Overexpression of IFIT2 inhibits the proliferation of chronic myeloid leukemia cells by regulating the BCR-ABL/AKT/mTOR pathway

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Abstract. Chronic myeloid leukemia (CML) is a myeloproliferative disorder that accounts for ~10% of all newly diagnosed leukemia cases. Early diagnosis is essential for long-term beneficial outcomes. The present study observed that interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) expression levels were reduced in bone marrow samples from CML patients compared with control samples using RNA sequencing and reverse transcription-PCR. IFIT2 expression levels were restored in patients treated with tyrosine kinase inhibitors. To investigate the effect of IFIT2 on CML patients, a stable IFIT2 expressing K562 cell line was established. It was demonstrated that IFIT2 overexpression in K562 cells inhibits cell proliferation and arrests the cell cycle at the G1 phase. In addition, it was demonstrated by western blotting that IFIT2 inhibits the BCR-ABL oncoprotein and regulates its downstream AKT/mTOR signaling pathway. IFIT2 could induce cell cycle arrest-associated gene p27kip1 by degrading cullin1-mediated E3 ligases. In summary, the present study demonstrated that IFIT2 was efficacious in inhibiting CML and is a potential therapeutic target.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease that is characterized by an increase in immature myeloid cells in the bone marrow and peripheral blood (1). CML is associated with a reciprocal translocation t(9;22)(q34;q11), which results in the generation of a BCR-ABL fusion gene (2). The BCR-ABL fusion gene encodes a tyrosine kinase oncoprotein that induces cell proliferation and leukemogenesis by activating the RAS, PI3K/AKT, ERK, MYC and JAK/STAT signaling pathways (3). The tyrosine kinase inhibitor (TKI) imatinib (IM) has been demonstrated to inhibit the kinase activity of BCR-ABL and has been used as a first-line treatment strategy for CML (4). However, ~35% of CML patients exhibit disease progression, relapse and/or intolerance to IM (5). The majority of primary and secondary resistance mechanisms include the amplification of BCR-ABL and point mutations within the ABL kinase domain (6-8). Second and third generation TKIs, such as dasatinib and ponatinib, are able to overcome IM resistance in some patients with T315I mutations (9-11). However, these TKIs have severe side effects, such as cardiovascular thrombotic events, and have therefore been removed from the market. There is an urgent need to develop novel therapeutic strategies with high efficacy and low toxicity for the treatment of patients with CML.

Interferon α (IFNα) has been the treatment choice for certain patients with CML (12). Therefore, TKIs, IFNα or a combination strategy may emerge as a treatment option for CML (13). Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), also known as ISG54, is a member of the IFN-stimulated genes. IFIT2 was originally identified as a direct response target to type I IFN, and it has been reported to inhibit the antiviral activity of BCR-ABL and has been shown to be a potential therapeutic target for CML (14). In addition, IFIT2 has been demonstrated to be associated with clinical parameters and therapeutic outcomes (15,16). Our previous study (16) revealed that IFIT2 overexpression inhibits the proliferation of chronic myeloid leukemia cells by regulating the BCR-ABL/AKT/mTOR pathway.
could enhance curcumin-induced apoptosis in the CML cell line K562. However, whether IFIT2 expression inhibits CML progression remains to be determined.

Using RNA sequencing arrays, the present observed that specific interferon signaling pathway-associated genes, including IFIT2, had reduced expression levels in CML patients compared with healthy controls. Therefore, the current study investigated the role of IFIT2 in CML.

Materials and methods

CML and normal bone marrow cells. Bone marrow cells that were isolated from 26 patients with primary CML were obtained from the Department of Hematology, First Affiliated Hospital of Nanchang University (Nanchang, China) between January 2016 and December 2017. All patients were diagnosed with CML during the chronic phase using the Morphology, Immunology, Cytogenetics and Molecular (MICM) criteria of the World Health Organization classification of myeloid neoplasms (17). Patients were identified as harboring BCR-ABL transcripts using karyotype analysis, fluorescence in situ hybridization and/or reverse transcription (RT)-PCR. All patients included in the study were administered IM (400 mg p.o. q.d.) as a front-line treatment, and the copies of BCR/ABL were monitored by PCR every 3 months. Additionally, 16 bone marrow samples as controls were collected from patients with normal bone marrow, diagnosed by the MICM criteria, at the Department of Hematology, First Affiliated Hospital of Nanchang University between January 2016 and December 2017. No significant differences were identified between the sex and age of the two groups (Table I). The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China). All the patients provided written informed consent.

Library construction and RNA sequencing. Total RNA from the bone marrow of patients was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA integrity was determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). RNA-sequencing library construction was performed using the standard TruSeq RNA sample preparation v2 protocol (Illumina, Inc.). Libraries were then sequenced using the Illumina HiSeq2500 platform at Shanghai Biotechnology Corporation (Shanghai, China). Sequencing and real-time data analyses were performed using the StringTie software (version 1.3.0) provided by Illumina.

Cell culture and reagents. The human CML cell line K562 was cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences). The 293T cell line was cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum. Cells were cultured in a 37°C incubator with 5% CO2. Doxycycline (DOX) and puromycin were purchased from Sigma-Aldrich; Merck KGaA and Invitrogen; Thermo Fisher Scientific, Inc., respectively. RT reagent (cat. no. KR103), Super-Real qPCR PreMix (SYBR-Green) reagent kit (cat. no. FP205) and PCR 2xMix reagent (cat. no. KT201) were purchased from Tiangen Biotech Co., Ltd. pLVX-tetone-puro lentiviral expression vector and the lentiviral packaging plasmids were purchased from Clontech Laboratories, Inc. Antibodies against p27 (cat. no. sc-53906; 1:500) and IFIT2 (cat. no. sc-390724; 1:500), goat anti-mouse IgG-HRP (cat. no. sc-2005; 1:10,000) and goat anti-rabbit IgG-HRP (cat. no. SC-2004; 1:10,000) were purchased from Santa Cruz Biotechnology. Anti-cullin-1 (cat. no. 71-8700; 1:1,000) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Anti-CSN3 (cat. no. GTX33109; 1:5,000) and anti-CSN5 (cat. no. GTX70203; 1:1,000) were purchased from GeneTex, Inc. Antibodies against mTOR (cat. no. 2972; 1:1,000), phosphorylated (p)-mTOR (cat. no. 2971; 1:1,000), AKT (cat. no. 9272; 1:1,000) and p-AKT (cat. no. 9271; 1:1,000) were purchased from Cell Signaling Technology, Inc. Antibodies against hemagglutinin (HA) (cat. no. AE008; 1:5,000), ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1; cat. no. A0282; 1:500), c-Jun (cat. no. A0246; 1:500), p-c-Jun-ser63 (cat. no. AP0048; 1:500) and β-actin (cat. no. AC038; 1:50,000) were purchased from Abclonal Technology, Inc.

Plasmid construction, transfection and lentiviral transduction. The IFIT2 open reading frame (ORF) was cloned in-frame to the HA tag at the N-terminal to generate the pEGFP-C1-IFIT2 plasmid. The vector pEGFP-C1 was from Clontech Laboratories, Inc. The IFIT2 ORF was amplified using PCR with PCR 2xMix reagent and the following primers: Sense, 5'-AGTCCATTTGCAATTCGTAACCATACCGACGTCCAGACTACGCTAGTGAGAACAAT AAC-3' and antisense, 5'-TATGGATCTCAGCAGTGACGCATA-3'. The HA-IFIT2 complementary DNA (cDNA) was then inserted into the lentiviral vector pLVX-tetone-puro, and then transfected into 293T cells together with the packing plasmids pSPAX2 and pMD2G at a ratio of 5:3:2 using EndoFectin™ (GeneCopeia, Inc.) based on the manufacturer's protocol. A total of 48 h post-transfection, virus was harvested and used to infect K562 cells. Stable clones expressing HA-IFIT2 (IFIT2-K562) and negative control (K562-TETONE) were then selected using puromycin. Stable cell clones transduced with the empty vector were used as the controls. The expression of HA-IFIT2 was measured after treating with 2 µg/ml Doxycycline (cat. no. 24390-14; MedChemExpress).

Cell cycle analysis. Cell cycle analysis was performed as described previously (18). Briefly, 1x10^6 cells stably expressing IFIT2 or control cells were harvested and then stained using the COULTER DNA Prep Reagents kit (Beckman Coulter, Inc.), according to the manufacturer's protocol. Stained cells were analyzed using a flow cytometer and cell cycle analysis was performed using MODFIT LT 4.1 software (Verity Software House).

Immunofluorescence staining. Stable cells were transferred onto slides using cyto spun and fixed with 4% paraformaldehyde for 15 min at room temperature. Following permeabilization with 0.5% Triton X-100 in PBS for 5 min at room temperature, cells were incubated with primary antibody anti-IFIT2 (1:200) for 2 h at 37°C. Slides were then washed three times with PBST and incubated with FITC-conjugated anti-mouse IgG (1:100;
study performed RNA-sequencing analysis of bone marrows obtained from three primary CML patients and health donors. Gene expression analysis demonstrated that a number of IFN signal pathway-related genes were differentially expressed in CML compared healthy controls. This included several well-known IFN-induced genes, such as IFIT3, IFIT2, IRF1, IFITM3, OAS2 and OAS1 (Fig. 1A). Among them, IFIT2 mRNA levels were markedly downregulated in CML patients. To validate these results, IFIT2 mRNA expression levels were measured in bone marrow cells from 26 patients with primary CML and 16 healthy controls by RT-qPCR. As presented in Fig. 1B, IFIT2 expression levels were significantly lower in CML patients compared with controls. In addition, IFIT2 expression levels were measured in CML patients treated with TKI. In 6 patients with CML who had been treated with TKI for 6 months, IFIT2 expression levels were higher compared with newly diagnosed patients (Fig. 1C). These results indicated that IFIT2 levels were increased in CML patients and upregulated in patients treated with TKIs.

**Stable K562 cell lines expressing IFIT2.** To functionally investigate the role of IFIT2 in CML, the present study established a stable IFIT2-K562 cell line that overexpressed IFIT2 following DOX exposure. HA-IFIT2 ORF was cloned into pLVX-Tetone-Puro vector with the Tet-On system, which induced gene expression in the presence of DOX (Fig. 2A). Recombinant lentiviruses were produced using 293T cells that were subsequently used to infect K562 cells. Following puromycin selection, western blot and immunofluorescence assays were performed to confirm IFIT2 expression in the selected stable cell lines. As presented in Fig. 2B, the HA-IFIT2 protein was detected using either IFIT2 or HA antibodies in IFIT2-K562 cell lines after 24 h of DOX exposure (2 µg/ml). IFIT2 was not detected in the vector control cell lines K562-TETONE (Fig. 2B). Immunofluorescence staining using IFIT2 antibody also demonstrated increased IFIT2 protein expression in the cytoplasm of IFIT2-K562 cells following DOX exposure (Fig. 2C).

**IFIT2 inhibits cell proliferation by inducing G1 phase arrest in K562 cells.** The effect of IFIT2 overexpression on the proliferation of K562 cells was then measured. The proliferation rates of the K562-TETONE and K562-IFIT2 cell lines were similar (Fig. 3A). Following exposure to 2 µg/ml DOX, cell proliferation in K562-TETONE cells was

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**Table I. Age and sex distribution of controls and patients with CML.**

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CML, chronic myeloid leukemia.

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**Results**

**IFIT2 expression levels are downregulated in CML patients and upregulated following TKI administration.** To determine the mechanism of leukemogenesis in CML, the present

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**Statistical analysis.** Student's t-test was used to compare the expression of IFIT2 in patients and control groups. One-way ANOVA was used to analyze the expression of IFIT2 in the bone marrow of the patients following treatment with TKI for different durations. Two-way ANOVA was used to analyze the differences in proliferation and cell cycle of the cells following stable expression of IFIT2 and treatment with DOX. Following ANOVA, Bonferroni's post hoc test was used to determine significant differences. All data are presented as mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

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**Western blot analysis.** Total proteins were extracted using lysis buffer [1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1 mM Na3VO4 and protease inhibitor cocktail]. Protein concentrations were quantified using the Bio-Rad Protein assay kit II (cat. no. 5000002; Bio-Rad Laboratories, Inc.). Total protein (20 µg/lane) was separated using 10% SDS-PAGE gel, and then electro-transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature in Tris-buffered saline-0.05% Tween-20 (TBST) containing 5% non-fat dry milk. Subsequently, the membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed three times with TBS for 10 min, and then incubated with peroxidase-conjugated secondary antibody at room temperature for 1 h. Following three additional 10 min washes with TBST, target proteins were detected using enhanced chemiluminescence detection reagent (EMD Millipore) and imaged using the Bio-Rad ChemiDoc XRS+ chemiluminescence imaging system (Bio-Rad laboratories, Inc.).
only slightly reduced due to DOX cytotoxicity; however, proliferation of K562-IFIT2 cells was significantly reduced at 48 and 72 h compared with K562-TETONE cells treated with DOX (P<0.0001). Cell cycle analysis demonstrated that IFIT2 overexpression induced growth arrest at the G1 phase in K562 cells. The two cell lines had a similar cell cycle phase...
distribution in the absence of DOX (Fig. 3B and C). However, treatment of K562-IFIT2 cells with DOX for 48 h resulted in an obvious accumulation of the cells at G1 transition phase (P<0.01). Moreover, a significant increase of K562-IFIT2 cells was present in G1 phase following treatment with DOX for 72 h (P<0.001; Fig. 3D). These results demonstrated that IFIT2 inhibits cell proliferation and arrests cells at the G1 phase in CML cells.

Figure 3. IFIT2 overexpression inhibits K562 cell proliferation and arrests cells at the G1 phase. TETONE-K562 and IFIT2-K562 cells were treated with or without DOX (5 µg/ml) for the indicated times. (A) Growth curves of the stable K562 cell lines at 24, 48 and 72 h. (B) Cell cycle was analyzed using flow cytometry. Percentage of cells in G1, S and G2/M phases of the cell cycle following DOX treatment for (C) 48 and (D) 72 h. *P<0.05, **P<0.01, ***P<0.005. IFIT2, interferon-induced protein with tetratricopeptide repeats 2; DOX, doxycycline.
IFIT2 reduces BCR-ABL expression and inhibits phosphorylation of mTOR and c-Jun. K562-TETON and K562-IFIT2 cells were treated with or without DOX (2 µg/ml) for 72 h, and then the expression levels of ABL1, BCR-ABL1, AKT, p-AKT (ser472), c-Jun, p-c-Jun (ser63), mTOR and p-mTOR were analyzed by western blotting. β-actin expression levels were used as the loading controls. IFIT2, interferon-induced protein with tetratricopeptide repeats 2; DOX, doxycycline; p-, phosphorylated; ABL, ABL proto-oncogene 1, non-receptor tyrosine kinase.

IFIT2 reduces BCR-ABL expression and inhibits phosphorylation of AKT/mTOR. The present study then assessed the effect of IFIT2 on the expression of BCR/ABL fusion protein and its downstream targets in K562 cells. As presented in Fig. 4, the two cell lines had similar BCR-ABL expression levels in the absence of DOX treatment. However, BCR-ABL protein expression was markedly decreased in IFIT2-K562 cells following DOX exposure, while ABL1 expression levels were unchanged for both stable cell lines. It has been demonstrated that several signal transduction pathways are activated by BCR/ABL, such as the JAK/STAT/AKT, PI3K/AKT/mTOR and ERK/MAPK/JNK pathways (3). These are critical pathways required for CML cell proliferation. To determine whether IFIT2 affects any of these signaling pathways, western blot analysis was performed. IFIT2 overexpression markedly reduced the protein levels of c-Jun and p-c-Jun. However, it had no effect on AKT or mTOR protein levels, but reduced the phosphorylation levels of p-AKT and p-mTOR. These results demonstrated that inhibition of cell proliferation induced by IFIT2 in CML cells was associated with reduced expression of BCR/ABL and inhibition of the BCR-ABL/AKT/mTOR signaling pathway.

IFIT2 increases p27kip1 expression by inhibiting CRL1-E3 ligase. BCR-ABL inhibits p27kip1 via the MAPK and PI3K signaling pathways and accelerates abnormal cell proliferation leading to leukemogenesis (20). The present study hypothesized that IFIT2 could upregulate p27kip1 protein expression by inhibiting BCR-ABL. Using western blot analysis, it was observed that p27kip1 protein levels were markedly increased in K562-IFIT2 cells following DOX exposure (2 µg/ml). Cell-cycle inhibitor p27 is a known substrate of cullin1-RING ligase (CRL1). CRL1 belongs to a large family of ubiquitin E3 ligases that regulate protein degradation via the ubiquitin proteasome system (21). As presented in Fig. 5, CUL1 levels were markedly decreased in K562-IFIT2 cells following DOX exposure compared with control cells. However, CSN3 and CSN5 levels were similar between the two cell lines, in the presence or absence of DOX. These results demonstrated that IFIT2 promotes the accumulation of p27kip1 by inhibiting BCR-ABL tyrosine kinase activity and degrading cullin1-mediated E3 ligases to suppress CML cell proliferation.

Discussion

In humans, IFITs consist of four members, IFIT1, IFIT2, IFIT3 and IFIT5. In addition to antiviral activity, IFIT2 has been reported to inhibit tumor cell growth, migration and metastasis in a variety of tumor types (22-24). Our previous
study demonstrated that curcumin induces cell apoptosis via an IFIT2-dependent signaling pathway in U937 leukemia cells but not in K562 cells. In addition, our previous study demonstrated that upregulation of IFIT2 by exogenous methods or treatment with IFNγ in K562 cells increases apoptosis and enhances the anticancer effects of curcumin (25). As the extraction of bone marrow is an invasive method, it was difficult to collect bone marrow specimens from healthy volunteers as controls. In the present study, the expression of IFIT2 in the bone marrow of CML patients was compared with that in normal controls that were diagnosed with iron deficiency anemia and immune thrombocytopenia, and whose bone marrow samples were normal according to the MICM criteria. It was demonstrated that IFIT2 levels are decreased in the bone marrow of CML patients, while patients treated with TKI therapy exhibited increased levels. This suggests that IFIT2 may play an important role in leukemogenesis and could be a potential therapeutic target for CML.

The present study also demonstrated that IFIT2 overexpression induced an accumulation of p27kip1 protein and inhibited CRL1-E3 ligase. p27kip1 is a potent inhibitor of cyclin-dependent kinases that drives G1 to S phase transition (27). The degradation of p27kip1 protein is mainly regulated through ubiquitination proteasome dependent pathway associated with cullin 1-mediated E3 ligases (21). The current results demonstrated the involvement of cullin 1-mediated E3 ligases in the upregulation of p27kip1. A previous study has demonstrated that p27kip1 deficiency increases the HSC-containing Lin-Sca-1+c-Kit+ cell population and accelerates leukemogenesis in CML mouse models (28). Therefore, increased p27kip1 levels may inhibit K562 proliferation. Previous studies have reported that inhibition of the nuclear import of p27kip1 by protein kinase B/Akt-mediated phosphorylation is associated with poor prognosis in numerous breast cancer patients (29,30). Tomoda et al (26) demonstrated that EPS8 regulates the proliferation and adhesion of leukemic cells by regulating downstream pathways. Multiple intracellular signal transduction pathways, including the JAK/STAT, ERK/MAPK and PI3K/AKT pathways, have been implicated in this process. Huang et al demonstrated that EPS8 regulates the proliferation, apoptosis and chemosensitivity of CML cells via the BCR-ABL/PI3K/AKT/mTOR pathway. This suggests that IFIT2 may suppress the proliferation of CML cells by regulating the BCR-ABL/AKT/mTOR pathway.

In conclusion, the present study demonstrated that IFIT2 overexpression in K562 cells decreases c-Jun, p-c-Jun and p-AKT levels. c-Jun, a critical component of the transcription factor AP-1, plays an important role in cell cycle progression, differentiation and cell transformation, and is a downstream target of the PI3K/AKT signaling pathway (31). AP-1 blockade has been shown to arrest the cell cycle by inducing the expression of p27 in cancer cells (31,32). This suggests that the PI3K/AKT/c-Jun signaling pathway may be involved in the increased expression of p27.

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Availability of data and materials

The datasets generated and/or analyzed during this study are included in this published article.

Authors' contributions

AL contributed to the study design. ZZ, NL, SL, MJ and JW preformed the in vitro and in vivo experiments. YZ, LW and CX performed the data analysis. ZZ, CX and AL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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