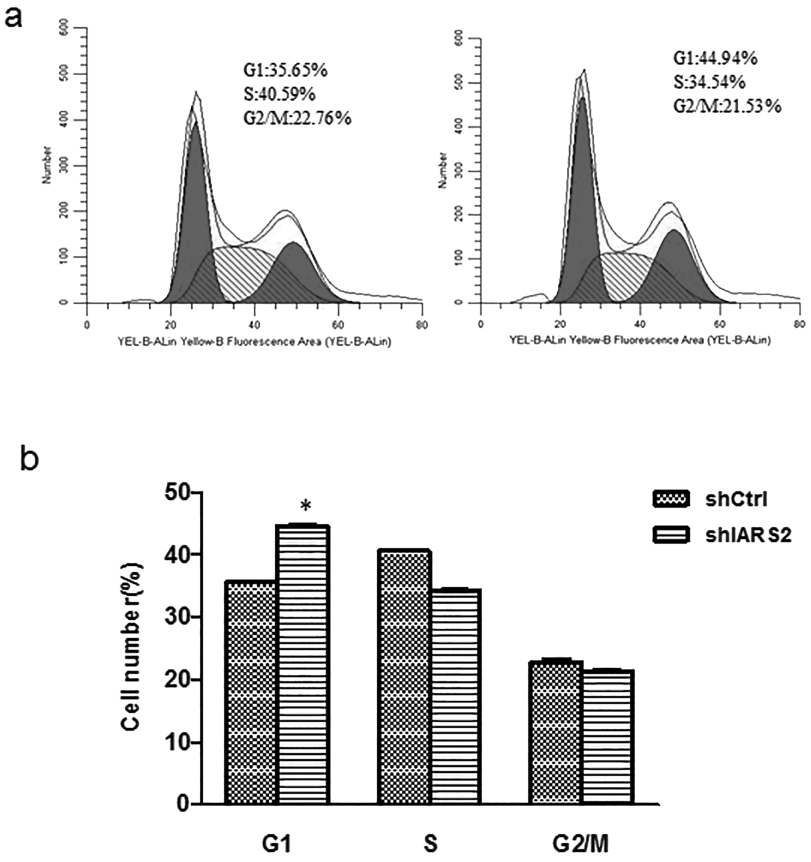

Figure 4. IARS2 knockdown alteration in cell cycling. (a) Cell cycle analysis of HL-60 cells after IARS2 knockdown. (b) Histograms of the distribution of cell cycle phases in HL-60 cells after lentivirus-mediated RNAi. *$p < 0.05$ versus shIARS2.

cytometer. The results indicated that, compared to the control group (shCtrl), HL-60 cells with knockdown IARS2 showed an increase in the percentage of cells in the G_1 phase from 35.6 ± 0.352 to 44.59 ± 0.312. The percentage of cells in the S phases decreased from 40.59 ± 0.455 in the control group (shCtrl) to 34.33 ± 0.159 in the IARS2 knockdown cells (Fig. 4).

IARS2 Knockdown Promoted the Activities of Caspase 3 in HL-60 Cells

Caspase-Glo®3/7 Assay (Invitrogen) was used to investigate the effect of IARS2 knockdown on caspase 3 activities in HL-60 cells infected with shRNA lentivirus after 4 days. The results indicated that the relative activity of caspase 3 in the experimental group was significantly higher compared to that in the control group ($p < 0.05$) (Fig. 5).

IARS2 Knockdown Regulation of Signaling Molecules

To further elucidate the molecular mechanisms by which IARS2 affects HL-60 cell growth, the PathScan Intracellular Signaling Array Kit was used to determine IARS2 knockdown involved in signaling molecules. The results showed that expression levels of p21, p53, caspases 3, 7, and 8 were unregulated in HL-60 cells after RNA interference with the IARS2 gene. In addition, expression of PCNA, eIF4E, and chk2 were significantly reduced in HL-60 cells after RNA interference with the IARS2 gene. Thus, these data indicated that IARS2 knockdown significantly inhibited the growth of HL-60

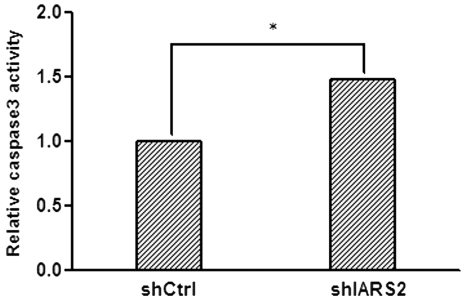

Figure 5. IARS2 knockdown activated caspase 3 in HL-60 cells. HL-60 cells after IARS2 knockdown were cultured, and the relative caspase 3 activity was determined. *$p < 0.05$ versus shIARS2.

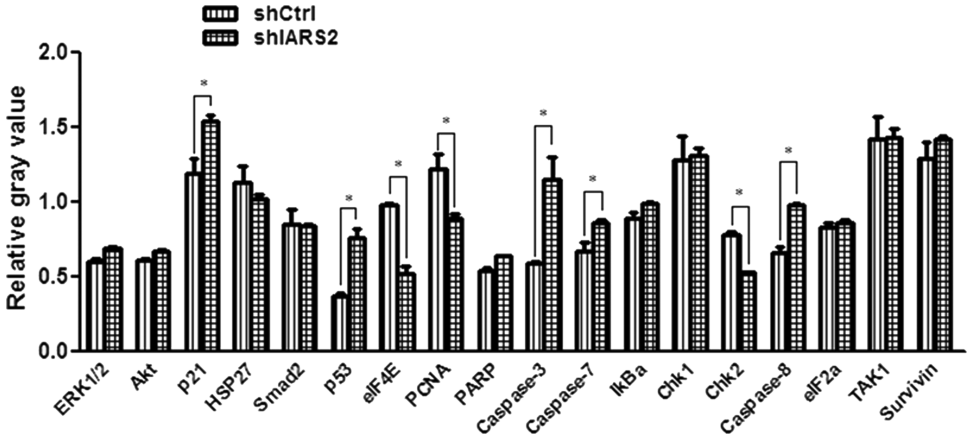

Figure 6. The apoptosis signaling pathway involved in IARS2. After lentivirus infection, HL-60 cells were cultured, and a PathScan Intracellular Signaling Array Kit was used to determine the changes in signaling molecules. *$p<0.05$ versus shIARS2.

cells by regulating protein expression of genes involved in proliferation (Fig. 6).

IARS2 Knockdown Regulation Expressions of p53, p21, PCNA, and eIF4E

To further reveal the mechanism of IARS2 knockdown inhibiting proliferation, various key effectors related to cell proliferation were quantified by Western blot analysis (Fig. 7). Our findings indicated that protein levels of p53 and p21 in HL-60 cells infected with Lv-shDGKZ were increased when compared to those of cells in the Lv-shCtrl group. Moreover, the expression of eIF4E and PCNA in HL-60 cells infected with Lv-shDGKZ was significantly decreased when compared to cells in the Lv-shCtrl group.

DISCUSSION

Recently, studies have suggested that aminoacyl-tRNA synthetase (AARS) participate in several vital activities, including apoptosis, angiogenesis, RNA splicing, and immunity[6]. In previous studies, it was shown that IARS2 plays a critical role in multiple system diseases, suggesting that this gene is relevant to the pathogenesis and prognosis of a variety of diseases[7,9–11]. Therefore, studying this emerging gene may bring hope to the serious ill.

In this study, we conducted a series of experiments related to IARS2 to investigate the expression level of IARS2 gene in leukemia cell and to evaluate the effect of IARS2 gene knockdown on proliferation, apoptosis, and cell cycle. Our findings demonstrated that IARS2

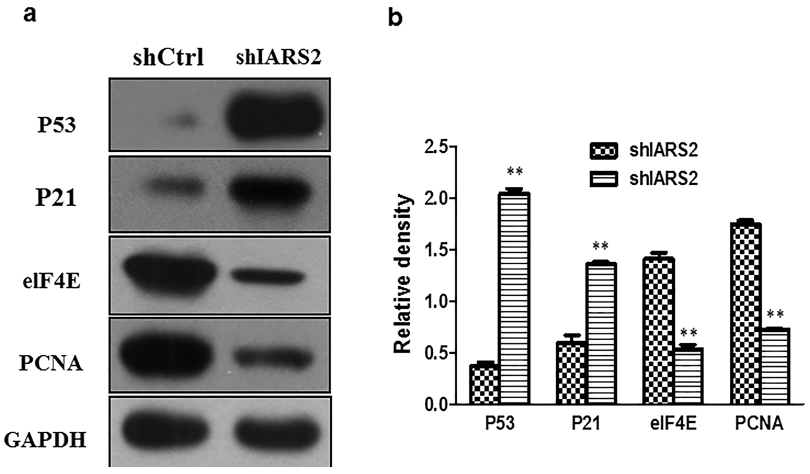

Figure 7. Proliferation-related protein expression in HL-60 cells. (a) After IARS2 knockdown, HL-60 cells were cultured, and the protein expressions of p53, p21, elF4E, and proliferating cell nuclear antigen (PCNA) were determined by Western blot analysis. (b) Histograms of the relative density proteins of p53, p21, elF4E, and PCNA after lentivirus-mediated RNAi. **$p<0.01$ versus shIARS2.

was stably and much higher expressed in HL-60, THP-1, K562 leukemia cells, and AML cells obtained from patients than in CD34 cells. Also it is shown that IARS2 knockdown inhibited the proliferation of HL-60 leukemia cells. Additionally, the relative activity of caspase 3 in HL-60 after IARS2 knockdown was significantly higher compared to that in the control group.

These data implied that IARS2 silencing can inhibit the proliferation of HL-60 cells and induce apoptosis.

The p53 gene is a tumor suppressor gene[17]; when DNA is damaged, the expression levels of p53 increase rapidly[18]. p53 plays a pivotal role in normal and leukemic hematopoiesis and is central in a complex web of AML-related signaling pathways[19]. Under normal conditions, p53 levels are kept low, mainly through inhibition by MDM2[20]. Activated p53 binds DNA and activates the expression of several genes including WAF1/CIP1 encoding for p21. p21 functions as a regulator of cell cycle progression at S phase[21]. p21 is a key factor regulated by p53 in response to DNA damage[22,23]. The tumor suppressor protein p53 can precisely control the expression of the p21 gene, which mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli[24]. Cell cycle arrest induces the function of p21 in promoting error-free replication-coupled DNA double-strand-break (DSB) repair[25], as well as inhibiting DNA replication by binding with the PCNA, DNA polymerase, and several other proteins involved in DNA synthesis[26,27]. eIF4E is a eukaryotic protein synthesis initiation factor[28]. It was reported that eIF4E gene amplification and overexpression appeared to progress from the "tumor-free" margin to the tumor core[24]. In this study, the expression of proteins related to apoptosis and cell cycle were investigated through PathScan Intracellular Signaling Array. The results showed that p21, p53, caspases 3, 7, and 8 were increased in HL-60 cells after RNA interference compared to shCtrl. Moreover, the expression of PCNA, eIF4E, and chk2 were significantly decreased in HL-60 cells after RNA interference with the IARS2 gene. These data indicated that IARS2 knockdown significantly inhibited the growth of HL-60 cells via regulation of protein expression of proliferation. Additionally the expressions of p21, p53, PCNA, and eIF4E were detected by Western blot. The result indicated that protein expressions of p21 and p53 increased, and PCNA and eIF4E were significantly decreased in the HL-60 cells after RNA interference with the IARS2.

In conclusion, our experimental results indicated that IARS2 knockdown in acute myeloid leukemia HL-60 cells inhibited proliferation and altered cell cycling by regulating the p53/p21/PCNA/eIF4E pathways. This suggests that IARS2 be a novel target gene for the treatment of leukemia, and its specific mechanism needs further investigations.

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