SRPK1-siRNA suppresses K562 cell growth and induces apoptosis via the PARP-caspase3 pathway

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Abstract. Serine-arginine protein kinase 1 (SRPK1) has been used as an important signal mediator, and is associated with cancer development. However, studies have yet to determine whether SRPK1 suppresses leukemia cell growth and induces apoptosis. Studies have also yet to reveal the underlying mechanisms. In the present study, the effects of downregulating SRPK1 gene expression on chronic myeloid leukemia cell lines (K562 cells) were investigated through RNA interference (RNAi) and the proliferation inhibition and apoptosis induction of SRPK1 in K562 cells were analyzed. K562 cells were transfected with two different concentrations of siRNA, and the transfection efficiency was detected via flow cytometry. The expression of SRPK1 was detected via reverse transcription-quantitative polymerase chain reaction. K562 cell proliferation and apoptosis were analyzed using MTT and flow cytometry respectively. The roles of caspase-3, poly (ADP-ribose) polymerase (PARP), p53 and B-cell lymphoma (Bcl)-2/Bcl-2 associated X, apoptosis regulator (Bax) proteins in the apoptosis of human K562 cells were further examined through western blot analysis. The SRPK1 expression was lower in the K562 cells transfected with SRPK1-siRNA compared with untransfected cells. The inhibition rate in the transfected groups was increased compared with the untransfected groups. Compared with control groups, the number of apoptotic cells in the SRPK1-silenced groups increased. The number of early apoptotic cells also increased. The cleaved caspase-3, cleaved PARP and p53 expression levels were significantly increased in the RNAi groups compared with control groups. Conversely, the Bcl-2/Bax rate was significantly lower. In conclusion, the knockdown of the SRPK1 gene by RNAi inhibited the proliferation of K562 cells and induced their apoptosis. Apoptosis was induced by the activation of the PARP-caspase3 pathway.

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Introduction

Serine-arginine protein kinases (SRPKs) family represents a class of enzymes that can phosphorylate a defined region in the RS domain in each SR protein. Phosphorylation and dephosphorylation cycle of SR proteins is essential for pre-mRNA splicing in cells (1). As a SR splicing factor phosphorylation protein, SRPK1 plays an important role in accumulating function signal and regulating the other members of SRPKs family. Recent study demonstrated that some SR proteins reveal altered expression in human cancers, over-expression of a specific SR protein, SF2/ASF, is sufficient to attract cellular transformation. Mammalian cells express two SRPKs and four members of the Clk/Sty family of kinases (2). It revealed that knocking down SRPK1 tumors grew significantly more slowly in comparison with SRPK1 expression counterpart tumors (3). The knockdown of SRPK1 expression through siRNA upregulates pro-apoptotic proteins, which subsequently sensitise breast, colorectal and pancreatic tumor cells to undergo apoptosis (4,5). Therefore, the expression level of SRPK1 may cause a molecular shift to influence tumorigenesis.

Small interfering RNA (siRNA)-mediated gene silencing shows an enhanced specificity and effectiveness and thus provides a powerful tool for many logical and therapeutic applications. In humans, several efficient siRNA sequences against members of apoptotic pathways, such as caspase-1, -2, -3, -8 and Fas, have been designed to examine the regulation of apoptosis (6-9).

Cell apoptosis plays a key role in the regulation of tissue turnover that integrates multiple physiological and pathological death signals. Many apoptotic signalling pathways, including Fas/FasL, caspase family, cytochrome *c* signalling and mitochondrial pathways, have been recognized (10-13). p53, a tumor suppressor, is involved in the apoptotic effects of cancer cells; it induces multiple cell death pathways. One key endpoint in this cascade is the activation of caspase-3, which cleaves several substrates, such as DNA repair enzyme poly (ADP-ribose) polymerase (PARP) or DNA fragmentation factor (DFF). Consequently, DNA strand breaks during apoptosis (14). B-cell lymphoma (Bcl)-2 family members are important regulators in the apoptotic pathway associated with individual components, such as Bcl-2 and Bcl-xL, which can suppress apoptosis, or other factors, including Bax and Bad, which can promote apoptosis.

In this study, the roles of caspase-3, PARP, p53 and Bcl-2/Bax proteins in the apoptosis of human chronic myeloid leukemia cell lines (K562 cells) were evaluated through interference by SRPK1-siRNA. First, the effect of downregulating SRPK1 gene expression in K562 cells by RNAi was analyzed and the proliferation inhibition and apoptosis induction of SRPK1 in K562 cells was confirmed. Second, the roles of caspase-3, PARP, p53 and Bcl-2/Bax proteins in the apoptosis of human K562 cells were investigated through western blot analysis. This study provided novel insights into the mechanism of apoptosis induced by human K562 cells via the PARP-caspase3 pathway.

Materials and methods

Subjects. K562 cells and HL-60 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (StemCell Technologies, Vancouver, BC, Canada) containing 10 ml of 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cultures were incubated at of 37˚C in a fully humidified atmosphere with 5% CO_2.

SRPK1 knockdown in K562 and HL-60 cells. SRPK1-siRNA was synthesised by RiboBio Biological Technology Co., Ltd. (Guangzhou, China). All of the transfections were conducted by using a C10511-1 riboFect™ CP transfection kit (RiboBio, Biological Technology Co., Ltd.) according to the manufacturer's instructions. The cells without the insert gene were used as control. The same volume-specific siRNA and control RNA were dissolved in RPMI-1640 medium at 100 μl and a certain volume of C10511-1 riboFect™ was dissolved in 100 μl of RPMI-1640 medium for 5 min at room temperature. After 5 min, RNA and C10511-1 riboFect™ were diluted, blended and incubated for 20 min at room temperature. The compound (200 μl) was added to the cell culture plate containing a serum-free medium, gently shaken and thoroughly incorporated. The cells were harvested after 4-6 h and replaced with a fresh medium. siRNA transfection efficiency was analysed by using a flow cytometer (FCM; Guava easyCyte 8HT) and Guava Incyte version 2.8 (both from EMD Millipore, Billerica, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the sample by using a TRIzol reagent (Invitrogen) and reversed-transcribed into cDNA by utilising an M-MLV reverse transcriptase kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The primer sequences were as follows: Human SRPK1 forward, 5'-CACGGCATG CATGGCCTTTGA-3' and reverse, 5'-CGGCGGCAGTGG CTCTCTTC-3'. Quantitative PCR was performed with an iCycleriQ real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Triplicate PCRs (20 μl) were performed using SYBR-Green Supermix (Toyobo Co., Ltd., Osaka, Japan). The reaction mixture was initially denatured at 95˚C for 10 min and then subjected to 45 PCR cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. mRNA levels were normalised to β-actin levels.

MTT test. The K562 and HL-60 cells were seeded in 96-well plates at a density of $1x10^5$/ml. Each plate was transfected with 50 and 100 nM siRNA according to the introduction above. Cultures were incubated at 37˚C in a fully humidified atmosphere with 5% CO_2 for 24 h. The plates were added with 10 μl of MTT (5 mg/ml) after 48 h and this procedure was continuously performed for another 4 h. After the supernatant was centrifuged, each plate was added with 100 μl of DMSO. The proliferation of K562 cells was quantified at an OD of 490 nM. Cell survival was calculated as the percentage of MTT inhibition, using the following formula: Inhibition rate (%) = (1 - treatment group/control group) x 100%.

Apoptosis detection assay. The cell apoptotic rate was evaluated by using a fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit (Becton-Dickinson and Co., Franklin Lakes, NJ, USA). The cells in the logarithmic phase were collected, centrifuged at 1,000 rpm for 5 min and washed with precooled PBS. The cells were suspended with 200 μl of buffer. Each pipe was added with 4 μl of Annexin V/FITC and 8 μl of 20 μg/ml PI for 15 min at room temperature. The sample was analysed with a flow cytometer.

Cell protein extraction. Total proteins were extracted from the cells by using a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM PMSF, 50 mM NaF, 1% NP-40, and 1 mM EGTA). The cells were lysed for 30 min in an ice bath and centrifuged at 13,000 rpm for 15 min at 4˚C. The supernatant was collected to quantify the proteins.

Western blot analysis. Whole-cell extracts were mixed in Laemmli loading buffer, boiled for 5 min and subjected to SDS-PAGE. After SDS-PAGE was performed, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and then incubated with specific antibodies. The membranes were washed with Tris-buffered saline and Tween-20 (TBST) and incubated with HRP-conjugated second antibody for 1 h at room temperature (25˚C). Immune complexes were visualised using an Anmbilon™ Western chemiluminescent HRP substrate system (EMD Millipore). The primary antibodies were partly from Cell Signaling Technology, Inc. (Beverly, MA, USA) including caspase-3 (1:1,000), cleaved caspase-3 (1:1,000), PARP (1:1,000), cleaved PARP (1:1,000), p53 (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000) while β-actin antibodies (1:500) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

Statistical analysis. Results were expressed as mean ± standard error (SE) of independent experiments performed in triplicate. SPSS (version 14.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were compared via one-way ANOVA followed by Tukey's post hoc test. Differences between values were considered significant at $P<0.05$.

Results

siRNA transfection efficiency. This study designed and synthesised scrambled siRNA with green fluorescent protein (GFP) to investigate whether siRNA can inhibit K562 cells *in vitro*. After the cells were transfected with

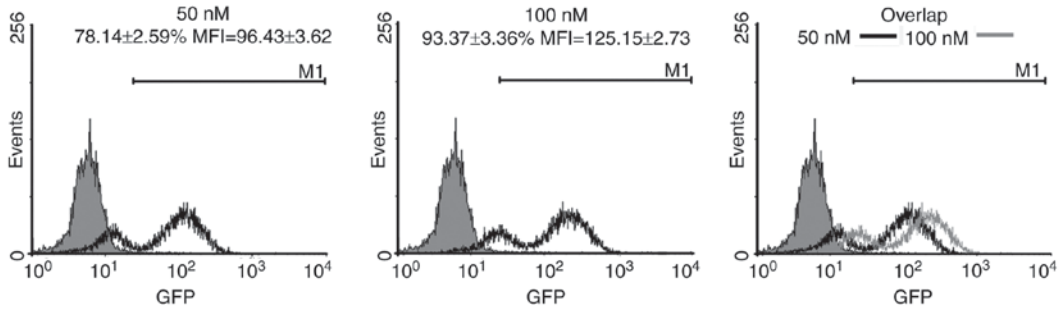

Figure 1. Efficiency of siRNA transfection examined through FCM. Results are presented as percentage (mean ± SD). Five independent experiments were performed. FCM, flow cytometric analysis.

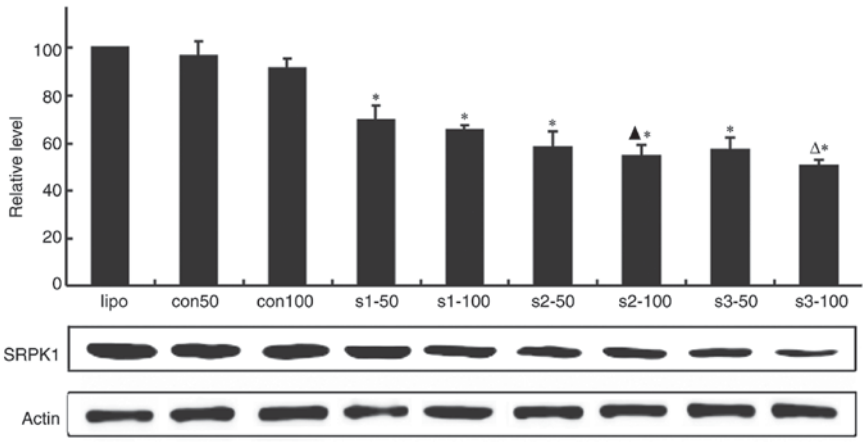

Figure 2. Suppression of the mRNA expression of SRPK1, as determined through real-time PCR and western blot analysis after transfection with SRPK1-siRNA. *P<0.05, compared with SRPK1 non-transfection groups; ▲P<0.05, the expression of SRPK1 on the synthesised sequence s2 at 100 nM siRNA was significantly lower than that at 50 nM; ΔP<0.05, the expression of SRPK1 on the synthesised sequence s3 at 100 nM siRNA was significantly lower than that at 50 nM.

50 and 100 nmol/l (nM) siRNA, the transfection efficiency was detected through FCM. The inhibition rate of 50 nM siRNA was 78.14±2.59%. By comparison, the inhibition rate of 100 nM siRNA was 93.37±3.36%. The mean fluorescent intensities (MFI) of 50 and 100 nM siRNA were 96.43±3.62 and 125.15±2.73, respectively. These results indicated that siRNA was transfected into the cells and the transfection induced by 100 nM siRNA was higher than that caused by 50 nM (Fig. 1).

Effect of SRPK1-siRNA on SRPK1 expression in K562 cells. To explore the effect of SRPK1 silenced by siRNA on K562 cells, we synthesised three SRPK1-siRNA (s1, s2 and s3) by company. After K562 cells were transfected with two concentrations of siRNA, the SRPK1 expression was detected through real-time polymerase chain reaction (RT-PCR) analysis and western blot analysis. The results showed that the mRNA levels of SRPK1 were lower in the transfected cells groups than in the transfection reagent and scrambled siRNA groups. SRPK1-siRNA at 100 nM was more effective than 50 nM in silencing the SRPK1 gene. SRPK1 protein was tested by western blot, the result was consistent with RT-PCR method (Fig. 2).

Suppression of tumor cells proliferation by SRPK1. The tumor cells with knocked down SRPK1 grew slowly. The transfected K562 and HL-60 cells were determined through MTT test to evaluate whether SRPK1 inhibits cell proliferation. In this study, 50 and 100 nM siRNA were transfected into K562 and HL-60 cells for 24, 48 and 72 h. The results revealed that the inhibition rate of the transfected groups was higher than that of the SRPK1 nontransfected groups. No statistical difference was found between the transfection reagent and siRNA scrambled groups. Compared with that of the SRPK1 nontransfection groups, the maximum inhibition rates of the transfection groups were s2 and s3 sequences at 100 nM siRNA cultured for 48 h. Therefore, 100 nM siRNA was superior to 50 nM. The inhibition rate increased at 48 h but slightly decreased at 48-72 h. These results indicated that s2 sequence was more effective than s3 in inhibiting cell proliferation in K562 and HL-60 cells (Fig. 3).

SRPK1-siRNA induced K562 cells apoptosis. In previous experiments, SRPK1 silencing can inhibit cell proliferation. The synthesised sequences s2 and s3 for 100 nM siRNA are better than the others. To investigate whether SRPK1 silencing accelerates K562 cell apoptosis, we chose the s2 and s3 sequences as experimental groups, the transfection regent group as control 1 and the scrambled siRNA group as control 2. Apoptosis was analysed through FCM. Compared with those in the control groups, the apoptotic cells in the SRPK1-silenced groups (s2 and s3) increased

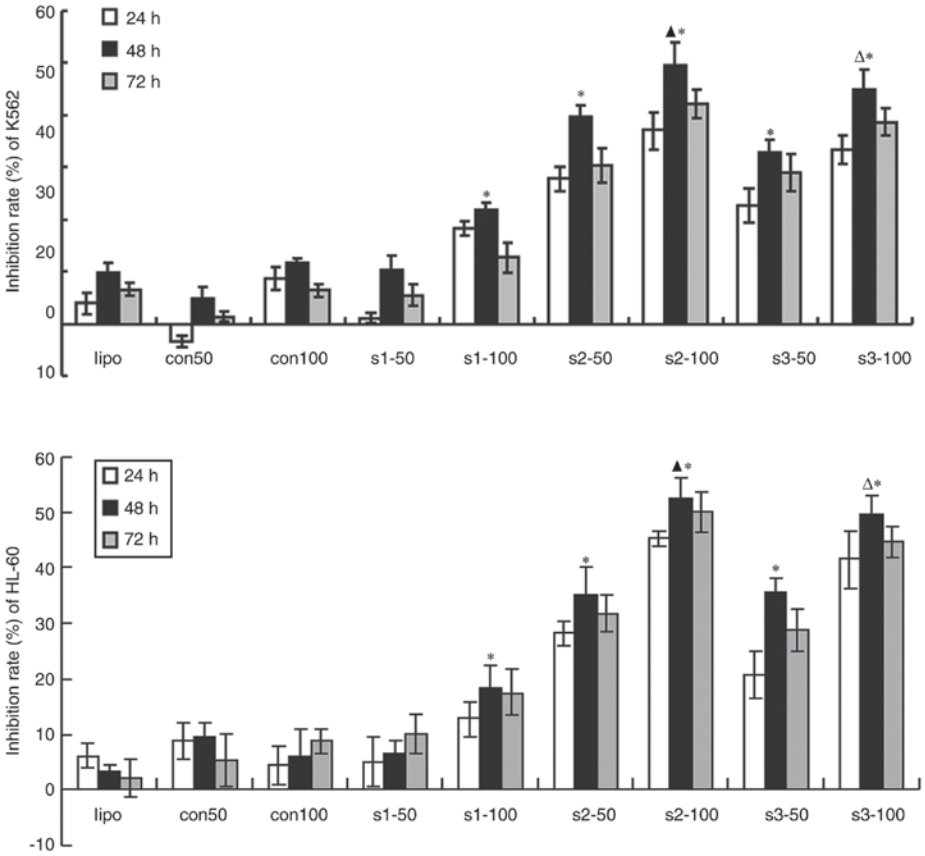

Figure 3. K562 and HL-60 cells viability were evaluated by MTT test. Data are representative of five separate experiments. *P<0.05, compared with SRPK1 non-transfection groups and synthesised sequence s1 for 50 nM siRNA; ▲P<0.05, the expression of SRPK1 on synthesised sequence s2 at 100 nM siRNA was significantly lower than that at 50 nM; ΔP<0.05, the expression of SRPK1 on synthesised sequence s3 at 100 nM siRNA was significantly lower than that at 50 nM.

by 29.95±3.27% and 31.4±2.85%. The early apoptotic cells also increased by 15.13±4.02% and 15.93±3.52% (Fig. 4). No significant difference in apoptotic cells was found between the control groups (2.09±1.98% vs. 3.14±2.16%). These results indicated that silencing SRPK1 induced K562 cell apoptosis (Fig. 4).

SRPK1-siRNA induced K562 cells apoptosis via the PARP-caspase3 pathway. To determine the possible mechanism of SRPK1 contributing to K562 cells, we examined caspase-3, PARP, p53, Bcl-2 and Bax protein expression through western blot analysis. The experiment was divided into four groups: Transfection regent group as control 1, scrambled siRNA group as control 2, synthesised sequence s2 for 100 nM siRNA as RNAi 1 and s3 100 nM siRNA as RNAi 2. The results revealed that the cleavage and activation of caspase-3 and PARP were significantly higher in the RNAi groups than in the control groups, whereas the expression levels of PARP and caspase-3 in the four groups did not significantly differ. The expression of RNAi 2 group was significantly higher than that of the RNAi 1 group. We further examined whether SRPK1 inhibition can modulate p53, pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins during induced apoptosis. The expression of p53 was significantly higher in the RNAi groups than in the control groups, while Bcl-2/Bax was significantly decreased. No significant difference was observed between the control and RNAi groups in Bcl-2/Bax (Fig. 5). These results

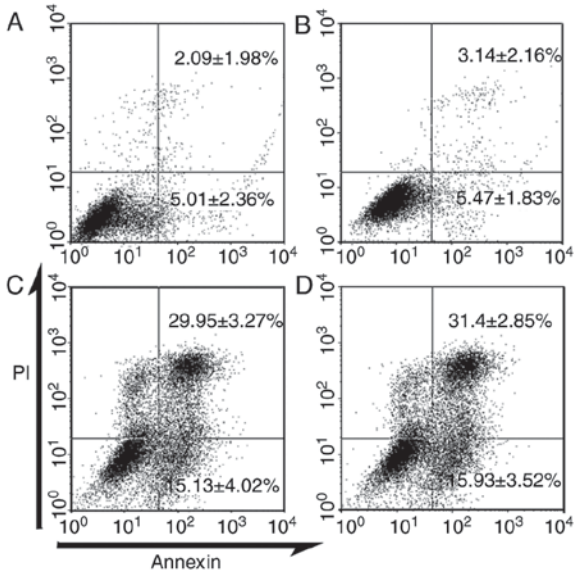

Figure 4. Apoptotic status of K562 cells was assayed through flow cytometry by using Annexin V-FITC. Data are representative of five separate experiments. (A-D) Transfection reagent group, scrambled siRNA group and synthesised sequence s2 and s3 at 100 nM siRNA. Results are presented as percentage (mean ± SD). Five independent experiments were performed.

suggested that the inhibition of SRPK1 could induce the apoptosis of K562 cells via the PARP-caspase-3 pathway.

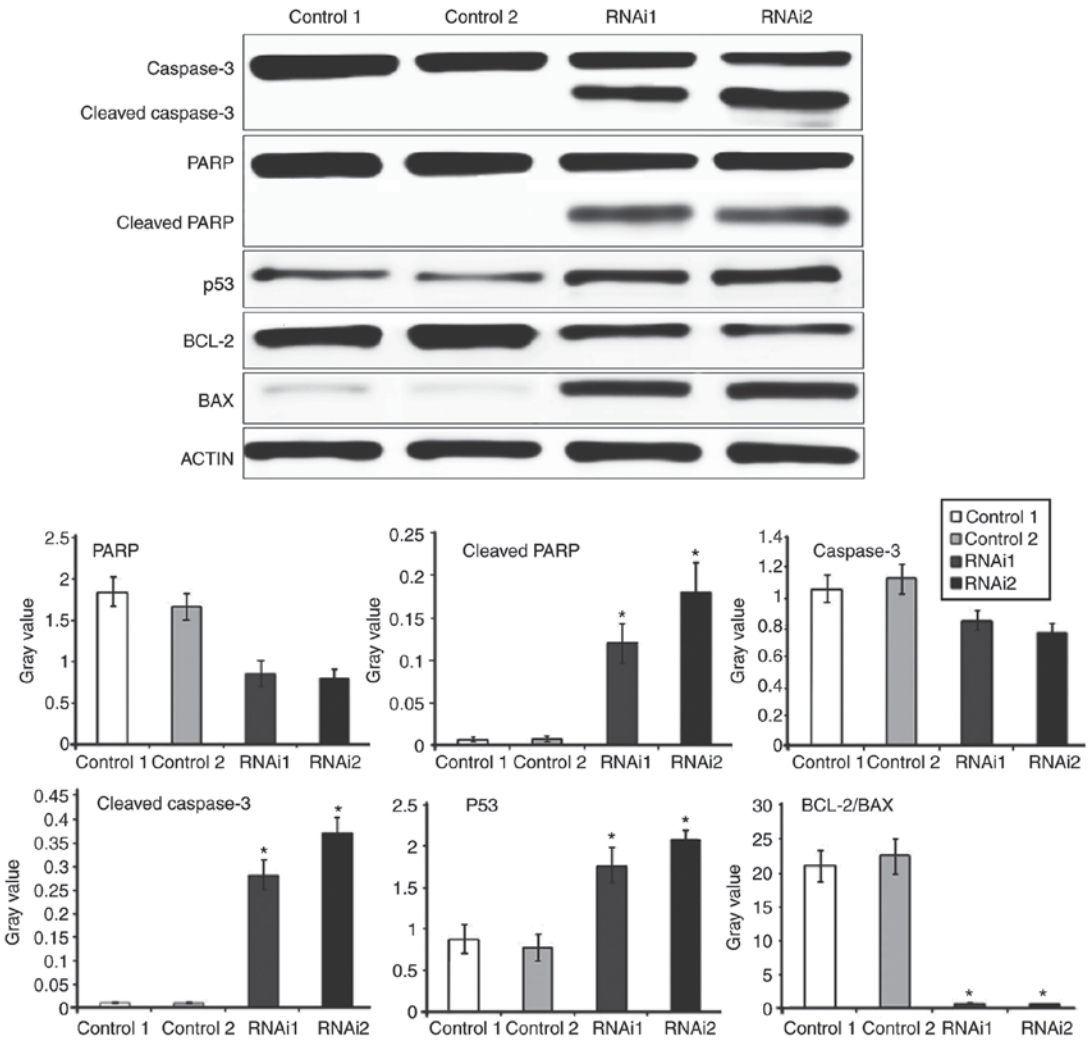

Figure 5. Western blot results showing the protein levels of caspase-3, cleaved caspase-3, PARP, cleaved PARP, p53 and Bcl-2/Bax. β-actin was used as an internal loading control. Data are representative of three separate experiments. *P<0.05, compared with control groups.

Discussion

Recent years, SRPKs were found as an important part of serine-threonine protein kinase family, which played an important role in specific phosphorylation of serine. SRPK1 is one of SRPKs that studied most deeply. As a SR splicing factor phosphorylation protein, SRPK1 plays an important role in accumulating function signal and regulating the other members of SRPKs family. SR protein kinase is a protein kinase family, all SRPKs show the ability of phosphorylating SR protein, regulating the alternative splicing of SR protein, influencing the distribution and location of SR protein in the nuclear and playing an important role in pre-mRNA splicing regulation. In addition, SRPKs can be involved in intracellular iron homeostasis, polyamine transportation, sperm development, paedomorphosis, cell cycle regulation, cell apoptosis and other important life activities by interacting with other proteins (15-17).

Current research has suggested that SRPKs could be potential to be the target of tumor therapy because of high expression in some tumor cells, such as prostate, breast, colon, and lung cancers (18-21). After interfering with SRPK1 gene, the proliferation of tumor cells was reduced, apoptosis potential increased and the sensitivity to chemotherapy drugs improved. This effect may be based on the mechanism of splicing. Sanidas *et al* (22) suggested that SRPK1a may play an important role in linking ribosomal assembly and/or function to elytroid differentiation in human leukemic cells. Wu *et al* (23) showed that knockdown of SRPK1 can inhibit tumor cells growth, invasion and migration in normoxic condition, but portion of the effect could be reversed in hypoxia. SRPK1 may be a new molecular player contributing to the early treatment of glioma. The study of van Roosmalen *et al* (24) provided comprehensive information on the molecular determinants of tumor cell migration and suggested that SRPK1 had potential for limiting breast cancer metastasis as a drug target. Aberrant SRPK1 expression in either direction can induce constitutive Akt activation and provide a mechanistic basis for previous observations that SRPK1 can be downregulated in some cancer types but upregulated in others (25). Ren *et al* (26) revealed that SRPK1 mediated TGF-Î²-induced proliferation and apoptosis by regulating AKT and JNK in ESCC. Thus, the TGF-Î²-SRPK1 pathway may be a useful target to affect the progression of ESCC. Sigala *et al* (27) had verified that the effect of SRPK1 knockdown on the viability of glioma cell lines was limited at least *in vitro*, whereas the *in vivo* effects of

this process can be attributed to the modulation of angiogenesis by SRPK1. Our study demonstrated that the mRNA levels of SRPK1 were lower in the transfected cell groups than in the transfection reagent and scrambled siRNA groups. The transfected K562 cells were determined through MTT and FCM test to evaluate whether SRPK1 can inhibit cell proliferation and induce apoptosis. The results showed that the inhibition rate in the transfected groups was higher than that in the SRPK1 nontransfected groups. HL-60 cells after transfection were tested by MTT, it revealed SRPK1-siRNA can inhibit HL-60 cells proliferation as well. Compared with that in the control groups, the apoptotic cells in the SRPK1-silenced groups (s2 and s3) increased, and early apoptotic cells also increased.

Apoptosis is a complex process regulated by various factors. For example, caspase-3 is generally considered as the main executor of apoptosis. One of the essential substrates cleaved by caspase-3 is PARP, an abundant DNA-binding enzyme that detects and signals DNA strand breaks (28). Caspase-3 and its substrate PARP are the key modulators of apoptosis, especially through the cleavage of PARP and caspase-3. p53 is a stress response protein, which is induced by DNA damage and deregulated oncogene expression (29,30). To confirm our findings, we analysed the protein levels of caspase-3, PARP, p53 and Bcl-2/Bax by western blot analysis. The results revealed that the cleavage and activation of caspase-3 and PARP were significantly higher in the RNAi groups than in the control groups, whereas the expression levels of PARP and caspase-3 in the four groups had no significant difference. The expression of the RNAi 2 group was significantly higher than that of the RNAi 1 group. These observations could imply that the inhibitory effects on the cyclic stretch-induced apoptosis of human K562 cells occurred primarily at the post-cleavage level. The lever of p53 protein was significantly higher in the RNAi groups than that in the control groups. The data suggested that p53 might mediate cellular sensitivity to apoptosis. The expression of Bcl-2/Bax was significantly decreased in the RNAi groups compared with that in the control groups. No significant difference was observed between the control and RNAi groups. p53 is implicated in the induction of two distinct apoptotic signaling pathways: The intrinsic and extrinsic pathways. The extrinsic pathway involves death receptors, which including caspase-3. The two pathways can trigger the activation of PARP and lead cells to apoptosis. This study showed that the expression of p53, cleaved caspase-3 and cleaved PARP increased while anti-apoptotic protein Bcl-2/Bax decreased in the RNAi groups, may indicate the apoptosis of tumor cells.

This study provided evidence revealing a novel function of SRPK1-siRNA in human K562 cells induced apoptosis by the activation of the PARP-caspase3 pathway. The limitation of the present study is the lack of a detailed molecular mechanism and *in vivo* experiments. In future studies, we will study further and related molecular mechanism will be identified to elucidate the potential role in tumor therapy.

Acknowledgements

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