

LIM kinase 1 serves an important role in the multidrug resistance of osteosarcoma cells

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Abstract. Multidrug resistance (MDR) is a major challenge for the management of the majority of cancers. The precise molecular mechanisms of MDR remain elusive. In a previous study, a multidrug resistant osteosarcoma model [MG63/vincristine (VCR)] was established by intermittent exposure of MG63 cells to gradually increasing concentrations of VCR. These cells exhibited cross-resistance to multiple structurally and mechanistically unrelated chemotherapeutic agents. The development of MDR was associated with increased expression of LIM kinase 1 (LIMK1). Compared with that in normal human fetal osteoblasts (hFOB) 1.19, the messenger RNA and protein expression of LIMK1 was significantly elevated both in MG63 and U2OS osteosarcoma cells. To observe the expression pattern of LIMK1 in osteosarcoma, immunohistochemical analyses were performed on specimens derived from 6 patients. The results indicated that LIMK1 was expressed to a greater extent in the tumor parenchyma than in the mesenchyme. The role of LIMK1 in MDR was confirmed by transfecting plasmids coding LIMK1-small interfering RNA (siRNA), wild-type-LIMK1 or empty vector into MG63/VCR cells, and measuring the expression of LIMK1 and multidrug resistance protein 1 (MDR1), also known as P-glycoprotein (P-gp). The results demonstrated that the level of MDR1/P-gp was positively correlated with the level of

LIMK1. This correlation was also shown with the doxorubicin efflux assay and by measuring apoptosis. Specifically, after 6 h of incubation with VCR, 25.6% of the cells transfected with the LIMK1-siRNA plasmid were apoptotic compared with 6.2% in the empty vector group and 1.3% in the group of cells transfected with the wild-type-LIMK1 plasmid. Thus, it was concluded that LIMK1 serves a key role in the MDR of osteosarcoma and functions through MDR1.

Introduction

Osteosarcoma is a highly malignant bone tumor with widespread histological heterogeneity, a lack of biomarkers, high local aggressiveness and a rapid metastasizing potential (1). Osteosarcoma is the most common primary bone malignancy and the third most common cancer in adolescents and young adults (2). Chemotherapy combined with surgery is the principal mode of treatment for osteosarcoma. However, tumor resistance to chemotherapy and molecularly targeted therapies limits their effectiveness (1,3). Tumor cells may become cross-resistant to a broad spectrum of chemotherapeutic agents with different mechanisms and structures following a single-drug treatment; this is known as multidrug resistance (MDR) (4). Within tumor cells, various MDR mechanisms can operate, including increased drug efflux, mutations of the drug target, enhanced repair of DNA damage, activation of alternative signaling pathways and evasion of cell death (3). Although the precise mechanisms of MDR remain unclear, several cell membrane transporter proteins are linked to the resistance observed with commonly used chemotherapeutic agents. For example, multidrug resistance protein 1 (MDR1), also known as P-glycoprotein (P-gp), belongs to the ATP-binding cassette (ABC) transporter family, and regulates the efflux of multiple structurally and mechanistically unrelated chemotherapeutic agents across the plasma membrane (3).

LIM kinase 1 (LIMK1) is a member of the serine/threonine protein kinase family. It is activated when the Thr508 residue is phosphorylated by Rho-GTPases such as Rho, Rac and cell division cycle 42 (5,6). LIMK1 functions through regulating actin cytoskeleton remodeling by phosphorylating and inactivating cofilin on the Ser3 residue (7,8). Increasing evidence suggests that LIMK1 serves an important role in

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Abbreviations: CCK-8, Cell Counting kit-8; DOX, doxorubicin; H-DMEM, high glucose-Dulbecco's modified Eagle's medium; hFOB, human fetal osteoblasts; LIMK1, LIM kinase 1; MDR, multidrug resistance; P-gp, P-glycoprotein; PI, propidium iodide; MTX, methotrexate; PTX, paclitaxel; THP, pirarubicin; VCR, vincristine

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tumor metastasis and invasion (9). However, few studies have addressed the role of LIMK1 in MDR in tumor cells (10). In a previous study, an osteosarcoma MDR model was established by using the human osteosarcoma cell line MG63 and vincristine (VCR), a drug commonly used in the treatment of osteosarcoma (11). Notably, the development of MDR was associated with increased expression of LIMK1. Therefore, the current study was designed to determine whether the over-expression of LIMK1 contributes to MDR and to identify its potential mechanism.

Materials and methods

Cells and cell culture. The human osteosarcoma cell lines U2OS and MG63 (purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in high glucose-Dulbecco's modified Eagle's medium (H-DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Human fetal osteoblastic (hFOB) 1.19 cells (donated by the Pathology Laboratory of Jilin University, Changchun, China) were cultured in DMEM-F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FCS. All three cell types were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Tumor samples from 6 patients with osteosarcoma were collected intra-operatively and stored at -80°C (March 2015 from the China-Japan Union Hospital of Jilin University, Changchun, China). Written consent was obtained from all patients or their families, and the study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of Jilin University.

Establishment of VCR-resistant cell sublines. To generate VCR-resistant sublines, MG63 cells were exposed to increasing concentrations of VCR as described previously (11). Briefly, parental MG63 cells were cultured initially in H-DMEM containing 10 ng/ml VCR (Shanghai New Hualian Pharmaceutical Co., Ltd., Shanghai, China) for 72 h. Surviving cells were collected and cultured in VCR-free medium for 1-2 weeks, and then further cultured with VCR-containing H-DMEM. This procedure was repeated ≥ 5 times until the majority of cells survived in the drug-containing medium. Cells were then exposed to increasing concentrations (10-500 ng/ml) of VCR following the same procedure. This process required 4-6 weeks to establish adequate growth at each VCR concentration. Eventually, a subline resistant to 500 ng/ml VCR was obtained, which was named MG63/VCR. Prior to each experiment, MG63 and MG63/VCR cells were maintained in drug-free media and subcultured ≥ 3 times.

Drug sensitivity assays. The Cell Counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was used to determine the sensitivities of cells to VCR, pirarubicin (THP), methotrexate (MTX), doxorubicin (DOX) (all from Hualian Pharmaceutical Co., Shanghai, China) and paclitaxel (PTX) (Laboratoire Pierre Fabre, Castres, Cedex, France). Cells in the logarithmic growth phase were trypsinized and plated onto 96-well plates at 8×10^3 cells/well, and cultured for 24 h in 100 μ l H-DMEM supplemented with 10%

FBS (Hyclone). The culture medium was then replaced with medium containing serial dilutions of each drug (125, 25, 5, 1, 0.2, 0.04, 0.008 and 0.0016 μ g/ml). Drug-free medium was added to the control and blank wells. After 48 h, the medium was replaced with 10% CCK-8. After incubation at 37°C for 2 h, the plates were analyzed in a plate reader at 450 nm. The percentage of inhibition for each treatment was calculated using the following formula: Inhibition = $(OD_{\text{control}} - OD_{\text{experimental group}}) / OD_{\text{control}} \times 100$. Based on this information, the 50% inhibitory concentration (IC₅₀) was calculated using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The resistance index (RI) was calculated as $IC_{50 \text{ MG63/VCR}} / IC_{50 \text{ MG63}}$.

Immunohistochemical staining. Immunohistochemical staining was performed as described previously (12). Briefly, tumor tissue sections were incubated with 3% H₂O₂ to inactivate endogenous peroxidases, followed by deparaffinization and rehydration. The antigen was released with 3% proteinase K, followed by washing with PBS and blocking with 10% goat serum (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The sections were then incubated for 1 h at room temperature with a mouse monoclonal antibody targeting β -actin (sc-58673, dilution 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or a rabbit polyclonal antibody targeting LIMK1 (sc-48346, dilution 1:100; Santa Cruz Biotechnology, Inc.). Biotinylated rabbit anti-mouse immunoglobulin (sc-2359, Santa Cruz Biotechnology, Inc.) diluted 1:100 in 3% normal goat serum (Santa Cruz Biotechnology, Inc.) was applied for 1 h at room temperature, followed by incubation with a horseradish peroxidase (HRP)-conjugated streptavidin-biotin complex (Santa Cruz Biotechnology, Inc.) for 10 min at room temperature. Paraffin-embedded sections were dehydrated, mounted with neutral gum and observed under a light microscope (Olympus Corporation, Tokyo, Japan).

Immunoprecipitation and western blot analysis. U2OS, MG63 and hFOB1.19 cells were lysed with lysis buffer [150 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0) containing 20 μ M phenylmethylsulfonyl fluoride] at 4°C for 30 min, and then centrifuged at 12,000 x g at 4°C for 15 min. The supernatant was stored at -70°C. Equal amounts of protein (100 ng), 15 μ l of protein A agarose beads (Santa Cruz Biotechnology, Inc.) and 1 μ l of anti-LIMK1 antibody (sc-48346, Santa Cruz Biotechnology, Inc.) were added to the tubes, which were rotated overnight at 4°C. The agarose-antibody-antigen complexes were collected by centrifugation for 20 sec at 12,000 x g and 4°C. The supernatant was removed carefully, and the complexes were washed twice with lysis buffer. The pellet was resuspended in gel-loading buffer (Santa Cruz Biotechnology, Inc.), and the proteins were denatured by boiling for 5 min. Protein A agarose beads were removed by centrifugation at 12,000 x g for 20 sec at 4°C, and the supernatant was transferred to a fresh tube. The proteins (20 μ l per lane) were separated by cellulose acetate membrane SDS-PAGE (5% acrylamide in concentration gel and 12% in separation gel) and analyzed by immunoblotting, as described previously (13,14), using primary antibodies against LIMK1. Immunodetection was accomplished using an HRP-conjugated goat anti-rabbit secondary antibody (BS-13278, 1:1,000; Bioworld Technology, Inc., St. Louis Park, MN, USA) rotated

for 1 h at room temperature. The protein bands were developed with a Novex ECL Chemiluminescent Substrate Reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) and analyzed by ImageJ software version 1.44 (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the cell lines was isolated using TRIzol reagent (Takara Bio, Inc., Otsu, Japan) and quantified using a NanoDrop ND-2000 (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and reversely transcribed into cDNA using the EX Tag kit (TaKaRa, Japan) according to the manufacturer's protocols. RT-PCR was performed utilizing TaKaRa EX Taq DNA Polymerase (Takara Bio, Inc.) under the following conditions: Denaturation at 98°C for 10 sec, annealing at 54-62°C for 30 sec and elongation at 72°C for 1 min for 20-30 cycles. The PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, Inc., Fremont, CA, USA). Images were obtained using the Gel Doc XR+ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using Image Lab™ software version 4.0 (Bio-Rad Laboratories, Inc.). The PCR primers used were: LIMK1, forward 5'-TGAGACAGGTGAGGTGATGG-3' and reverse 5'-AGGCTGAGTCTTCTCGTCCA-3'; MDR1, forward 5'-ATATCAGCAGCCCACATCAT-3' and reverse 5'-GAAGCACTGGGATGTCCGGT-3'; and β -actin, forward 5'-CTGGGACGACATGGAGAAAA-3' and reverse 5'-AAGGAAGGCTGGAAGAGTGC-3'.

Plasmid transfections. To assess the effect of LIMK1 in the multidrug resistance, four different plasmids were transfected into MG63/VCR cells using Lipofectamine™ 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. An expression plasmid coding for wild-type LIMK1-myc-enhanced cyan fluorescent protein (ECFP-LIMK1) and a short hairpin RNA (shRNA)-targeting LIMK1 construct, namely pSUPER-LIMK1-small interfering RNA (siRNA) (pSUPER-LIMK1), were constructed as described previously (11,12). The pSUPER-negative control-siRNA (pSUPER) vector containing a scrambled non-target sequence and an empty vector containing myc-ECFP, were used as negative controls. Transfected cells were cultured for 24 h prior to being transferred into 60-mm dishes or 96-well plates for subsequent experiments.

DOX efflux assay. Exponentially growing cells were plated onto 60-mm Petri dishes and incubated with 1.5 μ M DOX for 3 h at 37°C. Subsequently, cells were washed three times with ice-cold PBS, then centrifuged and suspended in ice-cold PBS and the mono-dispersed cells were analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA) using an argon laser of 15 mW at 488 nm (15). Cell fluorescence was evaluated in duplicate for each group, and all experiments were performed in triplicate.

Measurement of apoptosis. MG63/VCR cells in the exponential growth phase were plated onto 60-mm Petri dishes and incubated with 500 ng/ml VCR for 6 h at 37°C. Upon incubation, apoptosis was evaluated using fluorescein

isothiocyanate-Annexin V and PI (BD Biosciences) according to the manufacturer's protocol. The analysis was performed in a flow cytometer (BD Biosciences) using an argon laser of 15 mW at 488 nm. Cells ($\geq 10,000$ cells/sample) were collected and analyzed using Cell Quest software version 7.5.3 (BD Biosciences).

Statistical analyses. All data are expressed as means \pm standard deviation. Statistical significance was assessed by the unpaired Student's t-test when comparing means between two groups or one-way analysis of variance with post-hoc Dunnett's test to compare mean values among three or more groups. Statistical analysis was conducted with GraphPad Prism version 5.04 for Windows (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Elevated expression of LIMK1 in human osteosarcoma cells. To explore the role of LIMK1 in the progression of osteosarcoma, its expression *in vitro* was first assessed. Two osteosarcoma cell lines, U2OS and MG63, were used and compared with normal hFOB1.19 osteoblast cells. The expression of LIMK1 messenger RNA (mRNA) was significantly increased in both MG63 and U2OS cells compared with that in hFOB1.19 cells (by 2.6- and 4.2-fold, respectively) (Fig. 1A). A similar disparity was observed in western blot assays ($P < 0.01$) (Fig. 1B). The expression of LIMK1 was also analyzed in human osteosarcoma tissues by immunohistochemistry. LIMK1 was highly expressed in the tumor parenchyma compared with that in the mesenchyme (Fig. 1C) in 83.3% of the specimens derived from 6 clinical patients. The expression of β -actin was unchanged between the parenchyma and mesenchyme. These results indicated that LIMK1 was overexpressed in osteosarcoma parenchyma, and suggested that LIMK1 may affect the progression of this type of cancer.

LIMK1 and MDR1 are overexpressed in multidrug resistant MG63/VCR cells. In previous study (11), a multidrug resistant MG63/VCR subline was established by intermittent exposure of MG63 cells to gradually increasing VCR concentrations, as specified in the Materials and methods section. MG63/VCR cells were less sensitive to VCR-induced cytotoxicity than parental cells (Fig. 2A). The IC_{50} values of MG63 and MG63/VCR cells were 1.0 and 453.4 ng/ml, respectively. MG63/VCR cells also exhibited cross-resistance to other structurally and mechanistically different drugs. The RI values of VCR, MTX, DOX, PTX and THP were 476.2, 4.7, 46.5, 379.0 and 23.7, respectively (Fig. 2B). To explore the mechanism by which MG63/VCR cells exhibited increased drug resistance, the expression of LIMK1 and MDR1/P-gp was examined by RT-PCR and western blot analysis. The expression of LIMK1 and MDR1 mRNA was elevated in MG63/VCR cells by 2.4- and 5.1-fold, respectively, compared with that in MG63 cells ($P < 0.01$) (Fig. 2C). A similar result was obtained in the western blot assay, with increases by 4.7- and 6.6-fold in LIMK1 and MDR1 protein expression, respectively ($P < 0.01$) (Fig. 2D). These results indicated that LIMK1 and MDR1/P-gp may be important in the MDR of osteosarcoma.

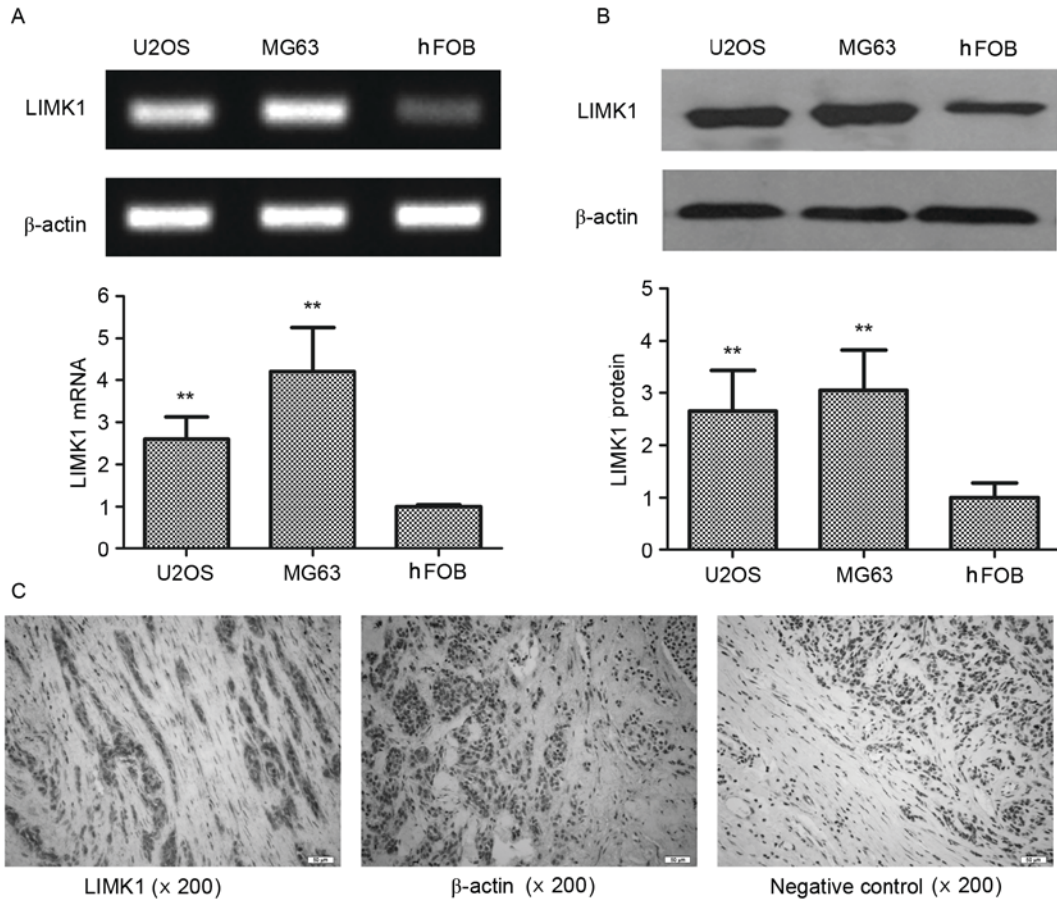


Figure 1. Expression of LIMK1 in human osteosarcoma (U2OS and MG63) and osteoblast (hFOB) cells. (A) Reverse transcription-polymerase chain reaction and (B) western blotting were performed to detect the levels of LIMK1 mRNA and protein, respectively. The expression of LIMK1 was normalized to that of β-actin, and is shown in the bar graphs as means + standard deviation (n=6; **P<0.01 vs. hFOB). (C) Immunohistochemistry staining using antibodies against LIMK1 and β-actin in human osteosarcoma tissue. Tumor samples from a total of 6 patients were evaluated, and a representative image of the 5 positive results obtained is shown (scale bar, 50 μm). LIMK1, LIM kinase 1; mRNA, messenger RNA; hFOB, human fetal osteoblasts.

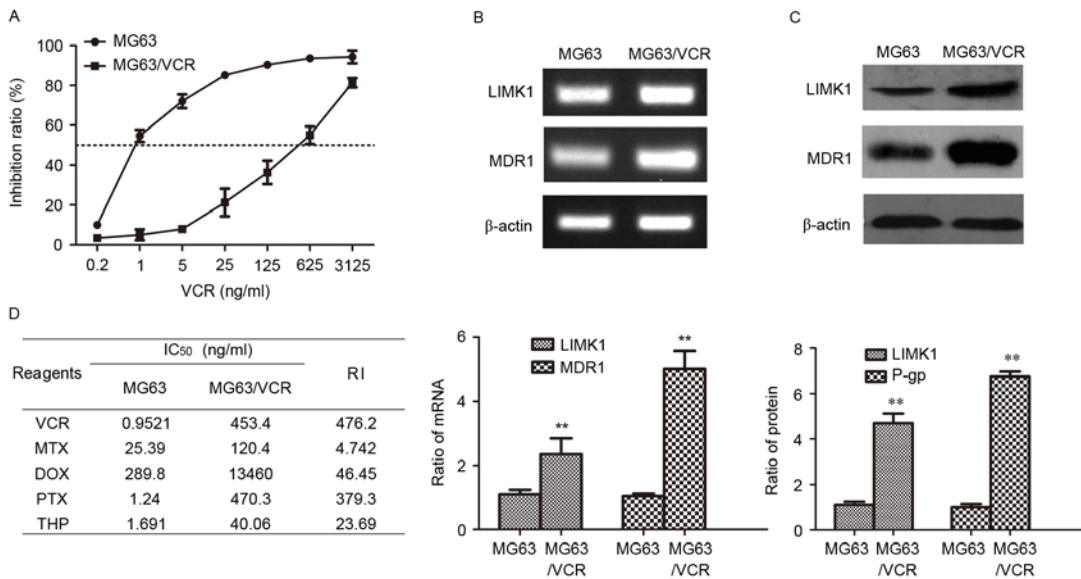


Figure 2. Expression of LIMK1 and MDR1 in MG63 and MG63/VCR cells. (A) Dose-dependent cell viability curves of cells treated with VCR. (B) Agarose gel electrophoresis of the reverse transcription-polymerase chain reaction products for LIMK1 and MDR1. (C) Western blotting was performed to detect the protein levels of LIMK1 and MDR1/P-gp proteins. (D) The concentration of each drug that produced 50% inhibition of growth (IC₅₀) and the RI values of various commonly used anticancer drugs in MG63 and MG63/VCR cells are shown. **P<0.01 vs. MG63 using the Student's t-test. All results are from three or four independent experiments. Data are expressed as means ± standard deviation. LIMK1, LIM kinase 1; MDR1, multidrug resistance protein 1; P-gp, P-glycoprotein; mRNA, messenger RNA; IC₅₀, 50% inhibitory concentration; RI, resistance index; MG63/VCR, multidrug resistant MG63 cells; VCR, vincristine; MTX, methotrexate; DOX, doxorubicin; PTX, paclitaxel; THP, pirarubicin.

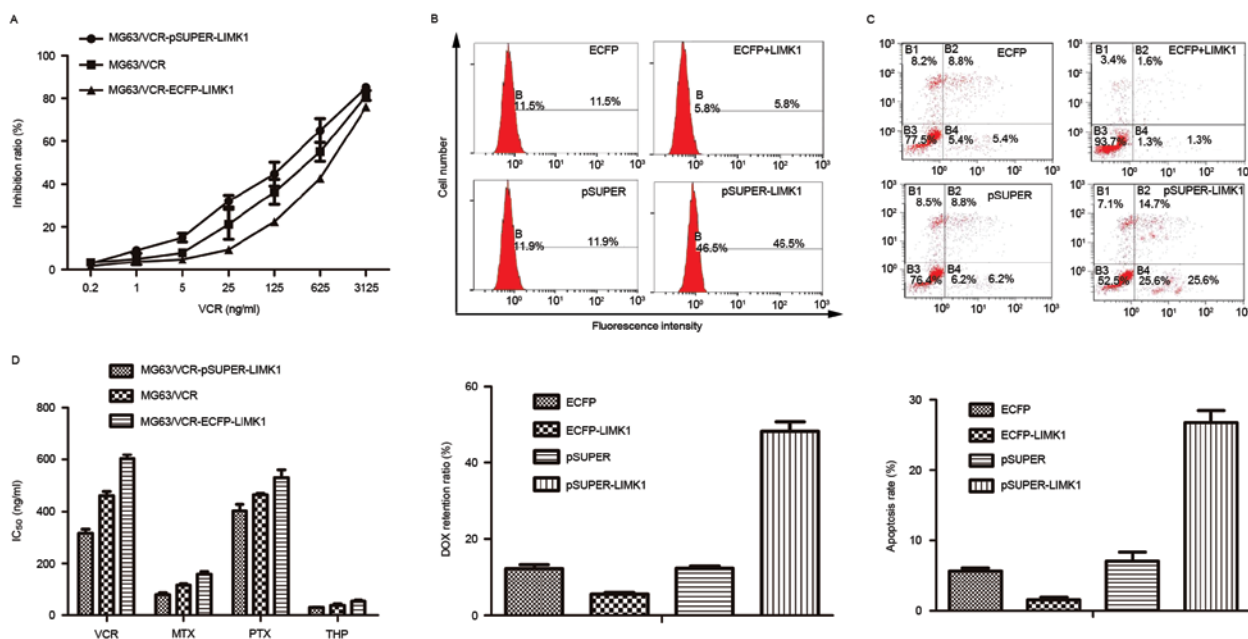


Figure 3. LIMK1 is critical for drug resistance in osteosarcoma cells. (A) MG63/VCR, MG63/VCR pSUPER-LIMK1-transfected and MG63/VCR ECFP-LIMK1-transfected cells were treated with different concentrations of VCR, and the cell viability was analyzed using the Cell Counting kit-8 assay. (B) The fluorescence intensity of DOX was analyzed quantitatively by flow cytometry. The mean fluorescence intensity + SD is shown in the bar graph. (C) Cell lines were incubated with VCR (IC₅₀) and assessed for early apoptosis by Annexin V/propidium iodide staining and flow cytometry analysis. (D) The concentration at which each drug produced 50% inhibition of growth (IC₅₀) is shown in the histogram. Representative data are shown. The histogram represents means ± SD of five independent experiments. LIMK1, LIM kinase 1; MG63/VCR, multidrug resistant MG63 cells; ECFP, enhanced cyan fluorescent protein; IC₅₀, 50% inhibitory concentration; VCR, vincristine; MTX, methotrexate; DOX, doxorubicin; PTX, paclitaxel; THP, pirarubicin; SD, standard deviation.

LIMK1 serves a key role in the MDR of MG63/VCR cells.

To assess whether LIMK1 was involved in the MDR of MG63/VCR cells, LIMK1 was knocked down by transfecting these cells with pSUPER-LIMK1 plasmids coding LIMK1 shRNA target sequences. In addition, LIMK1 expression was upregulated by transfecting the cells with ECFP-LIMK1 plasmids coding wild-type LIMK1. The viability of the transfected cells was evaluated upon treatment with a series of diluted drugs. A representative result with VCR is shown in Fig. 3A. The IC₅₀ values for VCR, MTX, PTX and THP were calculated and shown in Fig. 3B. Overall, cells expressing high levels of LIMK1 exhibited strong resistance to all chemotherapeutic drugs. DOX is a commonly used anticancer drug that has autofluorescence at the same wavelength as PI (15). The concentration of DOX was evaluated in cells transfected with different plasmids. The results revealed that DOX accumulation was the highest in MG63/VCR cells transfected with pSUPER-LIMK1 compared with in other groups, as evidenced by the increased DOX fluorescence intensity in these cells. This suggested that DOX efflux was downregulated along with LIMK1. By contrast, cells transfected with the ECFP-LIMK1 plasmid exhibited an increased efflux ability and reduced sensitivity to the drugs tested (P<0.01) (Fig. 3C). The results from the apoptosis assays were consistent with these findings. Cytometric dot-plot images of MG63/VCR cells obtained after 6 h of incubation with the IC₅₀ of VCR were used to evaluate the population of apoptotic cells in the lower right quadrant of the graphs (which corresponds to Annexin V-positive and PI-negative cells) (Fig. 3D). The apoptosis rate in cells transfected with the pSUPER-LIMK1 plasmid (25.6%) was higher than that in the empty vector group (6.2%) and in the cells

transfected with the ECFP-LIMK1 plasmid (1.3%) (P<0.01) (Fig. 3D). These results indicate that LIMK1 is important in the MDR of MG63/VCR cells.

LIMK1 functions through regulating the expression of MDR1/P-gp.

LIMK1 and MDR1/P-gp were expressed at higher levels in MG63/VCR cells than in MG63 cells (Fig. 2). To explore the connection between the two genes, the expression of LIMK1 and MDR1/P-gp was assessed in MG63/VCR cells following transfection with LIMK1 shRNA, wild-type LIMK1 or negative control vector. The results indicated that both LIMK1 mRNA and protein expression levels were increased in MG63/VCR cells following transfection with wild-type LIMK1, compared with those in cells transfected with the empty vector. LIMK1 mRNA and protein expression levels were significantly reduced in cells transfected with LIMK1 shRNA, compared with those in cells transfected with the empty vector or wild-type LIMK1. Notably, the change in MDR1 expression was completely consistent with that in LIMK1 expression (P<0.05) (Fig. 4A and B). Immunocytochemistry staining was performed to compare the expression of MDR1 in the same cells. The results demonstrated that MDR1/P-gp expression was increased significantly in MG63/VCR cells transfected with ECFP-LIMK1 plasmid, while transfection with LIMK1 shRNA decreased MDR1/P-gp expression (Fig. 4C). These data suggest that LIMK1 may affect MDR by regulating the expression of MDR1/P-gp.

Discussion

In the present study, a novel function of LIMK1 in regulating MDR was identified. Previous studies demonstrated that

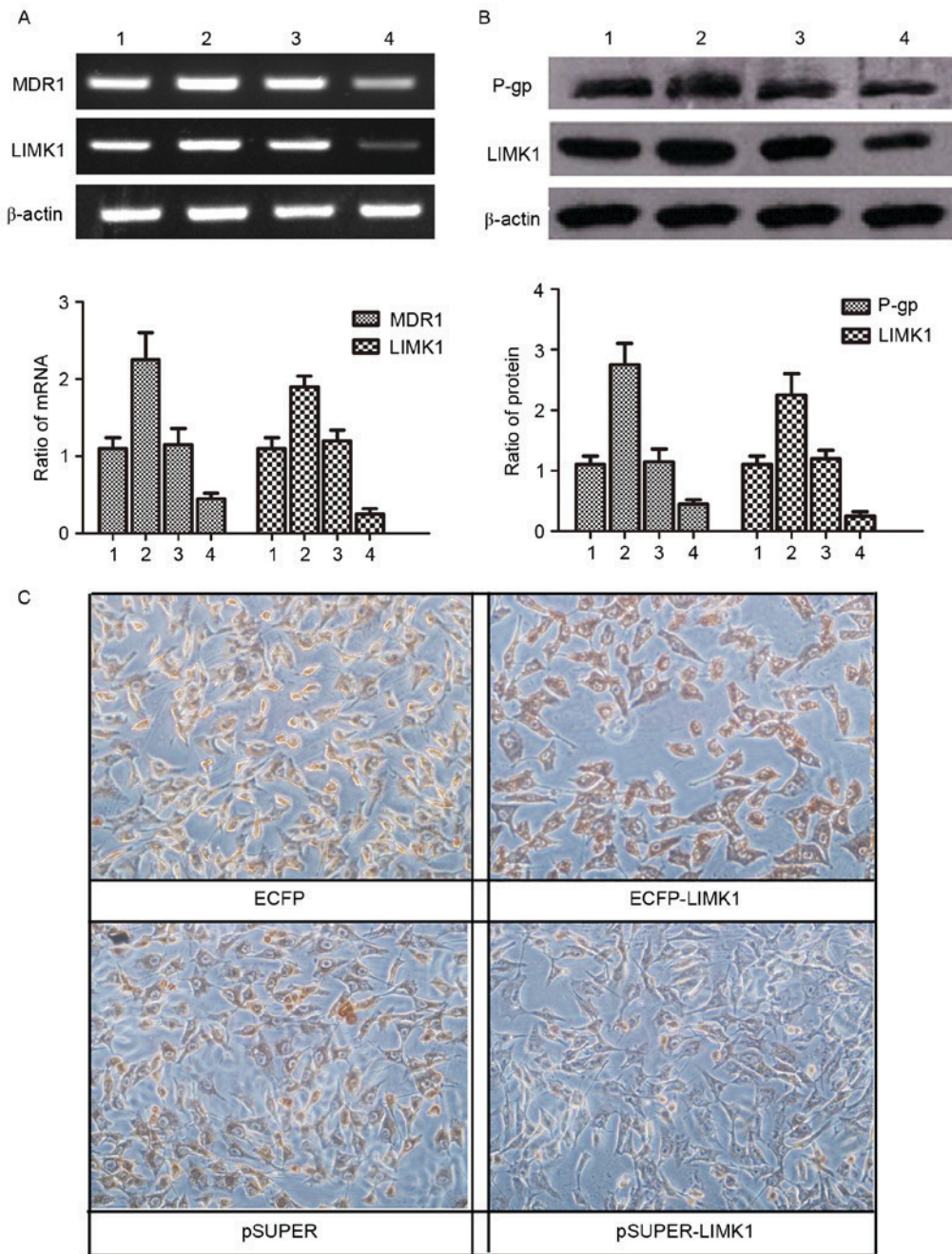


Figure 4. LIMK1 functions through regulating the expression of MDR1/P-gp. (A and B) Lanes 1, 2, 3 and 4 represent MG63/VCR cells transfected with plasmids for ECFP, ECFP-LIMK1, pSUPER and pSUPER-LIMK1 expression, respectively. (A) Agarose gel electrophoresis of the reverse transcription-polymerase chain reaction products for LIMK1 and MDR1 in MG63/VCR cells transfected with different plasmids. (B) Western blot analysis of LIMK1 and MDR1/P-gp proteins in MG63/VCR cells transfected with different plasmids. (C) Immunohistochemistry staining using antibodies against MDR1 in MG63 and MG63/VCR cells transfected with LIMK1 short hairpin RNA (pSUPER-LIMK1) or a control pSUPER-negative control-small interfering RNA vector (magnification, x200; scale bar, 50 μ m). LIMK1, LIM kinase 1; MG63/VCR, multidrug resistant MG63 cells; mRNA, messenger RNA; MDR1, multidrug resistance protein 1; P-gp, P-glycoprotein; ECFP, enhanced cyan fluorescent protein.

LIMK1 was overexpressed and highly active in cells and tissues of certain malignant tumors, including prostate and breast cancer (16,17). LIMK1 was considered to be a key molecule that stimulated malignant tumor cell invasion and metastasis, or possibly a new ‘oncogene’ (18,19). However, few studies linked LIMK1 with MDR, which represents a major challenge for managing the treatment of the majority cancers (20). In the present study, the expression of LIMK1 mRNA and protein in two human osteosarcoma cell lines (MG63 and U2OS) was markedly higher than that in hFOB

cells (Fig. 1A and B). Furthermore, the expression of LIMK1 in tumor parenchyma cells was significantly higher compared with that in mesenchymal cells (Fig. 1C), suggesting that LIMK1 was responsible for the genesis and development of osteosarcoma.

A multidrug resistant MG63/VCR subline was established by intermittent exposure of MG63 cells to VCR. This subline exhibited cross-resistance to other commonly used drugs to treat osteosarcoma, but which are structurally and mechanistically different to VCR (Fig. 2B) (1,21). These drugs included

an anti-microtubule agent (PTX), an antimetabolite (MTX) and two topoisomerase II inhibitors (DOX and THP) (22). Notably, the mRNA and protein expression levels of LIMK1 and MDR1/P-gp were higher in MG63/VCR cells than in MG63 cells (Fig. 2C and D). Therefore, it was hypothesized that there was a correlation between LIMK1 and MDR in osteosarcoma.

In order to verify this conjecture, additional experiments were conducted by upregulating and downregulating LIMK1 in MG63/VCR cells. Downregulation of LIMK1 inhibited the efflux of DOX and decreased the MDR of these cells compared with that of the controls. In addition, the sensitivity of these cells to VCR was elevated (Fig. 3). Of note, the expression of MDR1/P-gp, which was also increased in MG63/VCR cells compared with that in MG63 cells, was changed in accordance with the expression of LIMK1. MDR1 belongs to the ABC transporter family that regulates the efflux across the plasma membrane of multiple structurally and mechanistically unrelated chemotherapeutic agents (3). MDR1/P-gp-mediated MDR has been long considered a classical mechanism of MDR in malignant tumors, including osteosarcoma (3,20). The expression of MDR1 in MG63/VCR cells was increased significantly in cells overexpressing LIMK1 compared with that in cells transfected with empty vector. In addition, downregulation of LIMK1 resulted in a significant decrease in the expression of MDR1. These results indicate that LIMK1 serves an important role in MDR through regulating the expression of MDR1/P-gp. The RhoA/LIMK1/cofilin signaling pathway may have certain connection with the ABC transporter family. However, understanding the precise molecular mechanism requires further investigation.

In summary, the overexpression of LIMK1 increases MDR1/P-gp expression and has major effects on the MDR of human osteosarcoma. The present study provides new insights into the function of LIMK1 and suggests that altering LIMK1 is a potential new therapeutic strategy for osteosarcoma.

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