RPS27a promotes proliferation, regulates cell cycle progression and inhibits apoptosis of leukemia cells

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ABSTRACT

Ribosomal protein S27a (RPS27a) could perform extra-ribosomal functions besides imparting a role in ribosome biogenesis and post-translational modifications of proteins. The high expression level of RPS27a was reported in solid tumors, and we found that the expression level of RPS27a was up-regulated in advanced-phase chronic myeloid leukemia (CML) and acute leukemia (AL) patients. In this study, we explored the function of RPS27a in leukemia cells by using CML cell line K562 cells and its imatinib resistant cell line K562/G01 cells. It was observed that the expression level of RPS27a was high in K562 cells and even higher in K562/G01 cells. Further analysis revealed that RPS27a knockdown by shRNA in both K562 and K562G01 cells inhibited the cell viability, induced cell cycle arrest at S and G2/M phases and increased cell apoptosis induced by imatinib. Combination of shRNA with imatinib treatment could lead to more cleaved PARP and cleaved caspase-3 expression in RPS27a knockdown cells. Further, it was found that phospho-ERK(p-ERK) and BCL-2 were down-regulated and P21 up-regulated in RPS27a knockdown cells. In conclusion, RPS27a promotes proliferation, regulates cell cycle progression and inhibits apoptosis of leukemia cells. It appears that drugs targeting RPS27a combining with tyrosine kinase inhibitor (TKI) might represent a novel therapy strategy in TKI resistant CML patients.

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1. Introduction

Chronic myeloid leukemia (CML), which is characterized by the formation of Philadelphia (Ph) chromosome and BCR-ABL fusion gene, is a common hematologic malignancy [1]. As a constitutively active tyrosine kinase, BCR-ABL protein gives rise to uncontrolled growth of myeloid cells in the bone marrow through a series of downstream pathways [2]. The tyrosine kinase inhibitor (TKI) imatinib is a specific molecular target-drug for the treatment of Ph chromosome-positive CML [3–6]. Before the era of TKIs, CML patients in chronic phase (CML-CP) progressed to a more accelerated phase (CML-AP) after a median interval of about 5 years. CML-AP patients might still respond to treatment for months or years, but eventually developed a very aggressive blast phase (CML-BP), after which the median survival was about 6 months.

Some patients progressed directly to CML-BP without an intermediate phase of acceleration. CML-BP is associated with dramatic changes in the leukemia cell phenotype: enhanced "stemness", uncontrolled proliferation and invasion, abrogated differentiation, and early resistance to TKIs [7,8]. Previous researches demonstrated that response to imatinib in patients with advanced CML was less prominent than that in CML-CP [9,10]. At present, the molecular mechanisms responsible for these extensive changes are still uncertain; most likely, they involve activation of oncogenic factors, inactivation of tumor suppressors, or both. K562/G01 is one of the successful TKI-resistant cell model. It was found that the mechanisms of K562/G01 cells resistance to TKI involved the increased expression of BCR/ABL and mdrl/P-Glycoprotein (P-gp) amplification of BCR/ABL fusion gene and increased activity of BCR/ABL [11]. And abnormal expression of some other genes or alternative signaling pathways activation may also contribute to imatinib resistance [12,13]. We found that the expression level of ribosomal protein S27a (RPS27a) was significantly higher in patients with CML-AP/BP than that in patients with CML-CP. It seems that RPS27a expression may be associated with clinical stages in CML patients.

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RPS27a is one of two (the other one is L40) ribosomal proteins naturally synthesized as an ubiquitin (Ub) C-terminal extension protein [14,15]. The Ub-RPS27a precursor protein is rapidly processed by hydrolysis to an individual Ub monomer and the RPS27a protein in mammalian cells [16]. The RPS27a gene has been reported to be over-expressed in breast fibroadenomas, colorectal and renal cancers [17–19]. In this study, we found that RPS27a was over-expressed in patients with CML-AP/BP and acute leukemia (AL). RPS27a was reported to interact with MDM2, suppresse MDM2-mediated P53 ubiquitination and lead to P53 activation and cell cycle arrest [20]. It seemed that, besides imparting a role in ribosome biogenesis and post-translational modifications of proteins, RPS27a could perform extra-ribosomal functions [21,22]. As the level of RPS27a expression was associated with clinical stages in CML patients in our study, it was speculated that RPS27a might be involved in the transformation of CML-CP to CML-AP/BP and implicated in the response to imatinib treatment. In this study, we explored the possible role of RPS27a in leukemia using CML cell line K562 cells and its imatinib resistant cell line K562/G01 cells as cellular model. We found that RPS27a was highly expressed in K562 cells and even higher in K562/G01 cells. Importantly, it was reported for the first time, that RPS27a could induce cell cycle arrest, promote the proliferation and inhibit the effect of imatinib on apoptosis of K562 and K562/G01 cells via Raf/MEK/ERK, P21 and BCL-2 signal pathways.

2. Materials and methods

2.1. Patients

Bone marrow samples were obtained from 15 healthy donors for hematopoietic stem cell transplantation as control, 26 CML patients including 16 CML-CP and 10 CML-AP/BP, and 20 newly diagnosed AL patients enrolled in the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College. All samples were collected under informed consent of the subjects. The diagnosis and leukemia classification were based on 2008 World Health Organization criteria.

2.2. Cell lines culture

Nalm6, NB4, HL60, K562, U937, Kasumi-1, Skno-1, HEK293T and K562/G01 cell lines were maintained in our laboratory. The HEK293T cell line was cultivated in Dulbecco's modified eagle medium and other cell lines in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Imatinib at concentration of 1 μM was added to the culture system of K562/G01 cells to maintain resistance activity.

2.3. RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent treated with DNase 1 and 2 μg RNA was reverse-transcribed using Superscript II RT following the manufacturer's instructions (Life technologies, USA). Primers for qRT-PCR were designed using Primer premier software 5.0. Human GAPDH primers used as an internal control were 5′-GAAGGTGAAGGTCGGAGTC-3′ (forward) and 5′-GAA-GATGGTGATGGGATTTC-3′ (reverse). Human RPS27a primers were 5′-AGAAGAAGTCTTACACCACTCCC-3′ (forward) and 5′-TGCCAT-AAACACCCCAGC-3′ (reverse). The qRT-PCR products were 226 and 158 bp, respectively. The qRT-PCR was performed with SYBR Green PCR kit (Takara, Japan) following the manufacturer's instructions on the ABI PRISM7500 real-time PCR system. Thermal cycling conditions were 95 °C for 5 min, followed by 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. The qRT-PCR reactions were performed in a total volume of 20 μl, containing 2 μl of sample cDNA, 0.2 μM of each primer.

2.4. Western blot analysis

Total protein was extracted using RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na-desoxycholate, 5 mM EDTA, 1 mM NaF, 25 mM Na_3VO_4, 0.1 mM PMSF and 2 mg/ml Aprotinin). Protein concentration was determined by the BCA assay (Solarbio, China). The whole-cell lysates were heat-denatured at 95 °C for 5 min before run on 10% SDS–PAGE. After SDS–PAGE, the proteins were electro-transferred onto nitrocellulose membranes, blotted with each primary antibody, incubated in secondary antibody and then detected with enhanced chemi-luminescence detection reagent (Pierce, USA).

2.5. Short hairpin RNA-mediated RNA interference studies

The cDNA sequence of RPS27a was obtained from GenBank (NM_002954.5). Two different RPS27a-specific targeting sequences were designed with the software from Ambion. The target sequences for RPS27a were 5′-TTAGTCGCCTTCGTCGAGA-3′ (sh-1) and 5′-CAGACATTATTGTGGCAAA-3′ (sh-2). The shRNA expressing plasmids specifically targeting RPS27a (termed as RPS27a-sh1 and -sh2) were cloned into pLKO-1 vector to obtain pLKO-sh1, pLKO-sh2 construct. A scramble shRNA sequence (AATAGACTCAG-CAAATGCG) with no homology to any human gene was used as a negative control (scr). For the production of lentivirus, HEK293T cells were co-transfected with pLKO-sh1, pLKO-sh2 or pLKO-scr and pCMV, pMDG by $Ca_3(PO_4)_2$ precipitation. Lentivirus supernatants were harvested 48 h after transfection, and used to infected K562 cells (termed as K562-sh1, K562-sh2 and K562-scr) and K562/G01 cells (termed as K562/G01-sh1, K562/G01-sh2 and K562/G01-scr). Infected cells were selected by puromycin 7 days after infection for at least 2 weeks to obtain stable clones. Inhibition of RPS27a expression was measured by qRT-PCR as well as by Western blot using a rabbit anti-RPS27a monoclonal antibody (abcam, UK).

2.6. MTT assays

5×10^3 cells per well were seeded into 96-well plates in 100 μl volume and grown in RPMI1640 medium supplemented with 10% fetal bovine serum at 37 °C. Cells were cultured for 24, 48 and 72 h, respectively. After indicated time of incubation, 10 μl of MTT reagent was added. Then the cells were incubated at 37 °C for an additional 4 h, and 100 μl of 10% sodium dodecyl sulfate–HCl 0.1 N was added to each well. The mixture was incubated at 37 °C overnight and then the absorbance of formazan product was measured by Versa Max tunable microplate reader (Molecular Devices, USA) at the wavelength of 546 nm. The viability and the in vitro cytotoxicity of K562, K562/G01 cells and their transfectants were assessed by MTT assays.

2.7. Cell cycle analysis by flow cytometry

Cells were fixed in 70% ethanol for 24 h at 4 °C, and stained with 20 μg/ml propidium iodide (PI) containing 10 μg/ml RNase A for 30 min at room temperature. Fluorescence cell analysis was performed with a FACS Calibur flow cytometer (BD, USA). Resulting DNA distributions were analyzed by Modifit (Verify Software House Inc., USA) for the proportions of cells in the different phases of the cell cycle.

2.8. Apoptosis analysis by flow cytometric assay

Phosphatidylserine externalization was analyzed with Annexin V-Alexa Fluor 647-A/PI Apoptosis Analysis Kit by a FACS Calibur flow cytometer (BD, USA) for cell apoptosis. Apoptosis was quantified as the percentage of Annexin V positive cells.

2.9. Statistical analysis

All experiments were conducted at least three times and data were presented as mean ± SD. Statistical analysis was performed with the SPSS software package (version 17.0; SPSS). $P < 0.05$ was deemed statistically significant.

3. Results

3.1. RPS27a is highly expressed in CML-AP/BP, AL, K562 and K562/G01 cells

To explore the possible role of RPS27a in leukemia, we first investigated the expression level of RPS27a in CML-CP, CML-AP/BP, newly diagnosed AL and healthy donors. Relative quantification using qRT-PCR revealed a striking increase of RPS27a mRNA expression in bone marrow samples from CML-AP/BP and newly diagnosed AL patients than that from CML-CP and healthy donors (Fig. 1A). In addition, the mRNA and protein levels were determined in leukemia cell lines Nalm6, NB4, HL60, K562, U937, Kasumi-1 and Skno-1 cells by qRT-PCR and Western blot. RPS27a expression was detected in all leukemia cell lines at an approximate level (Fig. 1B and C). We also compared the expression levels of RPS27a in K562 with that in K562/G01 cells in order to explore

whether RPS27a played a role in the responsiveness of cells to imatinib. It was found that K562/G01 cells expressed a significantly higher level of RPS27a compared with that of K562 cells (Fig. 1D and E).

3.2. Knockdown of RPS27a inhibits the proliferation, induces cell cycle arrest and potentiates the effect of imatinib on apoptosis of K562 cells

As the expression of RPS27a was up-regulated in leukemia cells, the role of RPS27a in leukemia cells was elucidated by using K562 cells. Two different RPS27a specific shRNAs were applied to down-regulate RPS27a expression in K562 cells. As expected, stable infection experiments showed both RPS27a-sh1 and RPS27a-sh2 could significantly down-regulate RPS27a expression in K562 cells in mRNA and protein levels (Fig. 2A and B). MTT assays showed that RPS27a knockdown inhibited the viability of K562 cells (Fig. 2C). Cell cycle analysis showed that RPS27a knockdown induced cell cycle arrest at S and G2/M phases (Fig. 2D and Fig. S1A).

Furthermore, we analyzed chemosensitivity of K562-sh1/2 and K562-scr cells to imatinib treatment. As the expression of RPS27a was down-regulated in K562-sh1/2 cells, MTT assays showed that the IC50s of imatinib at 48 and 72 h decreased (Fig. 2E and Table S1). The results indicated that down-regulation of RPS27a could enhance the sensitivity of K562 cells to imatinib treatment. Then we investigated whether the enhanced chemsensitivity could induce more RPS27a-knockdown K562 cells undergoing apoptosis by using Annexin V-Alexa Fluor 647-A/PI double staining. The results showed that RPS27a knockdown alone did not influence the percentage of apoptotic cells without imatinib treatment. Interestingly, RPS27a knockdown significantly increased the percentage of apoptotic K562-sh1/sh2 cells after incubation with 0.5 and 1 μM
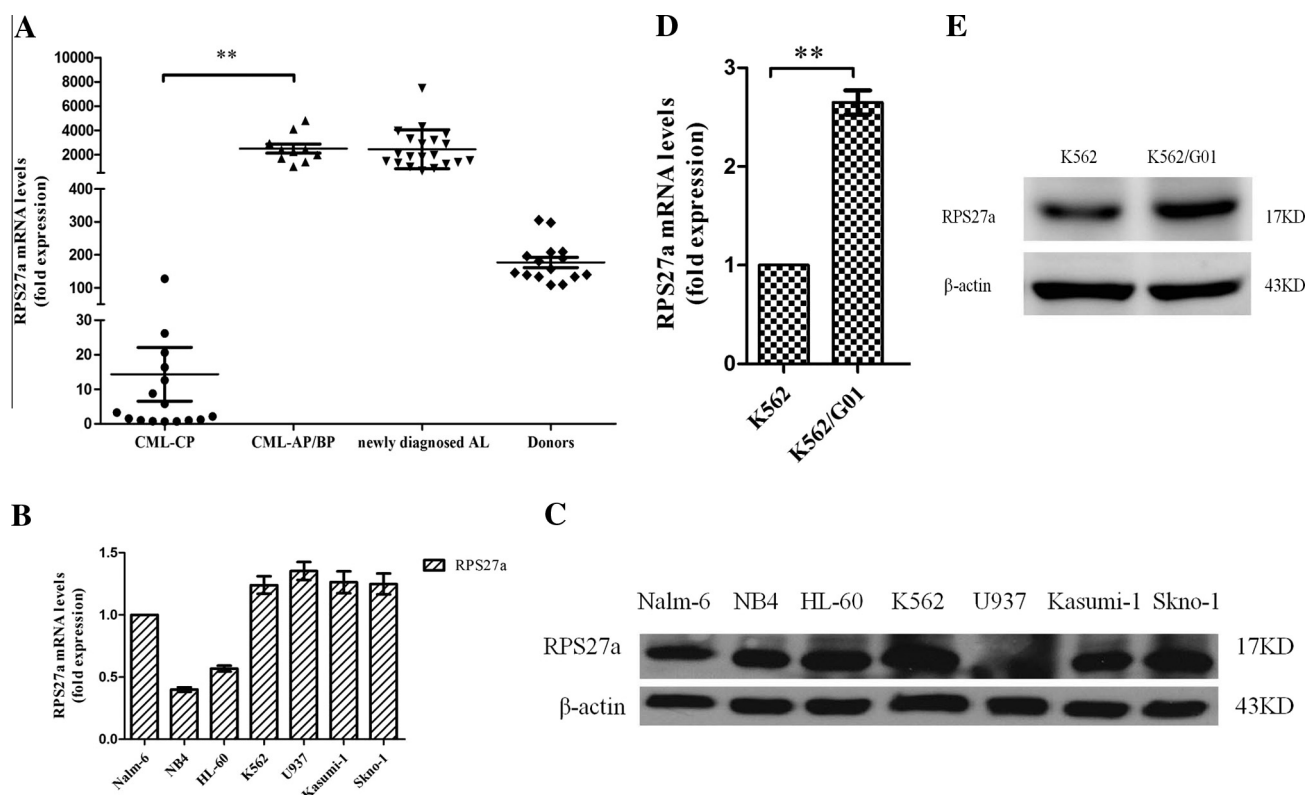

Fig. 1. Expression of RPS27a in leukemia patients and leukemia cell lines. (A) Differential expression of RPS27a mRNA in patients with CML-CP, CML-AP/BP, newly diagnosed AL and healthy donors was illustrated in scatter plots. (B and C) The qRT-PCR and Western blot were performed to determine the expression level of RPS27a in Nalm6, NB4, HL60, K562, U937, Kasumi-1 and Skno-1 cells. (D and E) The qRT-PCR and Western blot were performed to determine the expression level of RPS27a in K562 and K562/G01cells. Data are representative of at least three independent experiments. *$P < 0.05$; **$P < 0.01$.
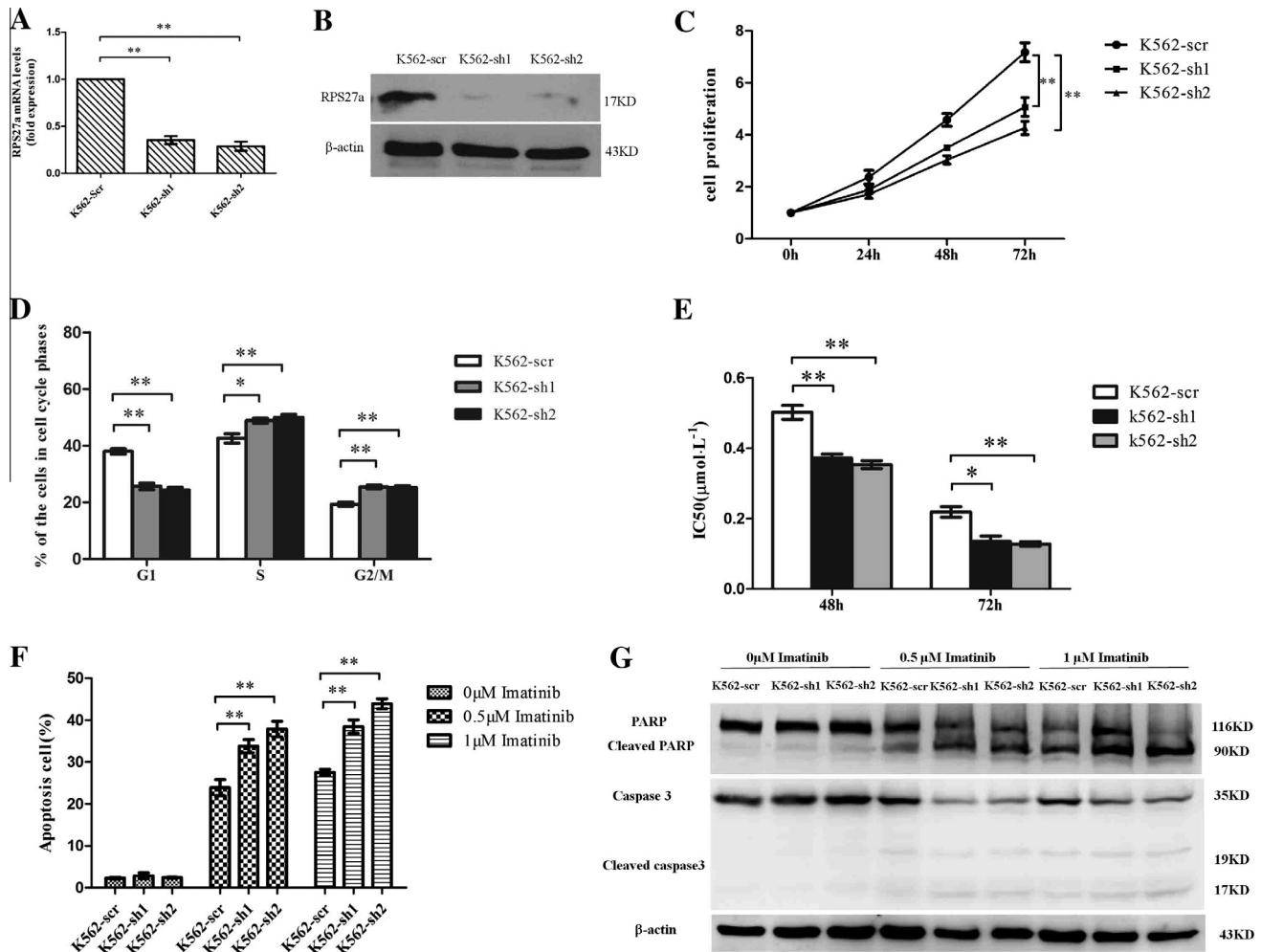
Fig. 2. Validation of RPS27a knockdown and its effect on cell viability, cell cycle arrest and apoptosis in K562 cells. (A) Relative RPS27a mRNA expression was assessed in K562 cells transfected with RPS27a shRNA plasmids by qRT-PCR. (B) Lysates of K562 cells transfected with indicated plasmids were analyzed for RPS27a protein by Western blot. (C) MTT assays were used to determine the cell viability after RPS27a knockdown. (D) The cells after RPS27a knockdown were stained with PI, and analyzed by flow cytometry. The percentage of cells in different cell cycle phases was indicated. (E) IC50 of K562-scr/sh1/sh2 to imatinib at 48 and 72 h was detected by MTT assays. (F) K562-sh1, K562-sh2 and K562-scr cells were treated with or without 0.5 μM imatinib and 1 μM imatinib for 48 h and subjected to cell apoptosis analysis by flow cytometry analysis of Annexin-V labeling. (G) Western blot was performed to detect the expression of cleaved PARP and cleaved caspase-3 induced by imatinib in RPS27a knockdown K562 cells. Data are representative of three independent experiments. *$P < 0.05$; **$P < 0.01$.

imatinib for 48 h compared with that of K562-scr cells (Fig. 2F and Fig. S1B). PARP and Caspase-3 have been demonstrated as key mediators of apoptosis, and their activation are taken as a hallmark of apoptosis. To confirm our finding of RPS27a on apoptosis, Western blot was performed to assess activation of PARP and caspase-3. As shown in Fig. 2G, more cleaved PARP and cleaved caspase-3 expression were observed in K562-sh1/sh2 cells compared with that in K562-scr after 0.5 and 1 μM imatinib treatment for 48 h. Our data indicated that RPS27a involved in the responsiveness of K562 cells to imatinib treatment, it may reduce the sensitivity of K562 cells to imatinib.

3.3. Knockdown of RPS27a inhibits the proliferation, induces cell cycle arrest and potentiates the effect of imatinib on apoptosis of K562/G01 cells

Imatinib resistance is becoming increasingly prominent due to various mechanisms, especially in more advanced CML. To confirm whether RPS27a plays a role in imatinib resistant K562/G01 cells, the same two RPS27a specific shRNAs used in K562 cells were applied to down-regulate RPS27a expression in K562/G01 cells, and the results showed that RPS27a expression was reduced in both mRNA level and protein level (Fig. 3A and B). As expected, similarly

to that in K562 cells, MTT assays showed that RPS27a knockdown inhibited the viability of K562/G01 cells (Fig. 3C). RPS27a knockdown induced cell cycle arrest at S and G2/M-phase (Fig. 3D and Fig. S2A). Then we investigated the role of RPS27a in the sensitivity to imatinib in K562/G01 cells. After RPS27a was inhibited, MTT assays indicated that the IC50 of imatinib at 48 and 72 h for K562/G01 cells reduced (Fig. 3E and Table S2). RPS27a knockdown increased the percentage of apoptotic K562/G01-sh1/sh2 cells after incubation with 2 and 4 μM imatinib for 48 h compared with K562/G01-scr (Fig. 3F and Fig. S2B). And more cleaved PARP and cleaved caspase-3 expression were observed in K562/G01-sh1/sh2 cells compared with that in K562/G01-scr after 2 and 4 μM imatinib treatment for 48 h (Fig. 3G). The above data indicated that RPS27a could also reduce the sensitivity of K562/G01 cells to imatinib.

3.4. Knockdown of RPS27a inactivates the Raf/MEK/ERK, P21 and BCL-2 signal pathways

To elucidate the mechanism of RPS27a in K562 and K562/G01 cells, some key molecules involved in proliferation, cell cycle and apoptosis pathways were investigated by Western blotting assay in K562-sh1/sh2, K562-scr, K562/G01-sh1/sh2 and K562/G01-scr
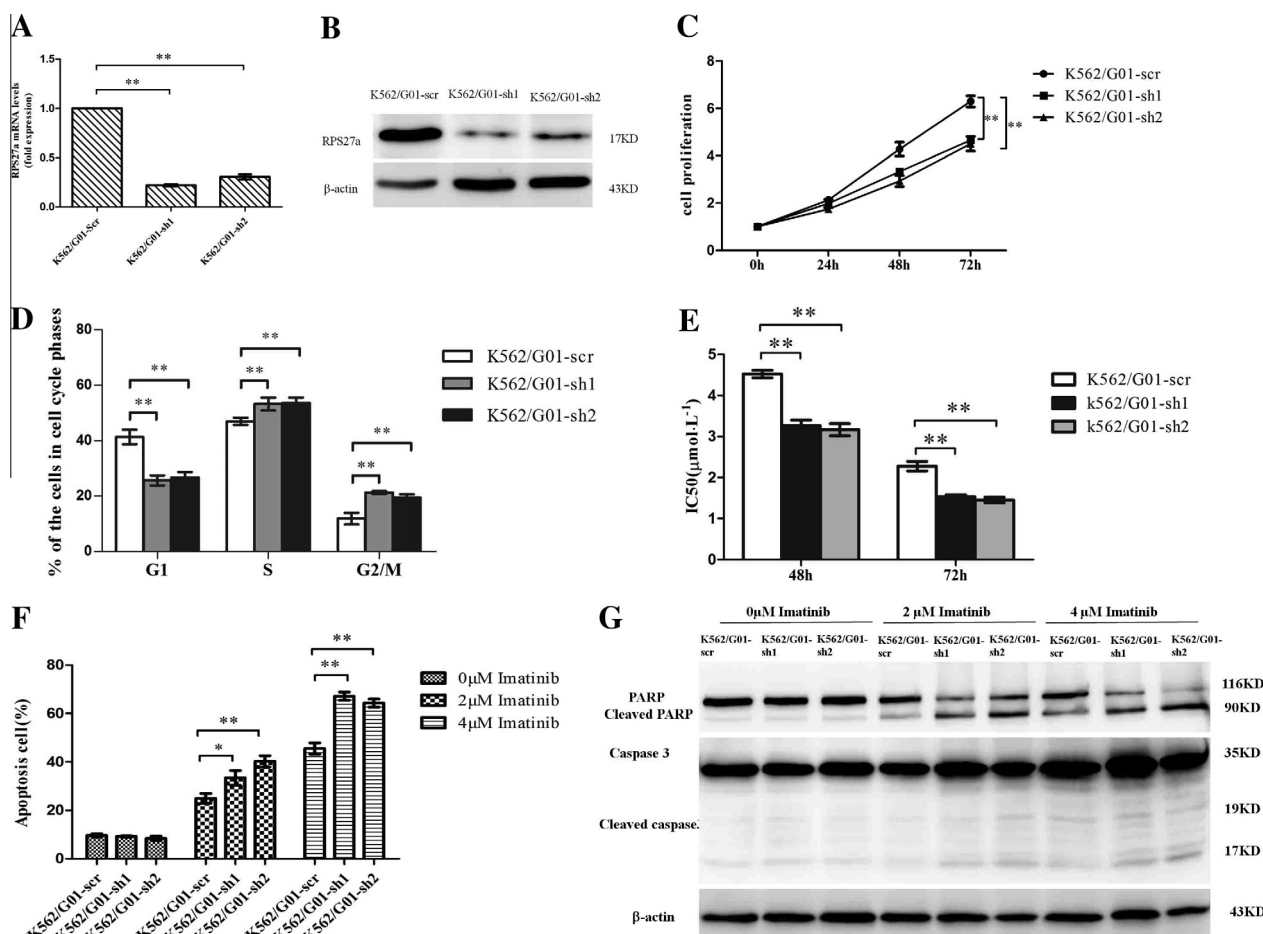
Fig. 3. Validation of RPS27a knockdown and its effect on cell viability, cell cycle arrest and apoptosis in K562/G01 cells. (A) Relative RPS27a mRNA expression was assessed in K562/G01 cells transfected with RPS27a shRNA plasmids by qRT-PCR. (B) Lysates of K562/G01 cells transfected with indicated plasmids were analyzed for RPS27a protein by Western blot. (C) MTT assays were used to determine the cell viability after RPS27a knockdown. (D) The cells after RPS27a knockdown were stained with PI, and analyzed by flow cytometry. The percentage of cells in different cell cycle phases was indicated. (E) IC50 of K562/G01-scr/sh1/sh2 to imatinib at 48 and 72 h was detected by MTT assays. (F) K562/G01-sh1, K562/G01-sh2 and K562/G01-scr cells were treated with or without 2 μM imatinib and 4 μM imatinib for 48 h and subjected to cell apoptosis analysis by flow cytometry analysis of Annexin-V labeling. (G) Western blot was performed to detect the expression of cleaved PARP and cleaved caspase-3 induced by imatinib in RPS27a knockdown K562/G01 cells. Data are representative of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.

cells, respectively. Compared with that in K562-scr and K562/G01-scr cells, p-ERK protein and BCL-2 protein expression were down-regulated and P21 protein was up-regulated in K562-sh1/sh2 and K562/G01-sh1/sh2 cells, while RPS27a knockdown did not affect the expression of p-AKT, BAX, and BCL-XL (Fig. 4A and B). The results showed that knockdown of RPS27a inhibited the proliferation of K562 and K562/G01 cells via down-regulating the p-ERK expression of MAPK signal pathway, induced cell cycle arrest via up-regulating P21 and potentiated the effect of imatinib on apoptosis of K562 and K562/G01 cells via inactivating BCL-2 protein.

4. Discussion

In this study, we found that the expression of RPS27a was significantly higher in CML-AP/BP patients than that in CML-CP patients. The expression level of RPS27a was high in K562 cells and even higher in K562/G01 cells. The ablation of RPS27a expression could inhibit the proliferation, induce cell cycle arrest and potentiate the effect of imatinib on apoptosis of K562 and K562/G01 cells partially through Raf/MEK/ERK, P21 and BCL-2 signaling pathways.

RPS27a, a small subunit ribosomal protein, could perform extra-ribosomal functions besides imparting a role in ribosome biogenesis and post-translational modifications of proteins. RPS27a is over-expressed in mouse liver cancer and some human tumors

[17–19]. The exact extra-ribosomal function of RPS27a is not entirely clear. In the present study, we found that RPS27a was highly expressed in CML-AP/BP, K562 cells and its imatinib resistant cells K562/G01 cells, and the ablation of RPS27a could inhibit the proliferation, induce cell cycle arrest and potentiate the effect of imatinib on apoptosis of both K562 and K562/G01 cells. This implies that RPS27a is a potential target for TKI resistant leukemia.

BCR-ABL oncogenic tyrosine kinase plays a critical role in the pathogenesis of CML [1]. Imatinib, which was developed as a small molecule inhibitor of the Abl tyrosine targeting the oncogenic fusion protein BCR-ABL, has been considered as an effective, frontline therapy for early CML-CP patients [4,5]. However, imatinib resistance is becoming increasingly prominent due to various mechanisms, especially in more advanced CML. The emergence of imatinib resistance points toward the need to develop novel therapeutic strategies for CML patients. Combining imatinib with other anticancer agents is one approach to overcome this problem [23]. Recent researches indicate that other relevant targets in the BCR-ABL pathways and strategies beyond targeting BCR-ABL needed to be explored for CML [24,25].

It has been described that targeting the Raf/MEK/ERK and PI3K/Akt/mTOR pathways may be an effective approach for therapeutic intervention in drug-resistant cancers [26,27]. And Raf/MEK/ERK signaling inhibition enhances the ability of dequalinium to induce apoptosis in the human leukemic cell line K562 [28]. The p21
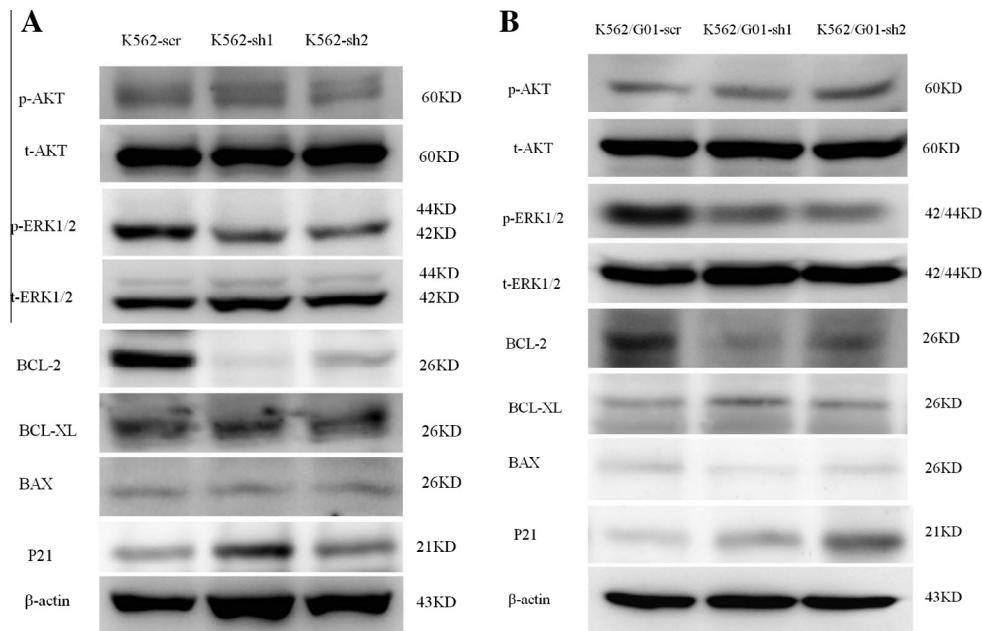
Fig. 4. Detection of activity of Raf/MEK/ERK, P21 and BCL-2 signaling pathways (A) Western blot was performed to examine the expression of Raf/MEK/ERK, P21 and BCL-2 signaling pathways related proteins in indicated cells (K562-scr, K562-sh1 and K562-sh2). (B) Western blot was performed to examine the expression of signaling pathways related proteins in indicated cells (K562/G01-scr, K562/G01-sh1 and K562/G01-sh2).

protein binds to and inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, and thus functions as a regulator of cell cycle progression at G1 and S phase. In addition, the BCL-2 family of proteins has been shown to be key regulators of apoptosis. In the present study, we investigated the changes of the Raf/MEK/ERK, PI3K/Akt and apoptotic signal transduction pathways in the RPS27a-knockdown cells and the control cells. Compared with that in K562-scr and K562/G01-scr cells, BCL-2 protein and p-ERK protein expression were down-regulated in K562-sh1/sh2 and K562/G01-sh1/sh2 cells. The results indicated that knockdown of RPS27a arrested the cell cycle at the S and G2/M phase, which was accompanied by an up-regulation of P21, and down-regulated the p-ERK expression of MAPK signal pathway and BCL-2 protein expression of apoptotic signal pathway, which contributed to inhibit the proliferation of K562 and K562/G01 cells and potentiate the effect of imatinib on apoptosis of K562/G01 cells.

In conclusion, RPS27a promotes the proliferation, regulates cell cycle progression and inhibits the effect of imatinib on apoptosis of CML cell lines at least partially through Raf/MEK/ERK, P21 and BCL-2 signaling pathways. It appears that drugs targeting RPS27a combining with TKI might represent a novel therapy strategy in some patients with TKI resistant CML.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.086.

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