

Overexpression of hepatocyte growth factor protects chronic myeloid leukemia cells from apoptosis induced by etoposide

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Abstract. Resistance to apoptosis induced by chemotherapy is still an obstacle for the treatment of chronic myeloid leukemia (CML). Numerous studies have indicated that upregulation of hepatocyte growth factor (HGF) protein expression reduced apoptosis induced by various factors. However, whether HGF has any effect on apoptosis induced by VP-16 (etoposide) in CML cells and its underlying mechanisms are unclear. HGF was overexpressed in the K562 cell line using transfection. The protein and mRNA expression levels, and the concentration of HGF were measured using western blot analysis, reverse transcription-quantitative (RT-qPCR) and ELISA respectively. Apoptosis in the K562 cell line was determined using flow cytometry and western blot analysis. Changes in cell viability were measured using a MTT assay. RT-qPCR and western blot analysis revealed that HGF was successfully upregulated at both the mRNA and protein expression levels in the K562 cell line, respectively. After VP-16 treatment, the number of apoptotic cells overexpressing HGF was lower compared with that in cells transfected with the empty vector. Mechanistic investigation revealed that overexpression of HGF led to the increase in Bcl-2 protein expression level and inhibition of caspase-3/9 activation. Furthermore, HGF overexpression resulted in activation of the PI3K/Akt signaling pathway. Therefore, the results of the present study revealed that targeting HGF could be used as a strategy to overcome VP-16 resistance in CML.

Introduction

Chronic myeloid leukemia (CML) is a hematological disease, that accounted for ~20% of adult leukemia worldwide in 2009 (1). CML is characterized by reciprocal translocation between the break-point cluster (BCR) gene on chromosome 22

and the Abelson leukemia virus oncogene (ABL) on chromosome 9, also termed the Philadelphia chromosome, which results in the formation of the BCR-ABL oncogene (2). Constitutive expression of the BCR-ABL1 fusion protein transforms hematopoietic stem cells into CML stem cells and ultimately leads to myeloproliferative disease (3). The BCR-ABL fusion protein also constitutively activates tyrosine kinase activity and various downstream signaling pathways, such as the AKT, JAK/STAT3 and MAPK pathways, contributing to cell proliferation, and resistance to apoptosis and disrupting genetic stability (3).

Hepatocyte growth factor (HGF) is a pleiotropic growth factor, that is secreted by mesenchymal stem cells and capable of inducing various physiological activities in different cells, such as proliferation, survival, migration and angiogenesis (4). The *HGF* gene is located on chromosome 7, which is a chromosome that is frequently altered in various hematological malignancies. HGF is initially produced in a one-chain inactive form and later cleaved into a two-chain (α , β) biologically active form by enzymes, such as HGF activator (4). HGF has been intensively studied, mainly due to its role in cancer development and progression. For example, it has been reported that primary CML cells express high levels of HGF mRNA and protein, as well as its corresponding tyrosine kinase receptor, MET (5). Furthermore, elevated serum HGF levels have been associated with poor prognosis in patients with CML (6). Notably, HGF has been found to be preferentially generated in CML basophils and basophil-derived HGF induces endothelial cell migration, and might be used as a target for CML (7). All these findings indicate that HGF might play an essential role in the development of CML.

Tyrosine kinase inhibitors (TKIs) are able to induce remission and improve survival in patients with CML; however, they are unable to eliminate leukemia stem cells (LSCs), as these cells do not depend on the kinase activity of BCR-ABL oncoprotein for survival (8). VP-16 (etoposide) is a widely used chemotherapeutic agent for various cancers, including CML. VP-16 has also been shown to enhance TKI-induced apoptosis in BCR-ABL-transformed CML cells (9). More importantly, VP-16 has also been found to effectively repress the growth of LSCs (10). In the current study, the effects of HGF on the cytotoxicity of VP-16 in a K562 CML cell line was investigated. It was found that overexpression of HGF significantly decreased

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VP-16-induced apoptosis. The results from the present study further highlight the potential value of HGF in the treatment of CML.

Materials and methods

Cells and reagents. The human K562 CML cell line was purchased from the Cell Bank of the Chinese Academy of Sciences. VP-16 was purchased from Jiangsu Hengrui Medicine Co., Ltd. (cat. no. 10092131). All other routine chemicals were purchased from MilliporeSigma.

LY294002 (PI3K/Akt-specific inhibitor) treatment. LY294002 was obtained from MilliporeSigma (cat. no. L9908). After the cells were cultured in a six-well plate at 4.0×10^5 /well for 24 h, the medium was discarded and the cells were treated for another 24 h with LY294002 at a final concentration of 20 μ M (dissolved in the fresh culture medium).

Cell culture and transfection. The cell line was cultured in Iscove modified Dulbecco's Media, with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. The pVITRO2-HGF plasmid was constructed using the pVITRO2 vector (Shanghai, GenePharma, Co. Ltd.). Small interfering (si)RNA against Bcl-2 (si-Bcl-2) and negative control (si-ctrl) were purchased from Shanghai, GenePharma, Co. Ltd. The following sequences were used: Bcl-2: 5'-AAG GUGUCUCCAGAUCCUGA-3'; Ctrl: 5'-AAAUGUGUG UACGUCUCCUCC-3'. Human HGF cDNA was subcloned into the pVITRO2 vector using the *SalI* enzyme. For over-expression of Bax, human Bax cDNA was subcloned into the pcDNA3.1 vector by Shanghai, GenePharma, Co. Ltd. The K562 cells were seeded, at a density of 4×10^5 cells/well in six-well plates and incubated for 24 h. si-Ctrl (200 pmol), si-Bcl-2 (200 pmol), pVITRO2-mcs (mock-transfectant; 3 μ g), pVITRO2-HGF (3 μ g) or pcDNA3.1 Bax (3 μ g) was transfected using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) for 24 h according to the manufacturer's instructions. The cells without transfection were used as a negative control (untreated).

Cell viability assay. Cell viability was analyzed as previously described (11). Briefly, the cells (2.0×10^3 /well) were seeded into 96-well plates. Cells were transfected with pVITRO2-HGF for 24 h, followed by another 24 h treatment of VP-16 (final concentration of 1 μ g/ml). Then cells were co-incubated with MTT reagent (20 μ l/well) for 4 h at 37°C, after which the supernatant was removed and cells were co-incubated with 200 μ l of DMSO for 20 min at 37°C. Absorbance at 490 nm was measured using a microplate reader (BioTek, USA).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.). cDNA was synthesized from total RNA (1 μ g) using a ThermoScript RT-PCR system (Thermo Fisher Scientific, Inc.). Reverse transcription reactions was performed with the following conditions: 37°C for 15 min, 42°C for 50 min

and 85°C for 5 min. The reverse transcription products were visualized using gel electrophoresis. The qPCR reactions were performed using the ABI7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and was performed with 20 ng cDNA, 2.5 pmol forward and reverse primers, 10 μ l of SYBR Green Fast qPCR Mix (2X; Takara Biotechnology Co. Ltd.) and the final volume was adjusted to 20 μ l with RNase-free water. The qPCR reaction was performed at 95°C for 5 min, 60°C for 30 sec, followed by 40 cycles at 95°C for 30 sec and 58°C for 30 sec. The Δ Ct value was calculated by the CT value of the target gene minus the CT value of endogenous control. The $\Delta\Delta$ Ct value (Δ Ct target- Δ Ct calibrator) was used to determine the fold changes in gene levels. The relative gene expressions were determined by the $2^{-\Delta\Delta C_t}$ method (12). GAPDH was used as an internal control. The following primers were used: HGF forward, 5'-GGATGGATGGTTAGTTTGAGATACA-3' and reverse, 5'-CTCTTCCGTGGACATCATGAAT-3'; Bcl-2 forward 5'-GTGGAGGAGCTCTTCAGGGA-3' and reverse, 5'-AGGCACCCAGGGTGATGCAA-3'; Bax forward 5'-TTT GCTTCAGGGTTTCATCCA-3' and reverse, 5'-CTCCAT GTTACTGTCCAGTTCGT-3'; and GAPDH forward 5'-GTG AGGAGGGGAGATTCAG-3' and reverse, 5'-GCATCCTGG GCTACACTG-3'. GAPDH was used as an internal control. Experiments were performed independently 3 times.

Western blot analysis. Western blot analysis was performed as previously described (13). The cells were lysed with RIPA buffer (Beijing Solarbio Science and Technology Co., Ltd.). Equal amount (20 μ g) of protein lysate was separated using 12% SDS-PAGE, then transferred onto PVDF membranes (Millipore Sigma). The PVDF membranes were blocked with 10% skimmed milk in TBS-Tween-20 (TBST, 0.1% Tween 20) at room temperature for 1 h, washed with TBST and incubated with a primary antibody in TBST, containing 5% BSA (cat. no. A1933; MilliporeSigma) overnight at 4°C. The following primary antibodies were used: Caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), Cleaved Caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), Caspase-9 (1:1,000; cat. no. 9502; Cell Signaling Technology, Inc.), Cleaved Caspase-9 (1:1,000; cat. no. 20750; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat. no. 4223; Cell Signaling Technology, Inc.), Bax (1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), phosphorylated (p)-PI3K (1:1,000; cat. no. 17366; Cell Signaling Technology, Inc.), PI3K (1:1,000; cat. no. 4255; Cell Signaling Technology, Inc.), p-Akt (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), Akt (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.) and GAPDH (1:2,000; G9545; MilliporeSigma). The membranes were washed three times with TBST, then incubated with anti-rabbit HRP-conjugated secondary antibody (1:4,000; A0545; MilliporeSigma) for 1 h at room temperature. Signals were visualized using an ECL reagent (Pierce; Thermo Fisher Scientific, Inc.). Band intensities were quantified using ImageJ software (<https://imagej.nih.gov/>) and GAPDH was used as the loading control.

Apoptosis assay. To measure apoptosis, an Annexin V-FITC Apoptosis Detection kit (MilliporeSigma) was used according to the manufacturer's instructions. Briefly, after treatment by VP-16 or transfection, the cells were washed with PBS and

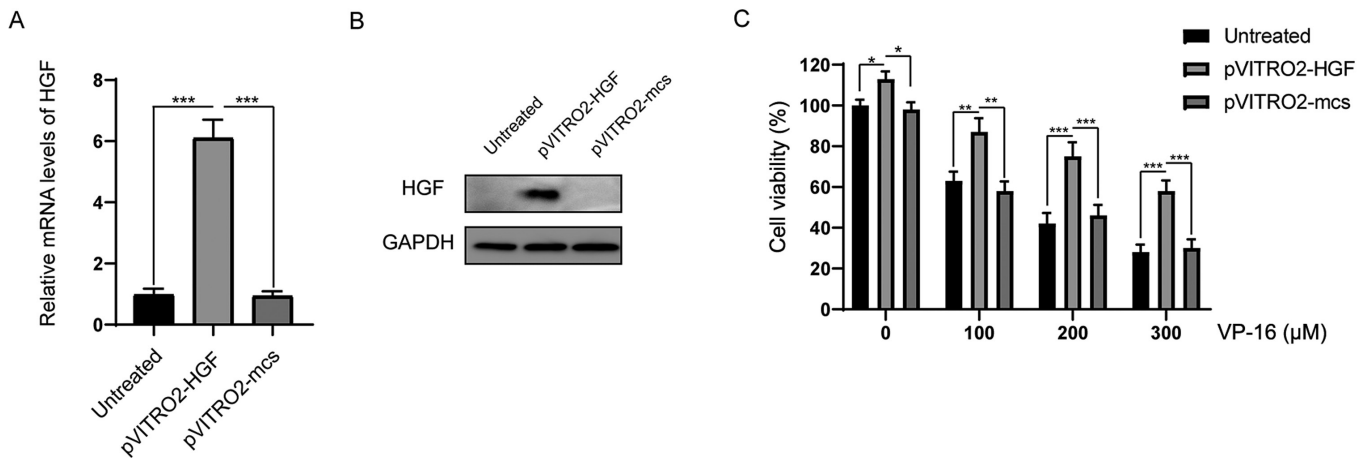


Figure 1. Overexpression of HGF inhibits the cytotoxicity induced by VP-16 in the K562 cells. The K562 cells were transfected with pVITRO2-HGF or pVITRO2-mcs for 24 h, and (A) mRNA and (B) protein expression levels of HGF were analyzed using reverse transcription-quantitative PCR and western blot analysis, respectively. (C) The K562 cells were transfected with pVITRO2-HGF or pVITRO2-mcs for 24 h, treated with various doses of VP-16 for another 48 h, then cell viability was analyzed. The data are shown as the mean and SD from three independent experiments, performed in triplicate. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. HGF, hepatocyte growth factor; mcs, mock.

resuspended in binding buffer, containing Annexin V and PI. The apoptosis rate was measured using an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Inc.) within 30 min of staining. The results were analyzed using the FlowJo v10.0 software (FlowJo, LLC).

Caspase activity assay. The activity level of caspase-3 and -9 was analyzed using the Caspase-3 and Caspase-9 Activity Assay kit (Fluorometric; Abcam) according to the manufacturer's instructions. After treatment by VP-16 or transfection, 100 μ l substrate was added to each well and the samples were incubated for 1 h at room temperature. Luminescence at 405 nm was measured using a BioTek 312e microplate reader (BioTek Instruments, Inc.).

Statistical analysis. All statistical analyses were conducted using SPSS v20.0 software (IBM Corp). All measurement data are presented as the mean $\pm$ SD. Multiple groups were analyzed using one-way ANOVA followed by the Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of HGF protects the K562 cells from cytotoxicity induced by VP-16. To investigate the protective role of HGF, the K562 cells were transfected with pVITRO2-HGF or pVITRO2-mcs (control). As indicated in Fig. 1A, after transfection for 24 h, the mRNA expression level of HGF was successfully upregulated in the pVITRO2-HGF transfection group compared with that in the pVITRO2-mcs and untreated groups. Western blot assays confirmed that the protein expression level of HGF was markedly increased following transfection with pVITRO2-HGF (Fig. 1B). Next, it was investigated whether the increase in HGF expression could affect the response of the K562 cells to VP-16. The K562 cells were treated with different concentrations of VP-16 (0, 100, 200 and 300 μ M) for 48 h, then the cell viabilities

were analyzed. As shown in Fig. 1C, overexpression of HGF significantly enhanced cell survival following treatment with VP-16.

Overexpression of HGF reduces apoptosis induced by VP-16 in the K562 cells. Then, it was investigated whether the protective role of HGF against VP-16 developed by affecting apoptosis in the K562 cells. Annexin V staining revealed that in the presence of VP-16 (300 μ M) for 24 h, the control group (without transfection) or cells transfected with pVITRO2-mcs had apoptosis rates of 20.8 ± 3 and $24.6\pm1.9\%$, respectively. Furthermore, overexpression of HGF significantly decreased apoptosis induced by VP-16 to $12.0\pm1.4\%$ (Fig. 2A). Consistent with the results from the apoptosis assay, the caspase activities also revealed that the activation of caspase-3/-9 induced by VP-16 was significantly inhibited following overexpression of HGF (Fig. 2B). In addition, western blot analysis showed that the cleavage of caspase-3/-9 was significantly inhibited by the overexpression of HGF (Fig. 2C). Taken together, these results indicate that HGF confers protection against VP-16-induced apoptosis via the regulation of caspase-3/9 activation.

Overexpression of HGF affects the expression level of Bcl-2 proteins. Since apoptosis can be regulated by the Bcl-2 protein family, it was investigated whether HGF could affect the expression level of the Bcl-2 protein family. First, the mRNA expression level of Bcl-2 and Bax was analyzed using RT-qPCR. The results revealed that VP-16 treatment led to the decrease of Bcl-2 mRNA expression level and the increase in the mRNA expression level of Bax in the pVITRO2-mcs and untreated groups compared with that in the pVITRO2-HGF transfection group (Fig. 3A). Notably, overexpression of HGF reversed the effects of VP-16 on the mRNA expression levels of both Bcl-2 and Bax (Fig. 3A). Western blot analysis demonstrated similar effects (Fig. 3B). To further investigate the role of the Bcl-2 proteins in the protective role of HGF, siRNA was used to knockdown the expression level of Bcl-2 (Fig. 3C). According to the results, the apoptosis induced by VP-16 was

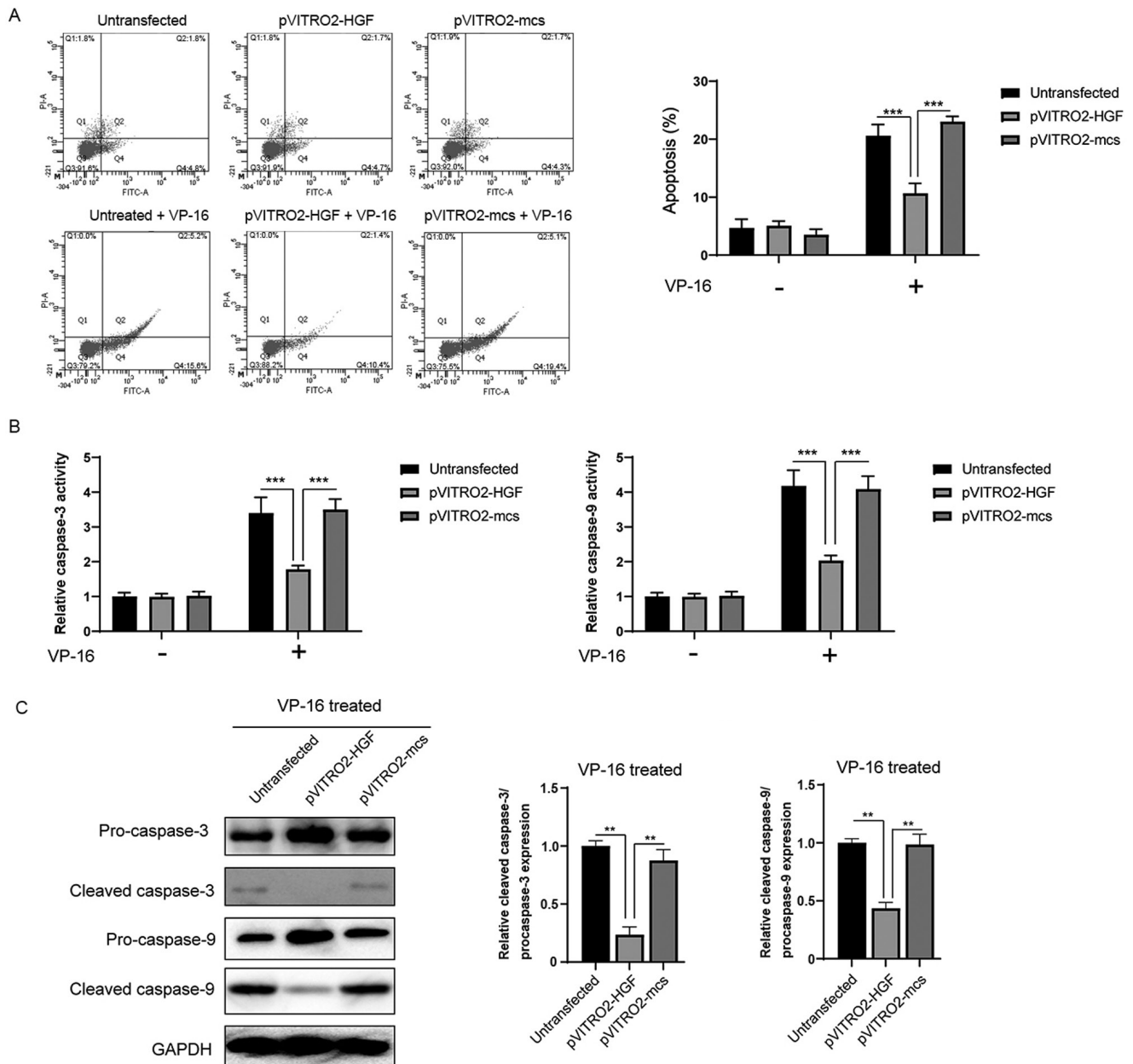


Figure 2. Overexpression of HGF inhibits apoptosis induced by VP-16 in the K562 cells (A) The K562 cells were treated as indicated for 24 h, then apoptosis was measured using Annexin-V staining and flow cytometry. (B) The K562 cells were treated as indicated, then caspase-3/-9 activities were analyzed using a colorimetric assay kit. (C) The K562 cells were treated as indicated for 24 h, then the protein expression level of caspase-3/-9 was analyzed using western blot analysis. The data are shown as the mean and SD from three independent experiments, performed in triplicate. ** $P < 0.01$, *** $P < 0.001$. HGF, hepatocyte growth factor; mcs, mock.

significantly enhanced in pVITRO2-HGF + si-Bcl-2 group compared with pVITRO2-HGF or pVITRO2-HGF + si-Ctrl group (Fig. 3D). At the same time, Bax was overexpressed in the K562 cells following transfection with pcDNA3.1 Bax plasmid (Fig. 3E). Compared with pVITRO2-HGF group or pVITRO2-HGF + pcDNA3.1 group, the VP-16-induced apoptosis was further enhanced in case of high expression of Bax in K562 cells (Fig. 3F). Taken together, these findings revealed that HGF exerts its protective effects at least partly via the regulation of Bcl-2 and Bax.

Overexpression of HGF leads to activation of the PI3K/Akt signaling pathway. To further investigate the

possible mechanisms involved in the protective role of HGF, it was investigated whether HGF affects the activation of the PI3K/Akt pathway. In the presence of VP-16, overexpression of HGF resulted in the activation of the PI3K/Akt signaling pathway compared with that in cells transfected with pVITRO2-mcs (Fig. 4A). LY294002 was used to further investigate the role of the PI3K/Akt signaling pathway (Fig. 4B). As indicated in Fig. 4C, inhibition of the pathway abrogated the protective effects of HGF against VP-16. Furthermore, inhibition of PI3K/Akt signaling enhanced the cleavage of caspase-3/-9 (Fig. 4D). The effects of HGF overexpression on the protein expression level of Bcl-2 and Bax were also reversed following inhibition of the PI3K/Akt

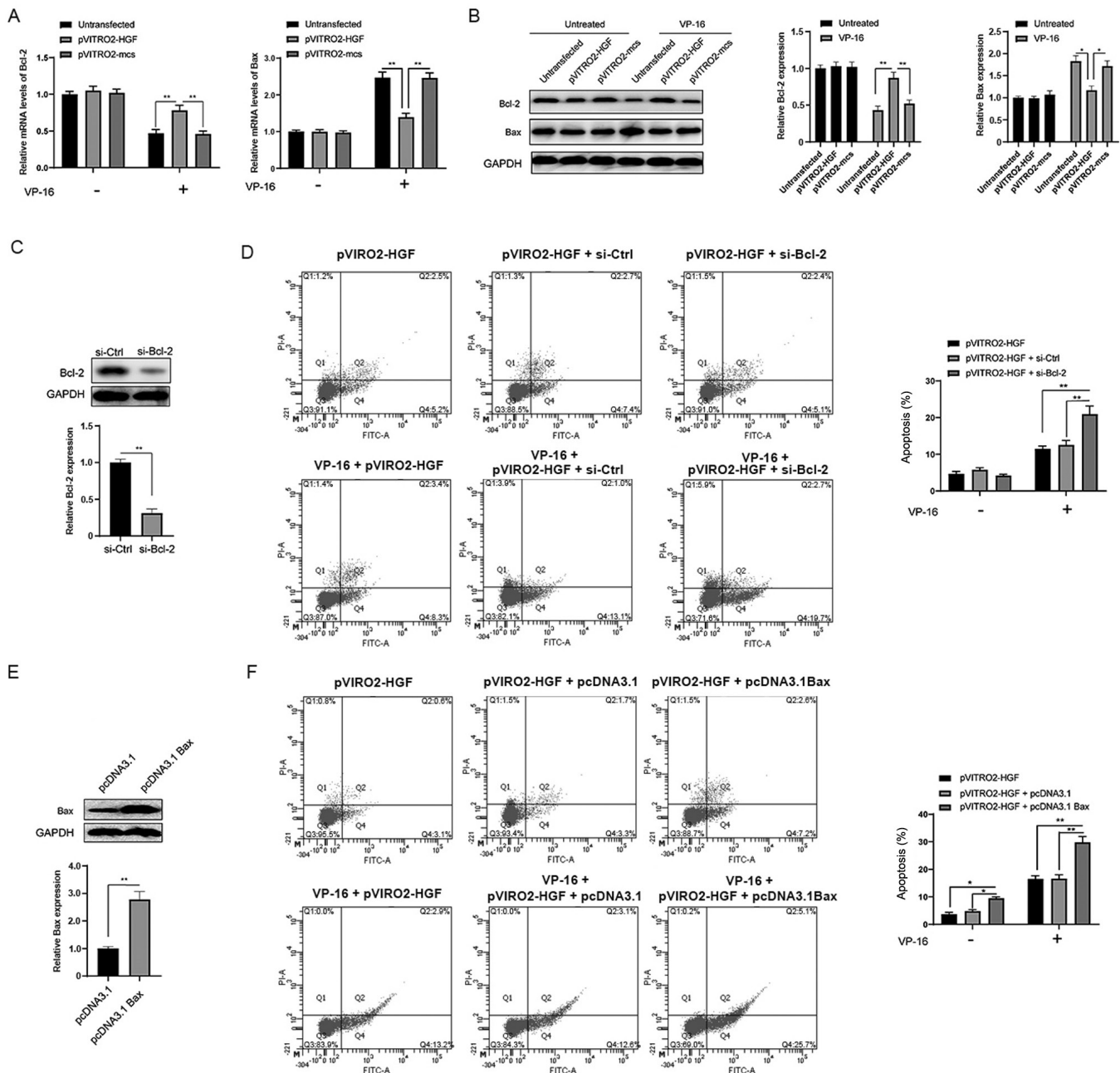


Figure 3. Overexpression of HGF affects the expression level of Bcl-2 proteins in the K562 cells. The K562 cells were treated as indicated for 24 h, then the (A) mRNA and (B) protein expression levels of Bcl-2 and Bax were analyzed using reverse transcription-quantitative PCR. (C) The K562 cells were transfected with siRNA targeting Bcl-2 for 24 h, then treated with or without VP-16 for another 24 h. The protein expression levels of Bcl-2 and rate of apoptosis was analyzed. (D) The K562 cells were transfected as indicated for 24 h, then treated with or without VP-16 for another 24 h. The protein expression levels of Bax and the rate of apoptosis were analyzed. The data are shown as the mean and SD from three independent experiments, performed in triplicate. * $P < 0.05$, ** $P < 0.01$. HGF, hepatocyte growth factor; mcs, mock; si, small interfering; ctrl, control.

signaling pathway (Fig. 4E). Taken together, these findings indicate that overexpression of HGF leads to the activation of the PI3K/Akt signaling pathway, which is essential for the protective role of HGF against VP-16-induced apoptosis in the K562 cells.

Discussion

CML is a malignant myeloproliferative disease, that occurs in pluripotent hematopoietic stem cells and is the third most common type of leukemia worldwide (14). Significant progress

has been made over the past few decades; however, there is still a lack of satisfactory treatment for CML.

In the present study, to the best of our knowledge, for the first time the role of HGF in the drug resistance of the K562 cells was analyzed, and the potential mechanisms were also investigated. It was found that overexpression of HGF significantly inhibited the cytotoxicity of VP-16 in the K562 cells. Further analysis into the molecular mechanism revealed that overexpression of HGF inhibited VP-16-induced apoptosis. Overexpression of HGF prevented the activation of caspase-3 and -9. The results from the present study are

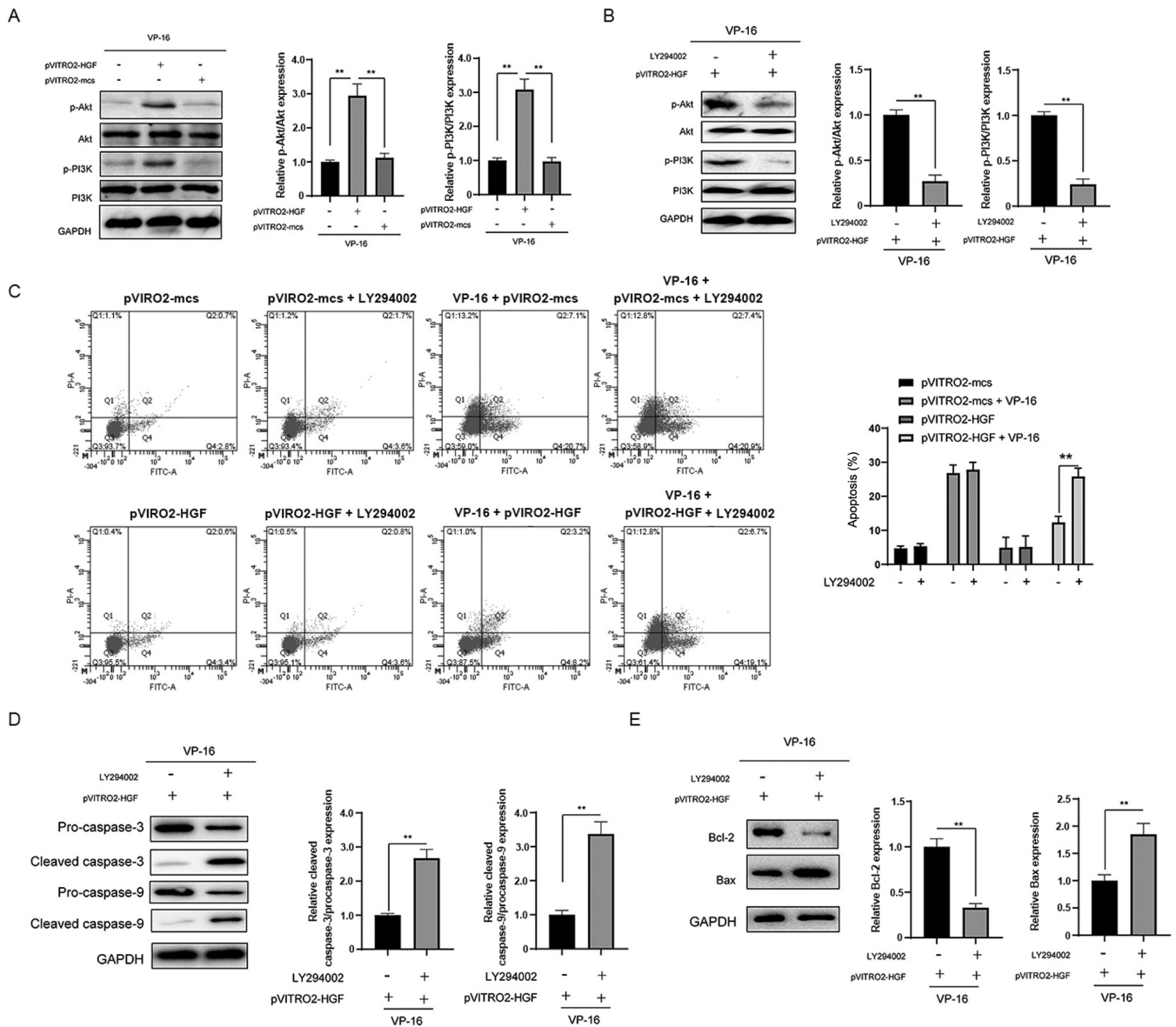


Figure 4. Overexpression of HGF leads to the activation of the PI3K/Akt signaling pathway in K562 cells. (A) The cells were transfected as indicated for 24 h, then treated with VP-16 for another 24 h. The expression level of proteins in the PI3K/Akt signaling pathway was analyzed using western blot analysis. (B) The cells were transfected with pVITRO2-HGF for 24 h, then treated with or without LY294002. The cells were treated with VP-16 for another 24 h, then the expression level of proteins in the PI3K/Akt signaling pathway was analyzed using western blot analysis. (C) The cells were transfected as indicated for 24 h with or without LY294002, then treated with or without VP-16 for another 24 h. Apoptosis was subsequently analyzed. The cells were transfected with pVITRO2-HGF for 24 h, treated with or without LY294002, treated with VP-16 for another 24 h, then the expression level of (D) caspase-3/-9 and (E) Bcl-2 and Bax was analyzed using western blot analysis. The data are shown as the mean and SD from three independent experiments, performed in triplicate. ** $P < 0.01$. HGF, hepatocyte growth factor; p, phosphorylated; mcs, mock.

consistent with previous studies, that also found that HGF inhibited caspase activation in hepatocytes and human proximal tubular epithelial cells (15,16). Notably, another study reported that HGF was able to activate the apoptosis signaling pathway by increasing caspase-3 activity in sarcoma cells (17). This discrepancy might be due to the different cell types and/or treatments used, and further investigation is required to evaluate the role of HGF in the progression of apoptosis.

It is well-known that there are two apoptotic pathways, namely, the extrinsic and intrinsic pathways (11). The extrinsic and intrinsic pathways are initiated by caspase-8 and -9, respectively, with both ultimately leading to the

activation of caspase-3 (11). As caspase-3/-9 activation was analyzed in the present study, the intrinsic pathway was triggered. It is also well-known that the intrinsic apoptosis pathway is subjected to the regulation of the Bcl-2 family of proteins (18). Therefore, it was investigated whether HGF could affect the Bcl-2 proteins. It was found that overexpression of HGF led to the increase in protein expression level of Bcl-2 and the decrease in Bax protein expression level. The results from the present study are consistent with previous studies that reported that HGF enhanced the protein and mRNA expression levels of Bcl-2 and inhibited the activation of Bax (15,19). Bcl-2 proteins are promising targets for the treatment of hematologic malignancies, and numerous small

molecule inhibitors have been designed to target the Bcl-2 proteins (20).

The PI3K/Akt pathway is activated in a wide variety of hematological malignancies, such as CML, acute myeloid leukemia, diffuse large B-cell lymphoma and chronic lymphoblastic leukemia (21). By promoting proliferation and/or inhibiting apoptosis, the PI3K/Akt pathway is considered vital for tumorigenesis (21), and several studies have suggested that targeting the PI3K/Akt pathway could overcome chemoresistance in CML cells (22,23). It was found that the resistant CML cell line K562/ADM presented higher PI3K/Akt activity compared with that in a sensitive cell line (23). In the present study, to understand why HGF overexpression attenuated VP-16-induced apoptosis, the expression level of proteins in the PI3K/Akt signaling pathway was further investigated. Notably, overexpression of HGF activated the PI3K/Akt signaling pathway. This finding is in accordance with previous studies, indicating that HGF could induce the activation of the PI3K/Akt signaling pathway (24,25). These results indicate that the HGF-mediated K562 cell response to VP-16 is, at least in part, PI3K/Akt dependent.

In conclusion, it was found that overexpression of HGF conferred resistance to VP-16 in the K562 cells. The inhibition of apoptosis caused by HGF overexpression was associated with suppression of caspase activation. Furthermore, the association between HGF and the PI3K/Akt signaling pathway were further elucidated, with the conclusion that overexpression of HGF could activate this pathway. These initial findings are promising; however, further comprehensive investigations are still required, such as animal models. Overall, targeting HGF could be a potential therapeutic target to overcome chemoresistance in the human CML K562 cells.

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Availability of data and materials

The datasets used and/or analyzed are available from the corresponding author upon reasonable request.

Authors' contributions

DC designed the experiments and revised the manuscript. XZ performed the experiments and wrote the manuscript. SH, HZ and ZG contributed to acquisition and analysis of data. XZ and DC confirmed the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and patients consent

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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