

ORIGINAL ARTICLE

Cysteine-rich protein 61 regulates the chemosensitivity of chronic myeloid leukemia to imatinib mesylate through the nuclear factor kappa B/Bcl-2 pathway

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Abstract

Although the targeted tyrosine kinase inhibitor imatinib mesylate (IM) has achieved significant responses against CML in the clinical setting, a small proportion of patients fail to respond to IM treatment and their disease continues to progress, indicating resistance to IM therapy. As a secreted extracellular matrix protein, cysteine-rich protein 61 (Cyr61) plays an important role in the resistance of solid tumors to chemotherapy, but its role in CML is unclear. In the present study, we observed that Cyr61 levels were upregulated in the plasma and bone marrow (BM) of patients with CML as well as in K562 cells. This upregulation of Cyr61 significantly decreased IM-induced cellular apoptosis of K562 cells through nuclear factor kappa B/B-cell lymphoma 2 pathways. Inhibition of Cyr61 restored the chemosensitivity of K562 cells to IM both in vitro and in vivo. Thus, our results showed for the first time that Cyr61 plays an important role in regulating the chemosensitivity of CML cells to IM, suggesting that selectively targeting Cyr61 directly or its relevant effector pathways may provide potential value in improving the clinical response of patients with CML to IM treatment.

KEYWORDS

apoptosis, chemosensitivity, chronic myeloid leukemia, Cyr61, imatinib mesylate

1 | INTRODUCTION

Chronic myelogenous leukemia is caused by the malignant transformation of a hematopoietic stem cell, which is driven by the constitutively active BCR-ABL fusion tyrosine kinase. Inhibition of BCR-ABL activity by TKI has revolutionized the treatment of CML.¹ Currently,

IM, a TKI, is the first-line treatment for CML and has been shown to elicit effective and durable responses in the majority of patients with CML.^{1,2}

Despite the fact that the introduction of IM has brought improvements in the long-term survival of a high proportion of CML patients, some studies have shown that a small number of patients

Abbreviations: BM, bone marrow; Cyr61, cysteine-rich protein 61; IM, imatinib; NF-κB, nuclear factor kappa B; TKI, tyrosine kinase inhibitor.

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fail to respond to IM treatment and their disease continues to progress, indicating resistance to IM therapy.^{3,4} The mechanism underlying resistance to IM is a complex network involving multiple cellular and molecular mechanisms.

Mutations of BCR-ABL do not account for all cases of resistance to IM therapy. Recently, attention has been drawn to the BM microenvironment, which plays an important role in the differentiation, migration, proliferation, survival, and drug resistance of leukemia cells.^{5,6} The chemoprotective effect of the BM microenvironment has been shown using *in vitro* CML cellular models under IM treatment.⁶⁻¹¹ For example, cytokines within the BM microenvironment, such as interleukin (IL)-3, IL-7, granulocyte-macrophage colony-stimulating factor, and C-X-C motif chemokine 12, can protect CML cells from IM-induced apoptosis and promote CML cell survival.⁷⁻¹¹ However, it remains unknown whether there are other soluble factors that are chemoprotective for CML cells under IM treatment.

Cysteine-rich protein 61 (Cyr61/CCN1), a member of the CCN protein family, is an extracellular matrix-associated protein that plays essential roles in cellular proliferation, survival, adhesion, migration, and differentiation.¹² As a secreted protein, Cyr61 plays unique roles in different tumor types. For example, in breast cancer,^{13,14} prostate cancer,^{15,16} and pancreatic carcinoma,¹⁷ Cyr61 functions as an oncogenic factor. In contrast, Cyr61 suppresses tumor growth in non-small cell lung cancer,^{18,19} endometrial adenocarcinoma,²⁰ and melanoma.²¹ Interestingly, more and more studies have shown that Cyr61 is linked to chemotherapy resistance. Specifically, Cyr61 protects tumor cells from chemo-induced apoptosis and increases cell survival in solid tumors, such as in breast cancer,^{22,23} ovarian cancer,²⁴ pancreatic adenocarcinoma,²⁵ and renal cell carcinoma.²⁶ Recent studies have indicated that Cyr61 is involved in stroma-induced chemoresistance in AML.²⁷ In our previous research, we observed that the levels of Cyr61 were increased in the plasma and BM of patients with acute lymphoblastic leukemia (ALL), and elevated Cyr61 promoted cell survival.²⁸ However, the level of Cyr61 in patients with CML and its involvement in IM-induced CML cellular apoptosis have not been reported.

In the present study, we examined Cyr61 levels in patients with CML and the function of Cyr61 in modulating the sensitivity of CML K562 cells to IM. We found that Cyr61 levels were elevated in the plasma and BM of patients with CML. Furthermore, we observed that Cyr61 effectively reduced IM-induced apoptosis by the NF- κ B/B-cell lymphoma 2 (Bcl-2) pathway. To our knowledge, this study is the first to show the expression and role of Cyr61 in patients with CML, suggesting that targeting Cyr61 or its effector pathways could improve the clinical response in patients with CML.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

Chronic myelogenous leukemia samples were obtained from Fujian Medical University Union Hospital, Fuzhou, China. The BM

supernatant ($n = 33$) and plasma ($n = 36$) samples were collected from newly diagnosed patients who had not received treatment. Normal BM ($n = 11$) and normal plasma ($n = 66$) samples from age-matched healthy transplant donors were included as controls. All BM supernatant and plasma samples were separated by centrifugation at $1000\times g$ for 10 minutes at 4°C and frozen until analysis. The CML K562 cell line and KCL22 cell line were maintained in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and 10% penicillin/streptomycin in a 37°C incubator supplied with 5% CO_2 . Primary leukemic cells from three patients with chronic-phase CML were isolated using Ficoll gradient as described previously^{29,30} and then were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotic/antimycotic solution. The research methods conformed to the standards stipulated in the Declaration of Helsinki and were approved by the Institutional Medical Ethics Review Board of the Fujian Medical University Union Hospital. Informed consent was obtained from all participants included in the study.

2.2 | Enzyme-linked immunosorbent assay

Concentrations of Cyr61 in the plasma and BM from CML patients were quantitated using the human Cyr61 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Three internal quality control serum samples or BM supernatants were tested in each assay to assess interassay precision.

2.3 | Cysteine-rich protein 61 knockdown

Lentivirus-based shRNAs, scramble (shNC) or against Cyr61 (shCyr61), were purchased from Shanghai GeneChem Co., Ltd. The target sequence of shCyr61 was 5'-CAACGAGGACTGCAGCAAA-3'. The viral particles were prepared with a standard method according to the manufacturer's instructions (GeneChem Co., Ltd, Shanghai, China). Viruses were collected at 72 hours post-transfection to infect K562 cells. Transduction efficiency of K562 cells was confirmed to be $>97\%$ before selection with $0.5 \mu\text{g}/\text{mL}$ puromycin (Sigma-Aldrich, St Louis, MO, USA) for 5 days. The knockdown efficiency of Cyr61 was evaluated by western blotting.

2.4 | Apoptosis assay

Apoptotic K562 cells were measured using Annexin V-FITC and propidium iodide (PI) double-staining Kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, 5.0×10^5 cells were washed with ice-cold PBS, resuspended in $195 \mu\text{L}$ binding buffer, and stained for 10 minutes at room temperature with $5 \mu\text{L}$ FITC conjugated anti-Annexin V antibody. Unbound Annexin V antibody was removed by washing with binding buffer. Percentage of apoptotic K562 cells (Annexin V positive) was determined by flow cytometry analysis. Flow cytometry was carried out using a FACSCalibur cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

2.5 | Real-time PCR analysis

Total RNA was extracted from specimens using a TriPure Isolation Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse-transcribed into first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was carried out using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers used in this study were as follows: Bcl-2, forward, CTGGTGGGAGCTTGCATCAC; Bcl-2, reverse, ACAGCCTGCAGCTTTGTTTC; Bcl-xl, forward, TCAGGCTGCTTGGGA TAAAGAT; Bcl-xl, reverse, AGAGGCTTCTGGAGACATTTG; XIAP, forward, TTGAGGAGTGCTGGTAAG; XIAP, reverse, CCATTCGTATAGCTTCTTGT; Survivin, forward, GGAAGAAGTAGCGTCACTC; Survivin, reverse, TGACGACC CCATAGAGGAACA; GAPDH, forward, CACATGGCCTCCAAGGAGTA; GAPDH, reverse, TGAGGGTCTCTCTTCTCTTGT. GAPDH was used as an internal control, and the relative expression of each mRNA was analyzed using the $2^{-\Delta\Delta C_t}$ method.

2.6 | Western blot analysis

Experimental cells were collected. In order to block secretion of Cyr61, K562, Jurkat, and Nalm-6 cells were treated with Brefeldin A (BD Biosciences, 5 μ L/mL culture medium) and monensin (BD Biosciences, 2.5 μ L/mL culture medium) for 5 hours. After washing with ice-cold PBS, cells were added to the RIPA lysis buffer for 20 minutes. Protein immunoblotting was done as described previously.²⁸ The following antibodies were used in this study: antihuman cyr61 monoclonal antibody (093G9) was kindly gifted by Dr Ningli Li (Shanghai Jiao Tong University School of Medicine, Shanghai, China), anti-NF- κ B p65 (4764; Cell Signaling Technology, Danvers, MA, USA), anti-Phospho-NF- κ B p65 (3033; Cell Signaling Technology), anti-Bcl-2(4233; Cell Signaling Technology).

2.7 | Mice and tumor xenografts

For tumor xenografts, K562-shCyr61 or K562-shNC cells were cultured in RPMI 1640 medium with 10% FBS in a 37°C incubator supplied with 5% CO₂. Before inoculation, the cells were concentrated by centrifugation and suspension in serum-free medium to 1.0×10^8 cells/mL. Six- to 8-week-old female NOD/SCID mice were maintained in pathogen-free conditions and injected s.c. with K562-shCyr61 or K562-shNC cells in the right flank (1.0×10^7 cells/mouse in 100 μ L). IM treatment was started at 10 days following inoculation. The mice were injected with IM dissolved in saline (10 mg/kg body weight per day) i.p. for 20 days. Control mice were injected with normal saline. Body weight was recorded every other day. The animals were observed daily for mortality and signs of health (weight loss, change in appetite, or behavioral changes). Tumor volumes were determined by measuring the length (l) and width (w) of the tumor and calculating the volume ($V = w^2 \times l/2$). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Fujian Medical University Union Hospital.

2.8 | Statistical analysis

Results are presented as mean \pm standard error of the mean (SEM) unless indicated otherwise. Statistical analyses were carried out using SPSS software version 13.0 (SPSS Inc.). Significance of differences between groups was assessed by Student's *t* test for single comparisons or by analysis of variance for multi-group comparisons. A value of $P < 0.05$ was considered to indicate statistical significance.

3 | RESULTS

3.1 | Cysteine-rich protein 61 levels are upregulated in CML patient samples

Increasingly more studies have demonstrated that Cyr61 plays an important role in the development of tumors. To explore the role of Cyr61 in the pathogenesis of CML, we examined its levels in plasma and BM samples from newly diagnosed, non-treated patients with CML. The results showed that Cyr61 levels were elevated in both plasma and BM samples of patients with CML compared to samples collected from healthy controls (Figure 1A), and further analysis showed that Cyr61 levels from the BM of CML patients in blast crisis (BC) were higher than in chronic phase (CP) (Figure 1B). Next, we analyzed Cyr61 expression in leukemia cell lines and found that CML K562 cells expressed Cyr61 at the highest level (Figure 1C,D,E). Taken together, these data indicated that Cyr61 is upregulated in CML, suggesting an essential role for Cyr61 in the pathogenesis of CML.

3.2 | Presence of Cyr61 effectively reduces the sensitivity of CML cells to IM

Previous studies have shown that Cyr61 plays an important role in resistance to chemotherapeutic drug-induced apoptosis in human breast cancer,^{22,23} ovarian cancer,²⁴ pancreatic adenocarcinoma,²⁵ and renal cell carcinoma.²⁶ Recently, Long et al²⁷ found that Cyr61 protects AML cells from chemotherapy-induced apoptosis. To explore whether Cyr61 could also protect CML cells from apoptosis induced by IM, CML K562 cells were exposed to a serial concentration of IM in the presence or absence of Cyr61. Apoptosis was analyzed by flow cytometry at 24 hours post-treatment. As shown in Figure 2A, the presence of exogenous Cyr61 partially rescued K562 cells from IM-induced apoptosis. To further identify the role of Cyr61 in protecting K562 cells from IM-induced apoptosis, we blocked the function of Cyr61 by either using anti-Cyr61 093G9 monoclonal antibody to neutralize Cyr61 protein (Figure 2B) or shRNA to knock down Cyr61 expression (Figure 2C). As shown in Figure 2B,D, both methods restored the sensitivity of K562 cells to IM treatment.

Further, we evaluated whether upregulated Cyr61 expression in CML BM could decrease CML cell apoptosis induced by IM. K562 cells were cultured in BM supernatants collected from newly diagnosed CML patients without prior treatment. Results showed that

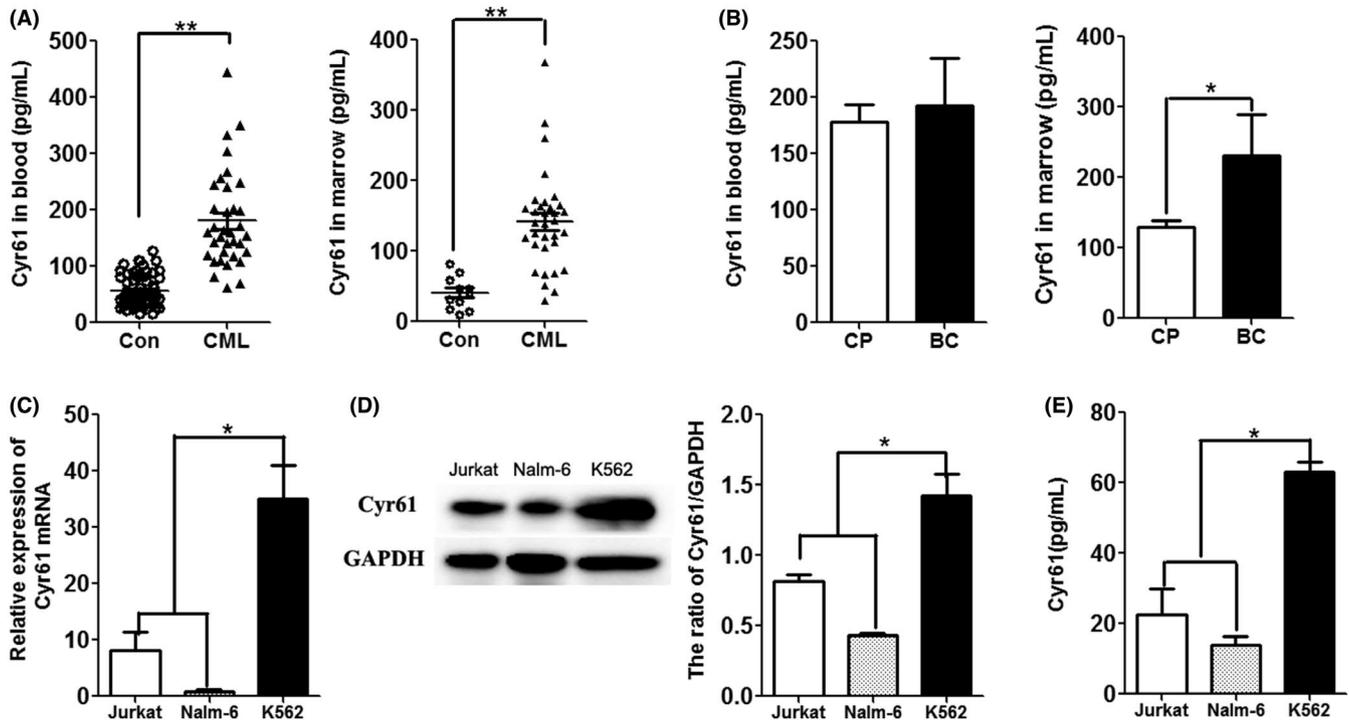


FIGURE 1 Cysteine-rich protein 61 (Cyr61) levels are upregulated in plasma and bone marrow (BM) samples from patients with CML. A, Left panel: Levels of Cyr61 in the plasma from CML patients ($n = 36$) and normal plasma from age-matched healthy individuals (CON; $n = 66$) were detected by ELISA. Right panel: Levels of Cyr61 in the BM supernatant from CML patients ($n = 33$) and the normal BM supernatant from age-matched healthy transplant donors ($n = 11$) were detected by ELISA. B, Levels of Cyr61 in the plasma from CML patients in blast crisis (BC) ($n = 5$) and in chronic phase (CP) ($n = 31$) were detected by ELISA. Right panel: Levels of Cyr61 in the marrow from CML patients in BC ($n = 4$) and in CP ($n = 29$) were detected by ELISA. C, Relative levels of Cyr61 mRNA in a T acute lymphoblastic leukemia (ALL) cell line (Jurkat), B ALL cell line (Nalm-6), and CML cell line (K562) were detected by qRT-PCR, and the level of Cyr61 mRNA in Nalm-6 cells was taken as the control to calculate the relative expression of Cyr61 in Jurkat and K562 cells. D, Levels of Cyr61 protein in Jurkat, Nalm-6, and K562 cells were detected by western blotting. Band intensity of Cyr61 was quantified by densitometry and normalized to GAPDH. E, Concentration of Cyr61 in the culture supernatant of Jurkat, Nalm-6, and K562 cells was detected by ELISA. Data represent mean \pm SEM of at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$

treatment with the anti-Cyr61 093G9 monoclonal antibody increased apoptosis in K562 cells induced by IM, suggesting that endogenous Cyr61 from the BM of CML patients decreased K562 cell apoptosis induced by IM (Figure 2E).

To investigate whether Cyr61 decreased IM-induced apoptosis is common for CML cells, we carried out the same experiments using another human CML cell line, KCL22 cells, and primary CML cells. After 24 hours of incubation with Cyr61, KCL22 cells and primary CML cells were treated with IM for 24 hours and the results showed that Cyr61 also partially decreased KCL22 cell and primary CML cell apoptosis induced by IM (Figure 2F,G).

3.3 | Cysteine-rich protein 61 activates Bcl-2 transcription in CML cells

As Bcl-2 family molecules are important regulators of cellular apoptosis, we examined Bcl-2, Bcl-xL, XIAP, and Survivin transcription in Cyr61-treated K562 cells by qRT-PCR. As shown in Figure 3A, Bcl-2 mRNA was significantly increased in Cyr61-treated K562 cells, whereas the expression of Bcl-xL, XIAP, and Survivin mRNA was not affected. Simultaneously, shRNA was

used to knock down Cyr61 expression in K562 cells. As expected, we observed that Bcl-2 transcription was markedly decreased in Cyr61-knockdown K562 cells (Figure 3B). In addition, Bcl-2 protein expression was significantly increased in Cyr61-treated K562 cells and decreased in Cyr61-knockdown K562 cells (Figure 3A,B). Last, a Bcl-2 inhibitor (ABT199) was used to block the function of Bcl-2 in Cyr61-treated K562 cells. We found that blocking Bcl-2 function restored K562 cell apoptosis induced by IM (Figure 3C). Together, these results suggested that the IM-induced apoptosis of K562 cells is regulated by Cyr61, possibly through the upregulation of Bcl-2.

3.4 | Cysteine-rich protein 61 inhibits IM-induced apoptosis through the NF- κ B signaling pathway

Previous studies have shown that NF- κ B,²² ERK1/2,²³ and PI3K-AKT³¹ are activated downstream of Cyr61 and are associated with innate chemoresistance in breast cancer cells. To address the mechanism by which Cyr61 protects CML cells from IM-induced apoptosis, we evaluated the profile of the AKT, NF- κ B, and ERK1/2 pathways using known inhibitors of these pathways,

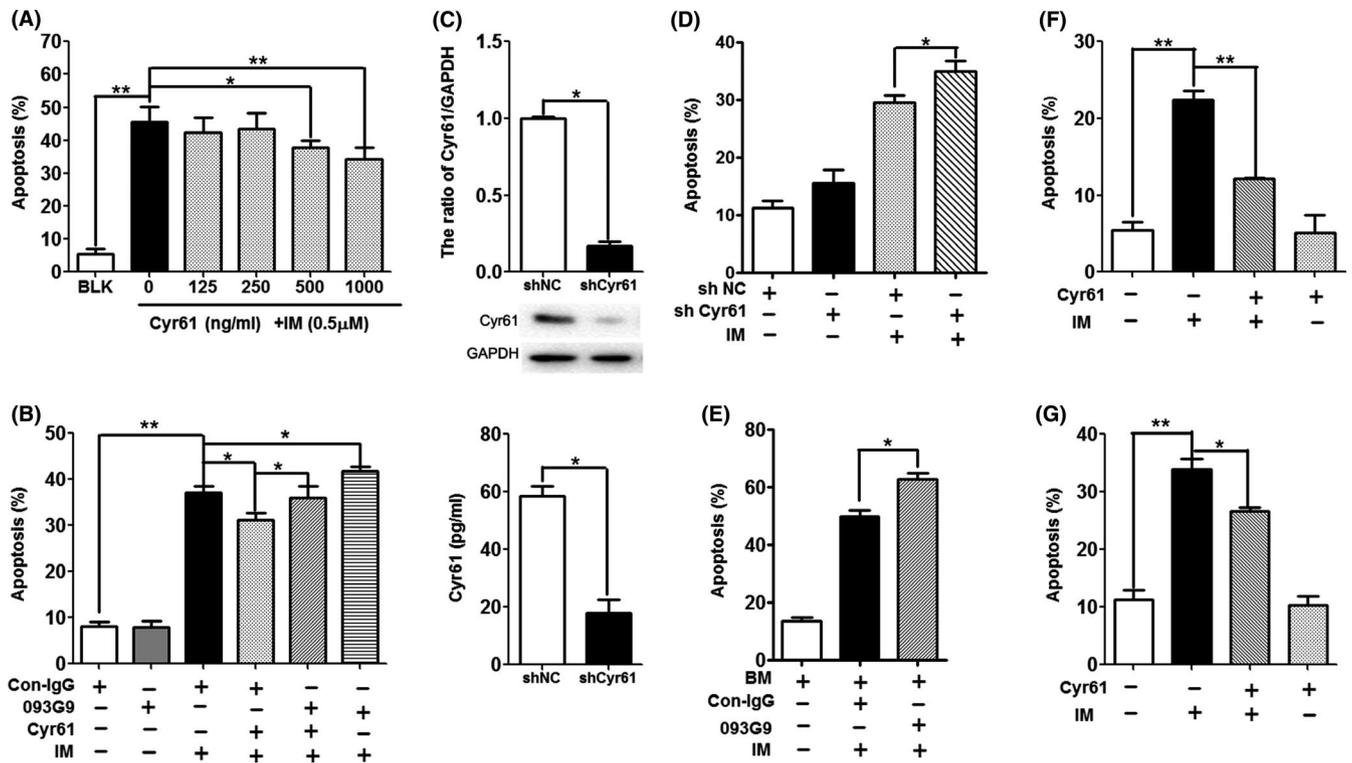


FIGURE 2 Role of cysteine-rich protein 61 (Cyr61) in the chemosensitivity of CML cells to imatinib mesylate (IM). A, K562 cells were treated with Cyr61 (125, 250, 500, 1000 ng/mL) for 24 h, and then treated with 0.5 μmol/L IM for 24 h; the percentages of apoptotic K562 cells were determined by flow cytometric analysis. Average percentage of apoptotic cells is shown. B, K562 cells were collected, incubated with Cyr61 (1000 ng/mL) preincubated with the antihuman Cyr61 093G9 monoclonal antibody (5000 pg/mL) or murine isotype-matched antibody (Con-IgG) (5000 pg/mL), and then treated with 0.5 μmol/L IM for 24 h. Cell apoptosis was determined by flow cytometric analysis. C, Cyr61 knockdown by shCyr61 or shNC (negative control) in K562 cells. Endogenous Cyr61 expression is shown in the upper panel, whereas the secreted Cyr61 level in culture medium was determined by ELISA and shown in the lower panel. D, Ratio of apoptotic K562-shCyr61 and K562-shNC cells was determined by flow cytometry at 24 h post-treatment with or without 0.5 μmol/L IM. E, K562 cells were incubated with BM supernatants from a mixture of different CML patients (Cyr61 concentration was 243 pg/mL) with preincubation with 1000 pg/mL 093G9 antibody or murine isotype-matched antibody (Con-IgG) for 2 h, and then treated with 0.5 μmol/L IM for 24 h. F, Human CML cell line KCL22 cells were treated with Cyr61 (1000 ng/mL) for 24 h and then treated with 0.5 μmol/L IM for 24 h; the percentages of apoptotic cells were determined by flow cytometric analysis. G, Primary leukemic cells from three patients with CP CML were isolated and treated with exogenous recombinant human Cyr61 (1000 ng/mL) for 24 h, and then treated with 0.5 μmol/L IM for 24 h. Data represent mean ± SEM of at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$

namely, PDTC (an inhibitor of NF-κB activation), LY294002 (an inhibitor of the PI3K/AKT pathway), and PD98059 (an inhibitor of ERK1/2). As shown in Figure 4A, compared with Cyr61 alone, IM-induced CML cellular apoptosis was markedly increased in the presence of Cyr61 together with the NF-κB inhibitor, whereas inhibition of AKT and ERK1/2 activity had no effect on apoptosis. In addition, the AKT inhibitor alone, ERK1/2 inhibitor alone, or NF-κB inhibitor alone had no effect on CML cellular apoptosis (data not shown). Further analysis showed that Cyr61 treatment led to a dramatic increase in the phosphorylation of the NF-κB p65 subunit in IM-treated K562 cells (Figure 4B). Next, we treated the cells with PDTC to determine whether NF-κB is associated with the Cyr61-mediated regulation of Bcl-2. Our results indicated that treatment with PDTC blocked the activation of Bcl-2 transcription by Cyr61 in IM-treated K562 cells (Figure 4C). To further examine the autocrine role of Cyr61 in the regulation

of NF-κB phosphorylation and Bcl-2 expression in K562 cells, we used specific siRNA to knock down Cyr61 expression in K562 cells. The results showed that NF-κB phosphorylation and Bcl-2 expression were markedly reduced in Cyr61-knockdown K562 cells (Figure 4D). In conclusion, our data showed that IM-induced CML cellular apoptosis is negatively regulated by Cyr61, likely through the NF-κB pathway by upregulating Bcl-2.

3.5 | Inhibition of Cyr61 restores the chemosensitivity of CML cells to IM in vivo

Finally, we examined the in vivo role of Cyr61 in CML cell chemosensitivity to IM using a mouse model of CML. K562-shNC and K562-shCyr61 cells were injected s.c. into NOD/SCID mice, respectively. At 10 days after cell inoculation, treatment with IM (10 mg/kg) or normal saline was started. IM dosage was chosen based on a

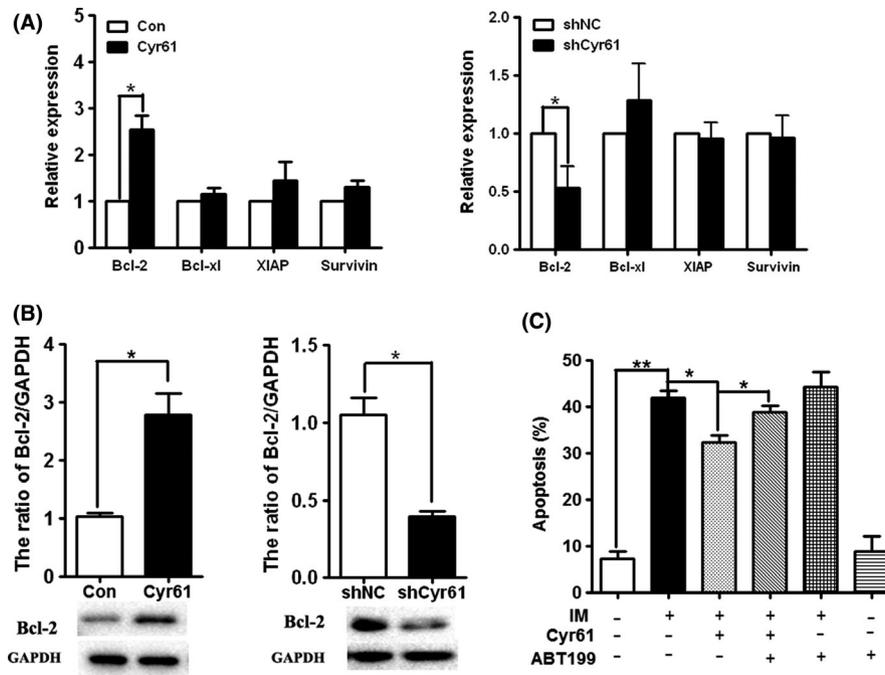


FIGURE 3 Cysteine-rich protein 61 (Cyr61) activates Bcl-2 transcription in CML cells. A, Left panel: Bcl-2, Bcl-xL, XIAP and Survivin mRNA expression in K562 cells treated by 1000 ng/mL Cyr61 for 8 h was detected by real-time PCR. Right panel: Bcl-2, Bcl-xL, XIAP and Survivin mRNA expression in K562-shCyr61 cells and K562-shNC cells was detected by real-time PCR. B, Left panel: Bcl-2 protein in K562 cells stimulated by 1000 ng/mL Cyr61 for 48 h was detected by western blotting. Right panel: Bcl-2 protein in K562-shCyr61 cells and K562-shNC cells was detected by western blotting. The band intensity of Bcl-2 was quantified by densitometry and normalized to GAPDH. C, K562 cells were treated with Cyr61 (1000 ng/mL), ABT199 (1 μ mol/L) (specific Bcl-2 inhibitor), Cyr61 + ABT199, or ABT199 for 24 h, and then treated with 0.5 μ mol/L imatinib mesylate (IM) for 24 h. Percentages of apoptotic K562 cells were determined by flow cytometric analysis. Data represent the mean \pm SEM of at least 3 independent experiments. * P < 0.05, ** P < 0.01

previous report showing that daily treatment with 10 mg/kg IM partially inhibited the tumor growth of K562 cells.³² Our results showed that IM treatment partially inhibited tumor growth in the K562-shNC xenograft model, achieving 45% and 48% suppression of tumor weight and size, respectively. In contrast, in the s.c. injected K562-shCyr61 cell xenograft model, IM treatment essentially abolished tumor growth, resulting in 76% and 71% reduction in tumor weight and size, respectively (Figure 5B,C). Together, these results showed that inhibition of Cyr61 increases the chemosensitivity of CML cells to IM *in vivo*.

4 | DISCUSSION

Imatinib mesylate is an effective treatment for CML as a result of its highly selective binding to the ATP-binding site of BCR-ABL protein and inhibition of BCR-ABL activation. Despite this, not all patients respond to the therapy, indicating that some factors potentially interfere with IM treatment in certain groups of patients. In the present study, we first showed that Cyr61 levels were increased in the plasma and BM of patients with CML. Furthermore, we provided key evidence showing that the increased levels of Cyr61 in BM protected CML cells from IM-induced apoptosis. These findings suggested that Cyr61 may interfere with IM treatment.

As mentioned earlier, Cyr61 overexpression promotes the growth of solid tumors.¹³⁻¹⁷ Our recent study showed that Cyr61 levels are increased in the plasma and BM of patients with ALL, and elevated Cyr61 levels promote cell survival.²⁸ In the present study, we discovered that Cyr61 levels were also upregulated in the plasma and BM of patients with CML, suggesting that Cyr61 may play an important role in the pathogenesis of CML.

Previous studies have shown that Cyr61 decreases the apoptosis induced by anticancer drugs in breast cancer cells,^{22,23} ovarian carcinoma cells,²⁴ renal cell carcinoma,²⁶ and AML,²⁷ suggesting that Cyr61 is linked to chemotherapy resistance in tumors. However, whether Cyr61 is involved in chemoresistance in CML has not been reported. In this work, we explored the function of Cyr61 in IM-induced CML cellular apoptosis and found that recombinant human Cyr61 decreased IM-induced CML cellular apoptosis. Moreover, shRNA targeting Cyr61 and a Cyr61-blocking antibody (093G9) restored the sensitivity of CML cells to IM. Considering that the effectiveness of Cyr61 may depend on the BM microenvironment, we focused on assessing the contribution of CML BM Cyr61 in protecting leukemic cells from the damage induced by IM. Our results showed that Cyr61 in CML BM decreased the apoptosis of CML cells induced by IM. The findings reported here are consistent with previous reports, in which Cyr61 blocks antitumor drug-induced apoptosis of cancer cells, resulting in chemotherapy resistance in breast cancer, cervical cancer, and AML.^{22-24,26,27} An

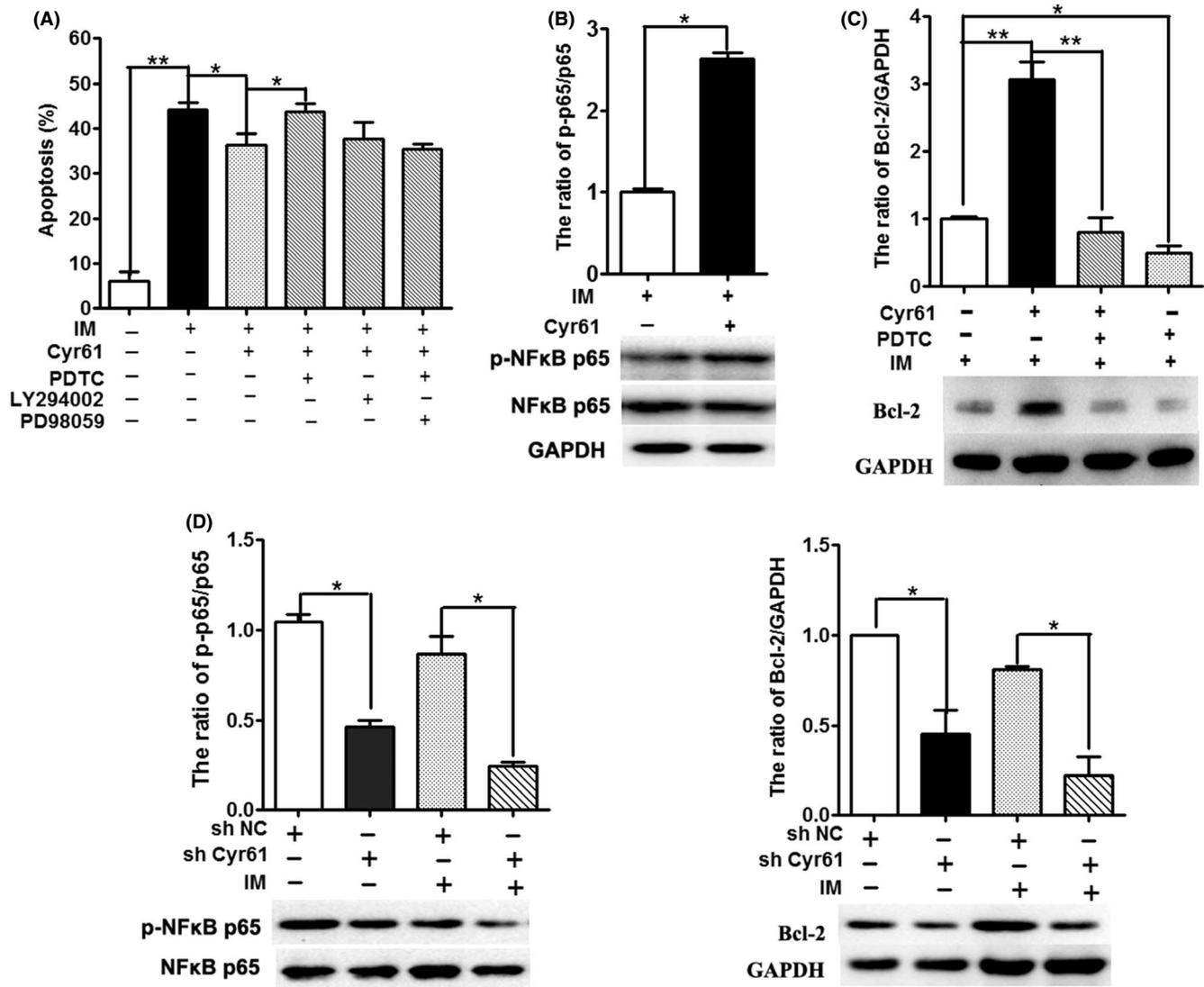


FIGURE 4 Cysteine-rich protein 61 (Cyr61) inhibits imatinib mesylate (IM)-induced apoptosis through the nuclear factor kappa B (NF- κ B) signaling pathway. A, Effect of the inhibitors of signaling pathways on Cyr61 decreased CML cell apoptosis induced by IM. K562 cells were treated with 20 μ mol/L LY294002, 1 μ mol/L PD98059 or 4 μ mol/L PDTC in combination with Cyr61 (1000 ng/mL) for 24 h and then treated with 0.5 μ mol/L IM for 24 h; the percentages of apoptotic K562 cells were determined by flow cytometric analysis. B, NF- κ B phosphorylation was detected by western blotting. Lane 1: stimulation of K562 cells with 0.5 μ mol/L IM for 10 min; lane 2: stimulation of K562 cells with 1000 ng/mL Cyr61 + 0.5 μ mol/L IM for 10 min. C, K562 cells were treated with 1000 ng/mL Cyr61 in combination with or without 4 μ mol/L PDTC for 24 h, and then treated with 0.5 μ mol/L IM for 24 h. Protein levels of Bcl-2 in K562 cells were detected by western blotting. D, K562-shCyr61 cells and K562-shNC cells were treated with 0.5 μ mol/L IM for 24 h. Left panel: NF- κ B phosphorylation was detected by western blotting. Right panel: Bcl-2 protein levels in K562 cells were detected by western blotting. Band intensity of Bcl-2 was quantified by densitometry and normalized to GAPDH. Data represent the mean \pm SEM of at least 3 independent experiments. * P < 0.05, ** P < 0.01

important observation was that inhibition of Cyr61 restored the chemosensitivity of CML cells to IM in a mouse model of CML. On the basis of these results, we propose that Cyr61 may interfere with IM treatment. Our previous findings also showed that blocking Cyr61 activity with specific antibodies ameliorates the severity of rheumatoid arthritis^{33,34} and psoriasis.³⁵ In addition, it has been reported that a secondary structure, called the G-quadruplex (G4) structure, can form on the 3'-untranslated region of Cyr61 mRNA, potentially modulating its translation.³⁶⁻³⁹ For example, therapeutic strategies using either G4 stabilizing small molecules or a

nucleic acid clamp approach could possibly be used to modulate the stability of this mRNA G4 structure, and thus the translation of Cyr61.⁴⁰⁻⁴³ In conclusion, Cyr61 plays important roles in the survival of CML cells and could potentially serve as a molecular target for the treatment of CML.

It is well known that the regulation of cellular apoptosis is determined by the balance of anti-apoptotic and pro-apoptotic factors of Bcl-2 family proteins.⁴⁴ We evaluated the effect of Cyr61 on the expression of Bcl-2, Bcl-xL, XIAP, and Survivin. Our findings showed that Cyr61 upregulated Bcl-2 expression

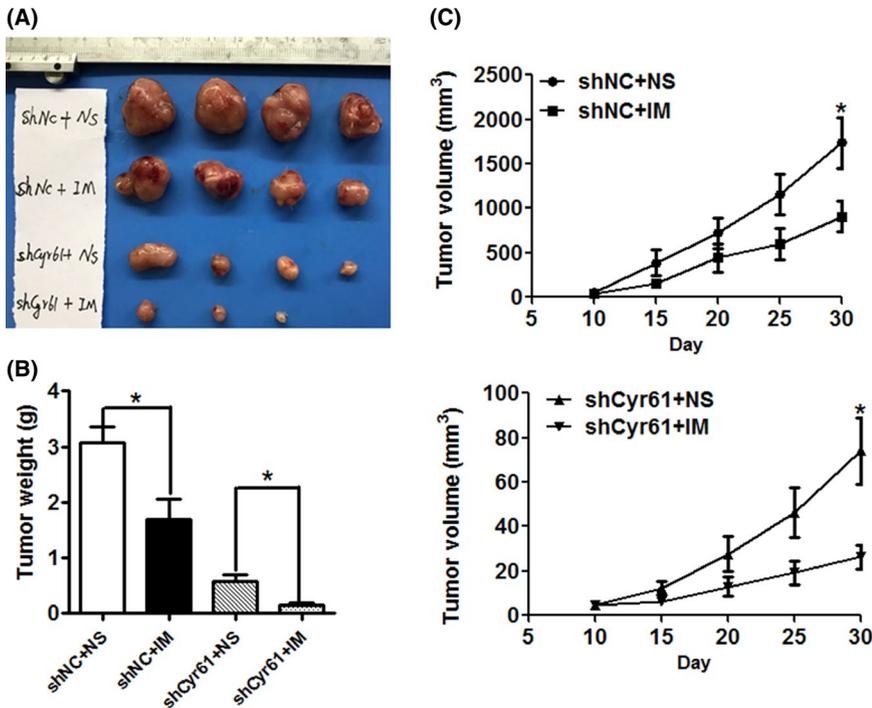


FIGURE 5 Inhibition of cysteine-rich protein 61 (Cyr61) restores the chemosensitivity of CML cells to imatinib mesylate (IM) in vivo. NOD/SCID mice bearing s.c. K562-shCyr61 or control K562-shNC cell xenografts ($n = 6$) were injected i.p. with IM or normal saline (NS) daily from 10 d after inoculation with 1.0×10^7 tumor cells for 20 d, and then the mice were killed. A, Representative images of tumors are shown. B, Tumor weight is shown. C, Average percentage of tumor volume is shown. * $P < 0.05$

without affecting Bcl-xL, XIAP, or Survivin, and blocking Bcl-2 function with a specific Bcl-2 inhibitor restored IM-induced CML cell apoptosis. The findings reported here are consistent with our previous results in which Cyr61 can promote the survival of ALL cells by upregulating Bcl-2 expression.²⁸ Considering that Bcl-2 acts as an anti-apoptotic factor, we propose that Cyr61 regulates the apoptosis of IM-induced CML cells through the Bcl-2 pathway.

Bone marrow

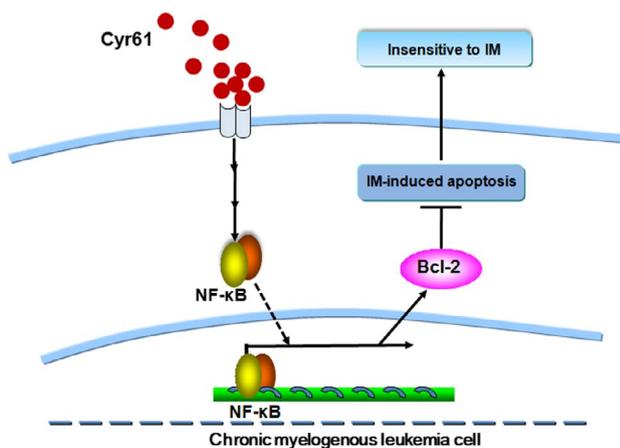


FIGURE 6 Proposed signaling pathway by which cysteine-rich protein 61 (Cyr61) reduces imatinib mesylate (IM)-induced CML cell apoptosis. Increased Cyr61 in the bone marrow from CML patients stimulates nuclear factor kappa B (NF-κB) phosphorylation, then upregulates Bcl-2 production, finally leading to decrease of IM-induced CML cell apoptosis and insensitivity to IM

Previous studies have shown that NF-κB,²² ERK1/2,²³ and PI3K-AKT³¹ are downstream targets of Cyr61 and are associated with innate chemoresistance in breast cancer cells. To elucidate the signaling pathway involved in the anti-apoptotic function of Cyr61, we evaluated the profile of the AKT, NF-κB, and ERK1/2 pathways. As expected, the NF-κB pathway contributed to the anti-apoptotic function of Cyr61. Recent evidence suggests that Cyr61 mediates ALL and AML cell survival by the AKT pathway²⁸ and by the ERK1/2 pathway, respectively,⁴⁵ however, we found that AKT and ERK1/2 were not involved in the ability of Cyr61 to inhibit the apoptosis of CML cells induced by IM. Further analysis showed that Cyr61 treatment led to strong activation of NF-κB signaling as well as Bcl-2 production. Together, we suggest that the anti-apoptotic effects of Cyr61 are derived from the activation of NF-κB signaling and the transactivation of Bcl-2. Considering the complexity of the BM microenvironment with a variety of cytokines, chemokines, and other growth factors,^{6-11,46} in vitro studies seem to be insufficient to elucidate the role that Cyr61 plays in inhibiting the apoptosis of CML cells induced by IM treatment. Therefore, further in vivo studies are required to identify the signaling pathways underlying the ability of Cyr61 to decrease IM-induced CML cell apoptosis.

Taken together, we first discovered that Cyr61 levels are up-regulated in BM and plasma samples of patients with CML and that this upregulation can effectively protect CML cells from IM-induced apoptosis, resulting in insensitivity to IM therapy (Figure 6). Our study indicates that Cyr61 plays an important role in regulating the sensitivity of CML cells to IM, and that targeting Cyr61 or its relevant effector pathways might dramatically improve the clinical responses of patients with CML to IM treatment.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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