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Upregulation of KCNMA1 facilitates the reversal effect of verapamil on the chemoresistance to cisplatin of esophageal squamous cell carcinoma cells

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Abstract. – OBJECTIVE: This study aimed to investigate the reversal effect of verapamil (VER) on the chemoresistance to cisplatin of esophageal squamous cell carcinoma (ESCC) cells.

PATIENTS AND METHODS: The reversal effect of VER on cisplatin resistance in ESCC cells was evaluated via CCK-8 assay, colony formation assessment, and flow cytometry. The key genes that mediate this effect were screened via high-throughput transcriptome se¬quencing. The mRNA and protein expression levels of potassium calcium-activated channel subfamily M alpha 1 (KCNMA1) in ESCC cells were examined via quantitative real-time PCR and Western blot analysis, respectively. The protein expressions of KCNMA1 in tissue samples from patients with either positive or negative responses to the therapeutic regimen of VER were determined via immunohistochemistry assay. Cell models with KCNMA1 knockdown and overexpression were es¬tablished to examine the role of KCNMA1 in mediating the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells.

RESULTS: Results revealed that VER significantly decreased the 50% inhibitory concentration of cisplatin, inhibited colony formation, and induced apoptosis in ESCC cells. The curative effects of VER combined with chemotherapeutic drugs in KCNMA1-positive patients were better than those in KCNMA1-negative patients. KCNMA1 upregulation enhanced the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells.

CONCLUSIONS: KCNMA1 facilitated the reversal effect of VER on cisplatin resistance in ESCC cells.

Key Words:
Verapamil, Reversal, Chemoresistance, Cisplatin, ESCC, KCNMA1.

Introduction

Esophageal carcinoma is a common malignant solid tumor with an estimated 570,000 new cases and 500,000 deaths in 2018, making it the leading cause of cancer-related deaths worldwide[1]. Esophageal squamous cell carcinoma (ESCC) is the major type of this disease in China[2]. The curative effects of recent advances in diagnostics and therapeutics on most patients did not achieve substantial improvements, and the 5-year survival rate is only approximately 15%-25%[3,4]. Currently, preoperative chemoradiotherapy with cisplatin and 5-fluorouracil is the standard treatment for advanced resectable ESCC, but the inherent or acquired chemoresistance is a major obstacle in ESCC treatment[5,6]. Therefore, exploring novel drugs or approaches for overcoming cisplatin resistance is imperative to improve the clinical outcomes for patients with ESCC.

Verapamil (VER) is an L-type calcium channel inhibitor mainly used for the treatment of cardiovascular diseases. However, recent studies demonstrated that it exerts reversal effects on multidrug resistance (MDR) in various human cancer cells[7-10]. An *in vitro* study showed that its effective dosage for reversing tumor MDR is 6.0-10.0 μmol/L[11]. The intravenous infusion may not only fail to achieve effective concentration but also lead to cardiovascular side effects, thereby limiting its applications in reversal agents of chemoresistance in clinical practice[12]. The concentration of drugs administered via arterial infusion in local tissues may reach 3-10-fold higher than that in peripheral

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blood[13]. In our previous clinical studies, we observed that arterial infusion of chemotherapy drugs combined with VER can improve the clinical efficacy of chemotherapy in patients with hepatocellular carcinoma[12], colorectal cancer[14], gastric cancer[15], lung cancer[16], and malignant ascites[17]. However, the role of VER in reversing chemoresistance in and the underlying molecular mechanism by which this drug exerts this effect on esophageal cancer remain unclear and thus must be investigated.

Previous studies suggested that VER is a P-glycoprotein inhibitor that can block drug efflux and improve the killing effect of chemotherapeutic drugs. Zhang et al[18] proved that its pharmacological effects are largely independent of P-glycoprotein; therefore, the detailed mechanism remains vague. In the present study, we evaluated the effects of VER on the reversal of cisplatin resistance in ESCC cells via cell viability analysis, clone formation, and apoptosis assays. We screened several target genes that may mediate this effect via high-throughput transcriptome sequencing. Among the best candidate genes was potassium calcium-activated channel subfamily M alpha 1 (KCNMA1). Thus, we investigated and validated its role in mediating VER reversal of cisplatin resistance in ESCC cells.

Patients and Methods

Patients and Tumor Samples

A total of 94 patients who agreed to the therapy in this study were selected between January 2008 and January 2014. The diagnosis of ESCC and the inclusion and exclusion criteria for patients with this disease followed the guidelines of the National Comprehensive Cancer Network. Patients were included on the basis of the following criteria: the patient had Karnofsky performance score of ≥80, 18-80 years old, expected survival time of >3 months, the heartbeat of ≥60 times/min, and voluntarily accepted the therapy and signed informed consent to participate in the combined treatment of VER with the arterial infusion. The diagnosis of ESCC was confirmed via esophageal barium meal X-ray, esophageal endoscopy, and CT examination and pathology. The patients had no application constraints for VER, and the feeding artery of the malignant tumors were accessed using a catheter to evaluate treatment efficacy. Participants were excluded on the basis of the following criteria: had undergone surgery; pregnant and lactating; with mental illness and mental retardation; with acute infections; with central nervous system symptoms; with atopy; WBC $<4.0\times10^9$ /L, BPC $< 10.0\times10^9$ /L, and Hb <60 g/L; and with disturbance of blood coagulation. Moreover, participants were excluded if therapeutic schemes could not be strictly enforced, could not tolerate the tests due to serious adverse reactions, and if their adverse reactions and efficacy could not be assessed because of incomplete information. Furthermore, participants with contraindications to cisplatin, lobaplatin, and 5-fluorouracil were excluded.

The treatment strategy consisted of 25 mg VER, 40-50 mg/m^2 cisplatin, and 1000 mg/m^2 5-fluorouracil. The curative effects of this strategy on ESCC were evaluated on the basis of the following clinical parameters: clinical cure or significant improvement (CR) was defined as tumor disappeared, metastatic lesions disappeared, metastatic lymph nodes completely disappeared, esophagus recovered to a normal state, and these symptoms were sustained for more than 1 month; improvement (PR) was defined as tumor lesion shrunk by over 50% or no new lesions or metastatic lesions formed, lymph nodes decreased by over 50%, and these symptoms were sustained for more than 1 month; stability (SD) was defined as tumor lesion shrunk by less than 50%, whereas the tumor increased by less than 25%, and these symptoms were sustained for more than 1 month; and progressive or worsening (PD) was defined as tumor lesions increased by over 25% or new lesions appeared. This study was approved by the Ethics Committee of The First Affiliated Hospital of the University of Science and Technology of China. Clinicopathological characteristics were evaluated, and all samples were confirmed by pathological analysis.

Chemicals and Reagents

Cisplatin (No. 15663-27-1) and Cell Counting Kit 8 (CCK-8, No. HY-K0301) were purchased from MedChem Express (Monmouth Junction, NJ, USA). VER (No. 152-11-4) was obtained from Topscience Co., Ltd. RNA extraction kit (DP451), reverse transcription kit, and 2 × SYBR Green Universal qPCR Master Mix (FP209-02) were acquired from Tiangen Biotech Co., Ltd. (Beijing, China). KCNMA1 antibody (ab192759) was bought from Abcam (Cambridge, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (sc-47724) was procured

from Santa Cruz Biotechnology (Santa Cruz, CA, USA). KCNMA1 siRNA and negative control were obtained from Ruibo Company (Guangzhou, Guangdong, China). KCNMA1 expression plasmid (RC215099) was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). Annexin V-FITC/PI kit (G003-1-2) was provided by Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Cell Lines and Culture Condition

The human ESCC cell lines KYSE150, KYSE180, and KYSE450 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100 U/mL penicillin sodium, and 100 μg/mL streptomycin at 37 °C, 5% CO_2, and saturated humidity.

Cell Viability Analysis

The ESCC cells (5×10^3) were seeded into 96-well plates in triplicates overnight and then treated with 0–40 μg/mL cisplatin and 4.91μg/mL VER for 48 h. CCK-8 was added to the wells (10 μL per well). After 2 h of incubation at 37 °C in 5% CO_2, the optical density (OD_{450}) of each well was determined using a Tecan Infinite M200 multimode microplate reader (Tecan, Basel, Switzerland). All experiments were performed in triplicate and repeated over three times, and the average of the results was calculated and used for each data point.

Colony Formation Assay

The ESCC cells were seeded into 6-well plates overnight and then treated with cisplatin and VER. After the cells were incubated for 9 days, the cells were washed with PBS and fixed by methanol for 10 min at room temperature. Crystal violet staining buffer (C0121, Beyotime, Shanghai, China) was then added into the plates and incubated at 37 °C for 30 min. The crystal violet staining buffer was subsequently removed, and the plates were washed with deionized water and dried at room temperature. Finally, the colonies in each plate were counted.

High-Throughput Transcriptome Sequencing

The cells were grouped into a negative control (NC) group, a VER alone (VER alone) group, cisplatin alone (DDP) group, and a cisplatin combined with VER (DDP+VER) group. Based on the toxicity of the drugs and the results of follow-up tests, the dose of cisplatin was chosen as half of the concentration of 50% inhibitory concentration (IC_{50}) in the cell lines. The dose of cisplatin was 10.0 μg/mL (IC_{50}: 20.96 μg/mL) in KYSE150 and 5.0 μg/mL (IC_{50}: 10.77 μg/mL) in KYSE180 cells, whereas that of VER was 4.91 μg/mL . Total RNA was extracted and quantified after the cells were incubated with the drugs at 37 °C for 24 h. High-throughput sequencing was conducted using the Illumina Hiseq 2000 sequencing platform (RuiboBio, Guangzhou, China). The method for screening differentially expressed genes adopted herein was the same as that in our previous studies[11,18].

Cell Transfection

KCNMA1 shRNA lentiviral transduction particles (KCNMA1 shRNA sequence: CCGGCCCAATAGAATCCTGCCAGAACTCGAGTTCTGGCAGGATTCTATTGGGTTTTT) and a negative shRNA control were obtained from Sigma-Aldrich (St. Louis, MO, USA). The KCNMA1 overexpression plasmid (PCMV6-AC-GFP) and an empty vector (PCMV6-Entry) were purchased from Origene Inc. (Rockville, MD, USA). The ESCC cells were seeded in 6-well plates and grown overnight to 80% confluence. Subsequently, the cells were transfected with shRNA-KCNMA1 or PCMV-KCNMA1 by using an Amaxa Nucleofector (Amaxa, Koeln, Germany) according to the manufacturer's instruction. Knockdown and overexpression efficiency was determined via Western blot assay.

Quantitative Real Time-PCR Assay

Total RNA extraction and quantification were conducted following the manufacturer's instructions. RNAs were reverse-transcribed into cDNA by using the PrimeScript™ RT reagent kit. Primers were designed using the Primer 5.0 software (Table I). The reaction system was prepared as follows: 12.5 μL of 2 × SYBR Green Universal qPCR Master Mix, 1.5 μL of each primer, 3 μL of cDNA, and distilled water were added to a final volume of 25 μL. The PCR was denatured at 95°C for 1 min and then amplified for 40 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 40 s on a StepOne™ RT-qPCR system. The mRNA levels of candidate genes were normalized to GAPDH and evaluated *via* the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate and repeated three times.

Table I. Primers used for qRT-PCR analysis.

Gene	Forward primer	Reverse primer
SLIT3	AGCGCCTTGACCTGGACA	TCGGCGTGCTCTGGAAAA
KCNMA1	CGAGGATGAAGAAGACCATGA	GGTTCATCCATTTGGTGGAG
NID1	TTATCCCCCTCCATCACTCA	TTATCCCCCTCCATCACTCA
KLK1	GCTCTGTACCATTTCAGCAC	GCTGTGTTTTCGTCGTCAAA
LPAR1	CTTTGCTGGGTTGGCCTACTT	GCCATGTGCTAACAGTCAGTCT
CHRDL1	CCTGGAACCTTATGGGTTGGT	AACATTTGGACATCTGACTCGG
GAPDH	ATCTCTGCCCCCCTGCTGA	GATGACCTTGCCCACAGCCT

Western Blot Assay

The total proteins of KYSE150 and KYSE180 cells were extracted using the EpiQuik Whole Cell Extraction Kit according to the manufacturer's protocol. The proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking with 5% nonfat dry milk for 1 h, the primary antibody (GAPDH, 1:1000; KCNMA1, 1:300) was added to incubate overnight at 4°C. The PVDF membranes onto which the proteins were transferred were washed with phosphate-buffered saline with Tween 20 (PBST) four times (10 min each time), incubated with a secondary antibody (1:5000) for 2 h, and washed with PBST three times (10 min each time). Protein bands were visualized using an enhanced chemiluminescence kit and quantified by densitometry by using the Image J software.

Apoptosis Analysis

The ESCC cells were cultured at a density of 3×10^5 cells/well in 6-well plates for 24 h and then incubated with cisplatin, VER, or vehicle (PBS) for 48 h. The cells were harvested using 0.25% trypsin, washed twice with cold PBS, and then resuspended in 300 μL of cold binding buffer. Both 3 μL of Annexin V-FITC and 2.25 μL of propidium iodide (PI) were added to stain at room temperature in the dark. The percentage of apoptotic cells was analyzed by flow cytometry.

Immunohistochemical Analysis

Paraffin-embedded sections of ESCC tissues were routinely processed by dewaxing hydration. The tissue slides were heated in citrate buffer for antigen retrieval. The slides were then incubated with KCNMA1 antibody overnight at 4°C, followed by goat serum blocking, SP immunohistochemical staining, DAB staining, and hematoxylin counterstaining. Known ESCC-positive sections served as a positive control, and PBS instead of the primary antibody served as a negative control. Semiquantitative results were used to determine the average optical density (IOD/area, density mean) of the positive expression area by using Image-Pro Plus 6.0 as in our previous study[11]. The average optical density values of each experimental group were calculated and statistically analyzed.

Statistical Methods

The IC_{50} of cisplatin to ESCC cells, colony number, KCNMA1 expression, apoptosis level, and other continuous variables were expressed as the mean ± standard deviation (SD). One-way ANOVA or Mann–Whitney U test was performed using Prism (Prism 5.0, GraphPad Inc., La Jolla, CA, USA) or SPSS software (version 24.0; IBM SPSS, Armonk, NY, USA) for data analysis. *p*-values less than 0.05 were considered statistically significant.

Results

VER Reverses Chemoresistance to Cisplatin of ESCC Cells

The effects of cisplatin on the cell viability of the ESCC cells were detected *via* CCK-8 assay. Results showed that the IC_{50} of cisplatin to KYSE150, KYSE180, and KYSE450 cells was 20.96, 10.77, and 8.60 μg/mL, respectively (Figure 1A). After VER (4.91 μg/mL) treatment, the IC_{50} of cisplatin to KYSE-150, KYSE180, and KYSE450 cells decreased to 5.67, 9.57, and 5.40 μg/mL, respectively ($p < 0.01$, Figure 1B). In the clone formation assay, the number of clones in the KYSE150 DDP+VER group was significantly fewer than that in the KYSE-150 DDP group ($p<0.01$) (Figures 1C and 1D). These results suggested that VER increased the sensitivity of cisplatin to ESCC cells, especially to KYSE150

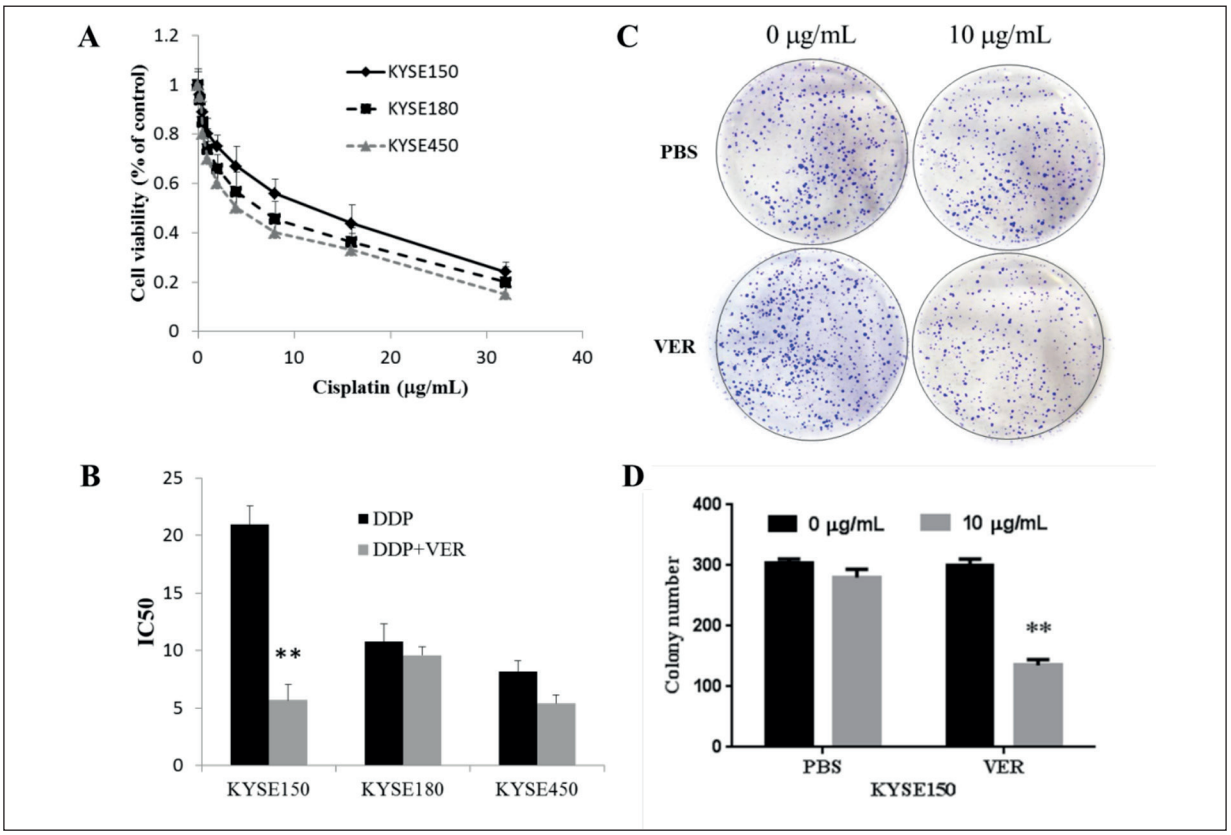

Figure 1. Evaluation of VER reversal of chemoresistance to cisplatin of ESCC cells. **A,** ESCC cells were treated with different cisplatin concentrations (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, and 32 μg/mL) for 48 h. Cell viability was measured via CKK-8 assay. Three independent experiments are shown as the mean ± SD. **B,** ESCC cells were treated with 4.91μg/mL VER and then with different cisplatin concentrations. Cell viability was measured via CKK-8 assay. Data are shown as the mean ± SD. $^{**}p<0.05$ versus the KYSE150 DDP group. **C,** KYSE150 cells were treated with 10.0 μg/mL cisplatin with or without VER. Colonies were stained with crystal violet, and images were obtained at 200× magnification. **D,** The number of colonies in each group was calculated and shown as the mean ± SD. $^{**}p<0.01$ versus the VER + 0.0 μg/mL cisplatin group.

cells. Afterward, we selected KYSE150 cells as the VER reversal-sensitive and KYSE180 cells as the reversal-resistant model cells for further study.

High-Throughput Transcriptome Sequencing Screens Differentially Expressed Genes in ESCC Cells

VER reportedly exerts remarkable reversal effects on HCC cells to doxorubicin[18]. However, the reversal effect of VER on ESCC and the underlying mechanism by which this drug exerts this effect remain unknown. In the present study, high-throughput transcriptome sequencing based on Illumina technology was performed to screen candidate genes that can potentially mediate this reversal effect. The expression levels of numerous genes substantially changed after cisplatin and VER treatments (Figure 2A). Among these genes, several differentially expressed candidate genes in ESCC cells, namely, SLIT3, KCNMA1, NID1, KLK1, LPAR1, and CHRDL1, were chosen and validated via qRT-PCR. As shown in Figure 2B, KCNMA1 expression was significantly upregulated in the KYSE150 DDP+VER group compared with that in the KYSE150 DDP group ($p<0.01$). Moreover, KCNMA1 expression level in KYSE150 cells was higher than that in KYSE180 cells. Furthermore, KCNMA1 expression was upregulated in both KYSE150 and KYSE180 cells after VER and cisplatin treatments (Figure 2C). These results suggested that KCNMA1 may mediate the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells.

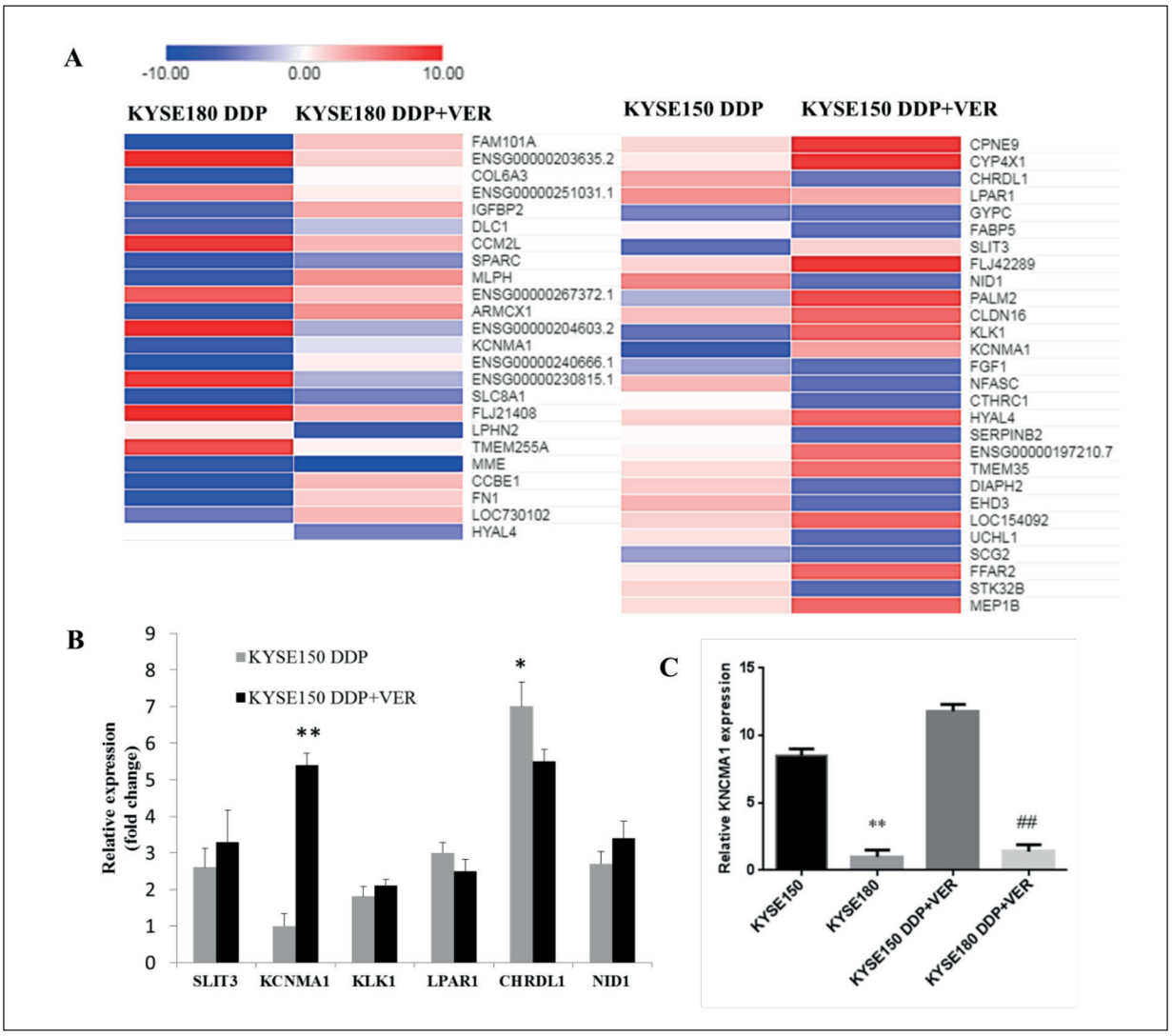

Figure 2. High-throughput sequencing and validation of differentially expressed genes associated with the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells. **A,** High-throughput transcriptome sequencing identifies the differentially expressed genes related to the reversal effect of VER on the chemoresistance to cisplatin of KYSE150 and KYSE180 cells treated with cisplatin and/or VER. **B,** The expression of candidate genes in KYSE150 cells was validated *via* qRT-PCR. *p<0.05; **p< 0.01 versus the KYSE150 DDP group. **C,** The relative KCNMA1 expression in KYSE150 and KYSE180 cells was examined via qRT-PCR. **p<0.01 versus the KYSE150 group, $^{\#\#}p$<0.01 versus the KYSE150 DDP+VER group.

Western Blot Assay Detects the Protein Expression of KCNMA1 in ESCC Cells

The protein expression of KCNMA1 in KYSE150 and KYSE180 cells was further measured to confirm its role in mediating the reversal effect of VER on the chemoresistance to DDP of ESCC cells. The protein expression of KCNMA1 did not show significant differences between the KYSE180 DDP group and the KYSE180 DDP+VER group, whereas KCNMA1 was significantly upregulated in the KYSE150 DDP+VER group (p<0.05). Therefore, we postulated that KCNMA1 may mediate this reversal effect (Figures 3A and 3B).

Protein Expression of KCNMA1 in the Tissue Samples from Patients as Detected by Immunohistochemistry Assay

A total of 94 patients who volunteered to accept therapy in this study were selected between January 2010 and January 2018. Among the pa-

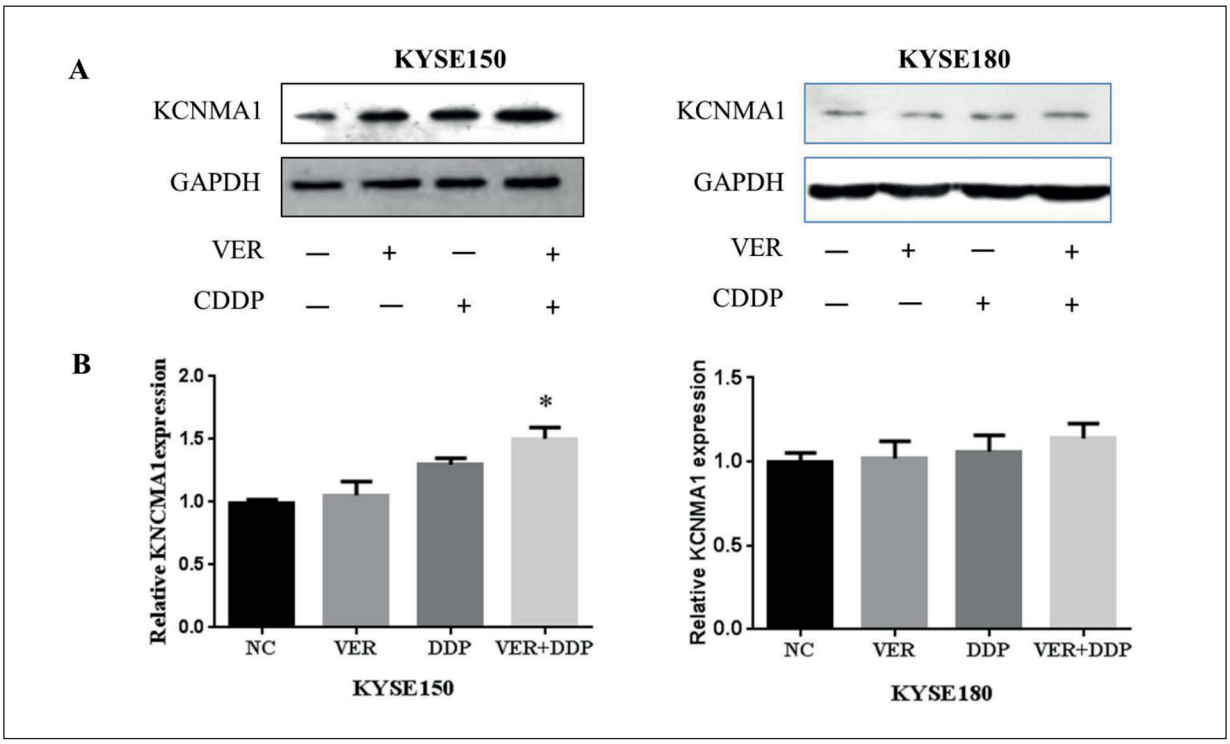

Figure 3. Protein expression of KCNMA1 in KYSE150 and KYSE180 cells treated with cisplatin and/or VER as detected by Western blot assay. *p<0.05 versus the KYSE150 DDP group.

tients, 59 were males, and 35 were females. Their age ranged from 42 to 78 years with a mean age of 60.94 years (Table II).

Targeted arterial infusion of VER combined with chemotherapeutic drugs by using the Selinger technique was conducted in the patients with intermediate or advanced stages of ESCC. Results showed that clinical staging significantly decreased, and short-term efficacy increased. Among the patients, 11 cases had complete remission (CR), 65 cases had partial remission (PR), 13 cases did not change (SD), and 5 had progressive disease (PD). The total response rate (CR+PR) was 80.85% (Figure 4A). KCNMA1 expression in the tissue samples from the patients presenting with positive (CR or PR) or negative (SD and PD) response to the reversal therapeutic regimen of VER was measured via immunohistochemistry assay (Figure 4B). Results indicated that KCNMA1 expression was significantly higher in the positive response group than that in the negative response group (Figures 4C and 4D).

Table II. Summary of clinicopathological variables.

Characteristic	Number of patients
Patients	94
< Gender	
< Male	85
< Female	9
Age (years)	42-78, median = 60.94
TNM stage	
I-II	13
III	45
IV	36
Differentiation	
Low	17
Median	51
High	26
Chemotherapy	
First	41
Non-First	53
Time of follow up (months)	n = 54, 4-31, median = 9

KCNMA1 Dictates the Reversal Effects of VER on the Chemoresistance to Cisplatin of ESCC Cells

KCNMA1-targeting siRNA or an overexpression vector was transfected into KYSE150 and KYSE180 cells to confirm further whether KCNMA1 is the critical gene that contributes to the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells. Transfection efficien-

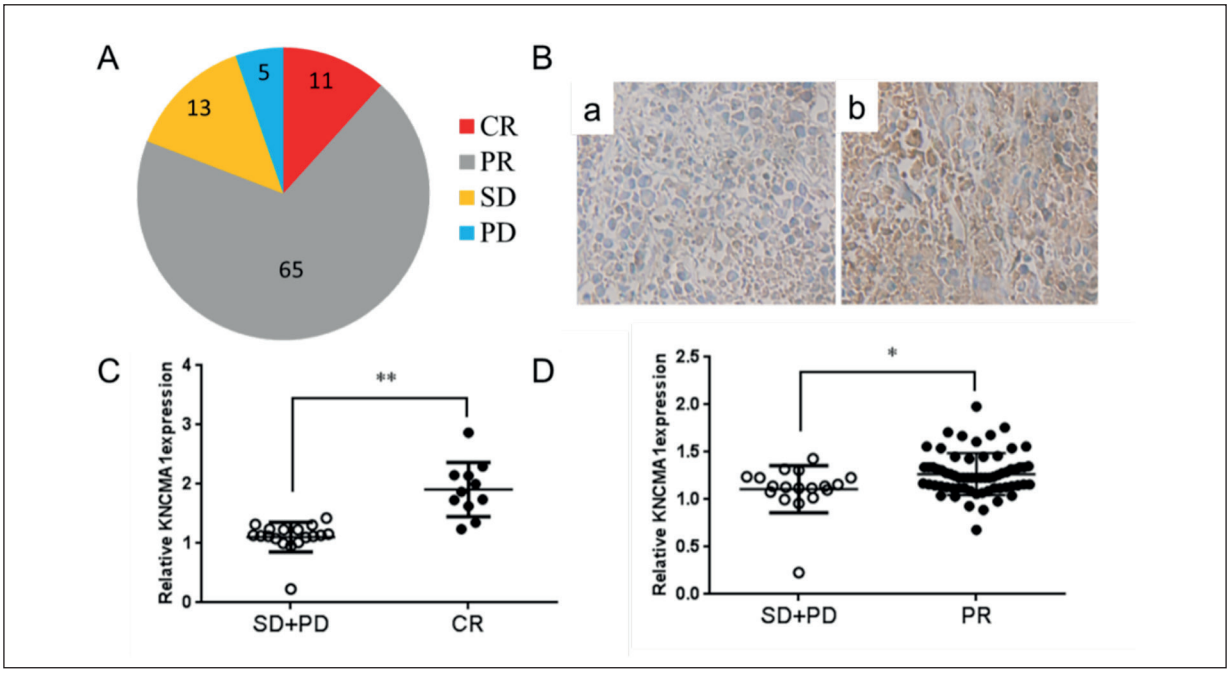

Figure 4. The expression level of KCNMA1 in the tissues of patients with ESCC was measured via immunohistochemistry assay. **A,** Results of the evaluation of the curative effects of targeted arterial infusion of VER combined with chemotherapeutic drugs on ESCC. Among the patients, 11, 65, 13, and 5 cases had CR, PR, SD, and PD, respectively. **B,** Representative images of KCNMA1 expression in the tissue samples from the patients presenting with a positive or a negative response to the reversal therapeutic regimen of VER. i. positive response, CR, 200×; ii. negative response, PD, 200×. **C,** KCNMA1 expression in the tissue samples from SD+PD and CR patients. $^{**}p<0.01$. **D,** KCNMA1 expression in the tissue samples from SD+PD and PR patients. $^{*}p<0.05$.

cy was then measured via Western blot assay. siR-KCNMA1 effectively silenced KCNMA1 expression in KYSE150 cells, and transfection of the overexpression plasmid PCMV-KCNMA1 upregulated KCNMA1 expression in KYSE180 cells (Figure 5A). IC_{50} values were then detected via CCK-8 assay. The IC_{50} of cisplatin in the siR-KCNMA1+VER group increased compared with that in the siR-NC VER group, suggesting that KCNMA1 knockdown dampened the reversal effect of VER on reversal-sensitive KYSE150 cells (Figure 5B). However, after PCMV-KCNMA1 transfection, the IC_{50} value decreased, indicating that KCNMA1 overexpression enhanced the reversal effect of VER on KYSE180 cells (Figure 5C).

KCNMA1 Enhances the Antiproliferation and Pro-Apoptotic Effects of VER on ESCC Cells

Cell proliferation in KYSE180 cells was also detected via clone formation assay with 5.0 μg/mL (about half of IC_{50}) cisplatin treatment. The number of clones was 278.7 in the PCMV-NC group (Figure 6A) and 222.7 in the PCMV-NC VER group (Figure 6B). The number of clones in the PCMV-KCNMA1 VER group was 139.0, which was fewer than that in the PCMV-NC VER group, indicating that KCNMA1 overexpression enhanced the antiproliferation effects of VER on ESCC cells. Cell apoptosis in KYSE180 cells was also measured via Annexin V-FITC/PI assay. As shown in Figure 6C, the apoptosis rate was 16.97% and 15.37% in the PCMV-NC VER and the PCMV-NC groups, respectively, but the difference was not significant. However, after KCNMA1 overexpression, the apoptosis rate increased to 23.3%, which was higher than that in the PCMV-NC VER group ($p < 0.05$). These results suggested that upregulation of KCNMA1 could enhance the pro-apoptotic effects of VER on ESCC cells.

Discussion

MDR is one of the main constraints of the clinical efficacy of ESCC chemotherapy[19,20]. P-glycoprotein, also known as ABC transporter Mdr1 (ABCB1), can induce ATP hydrolysis to

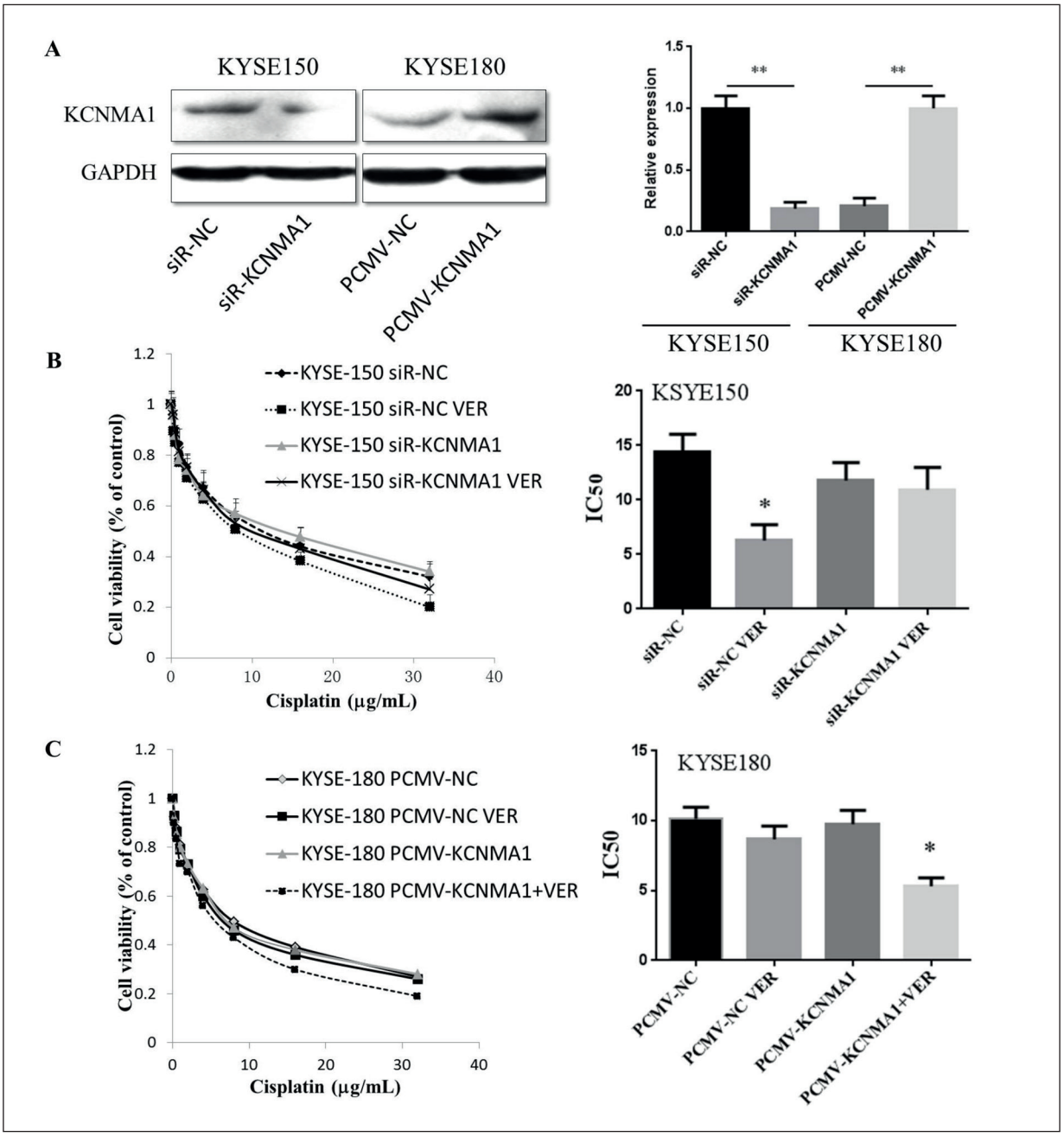

Figure 5. KCNMA1 mediates the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells. **A,** Transfecting ESCC cells with KCNMA1-targeting siRNA or an overexpression vector, a control siRNA, and Western blot assay detect KCNMA1 expression. $^{**}p < 0.01$. **B,** The IC_{50} of cisplatin against KYSE150 cells transfected with siR-KCNMA1 and siRNA control was detected via CCK8 assay. $^{*}p<0.01$. **C,** The IC_{50} of cisplatin against KYSE180 cells transfected with PCMV-KCNMA1 and PCMV control was detected via CCK8 assay. $^{*}p<0.01$.

generate ADP and release energy. Moreover, with the participation of calcium ions, it can combine with chemotherapeutic drugs within the cell to pump them out of the cells. Consequently, drug concentration within the cell declines and the toxic effect of the drug on tumors diminishes, thereby resulting in MDR[21]. VER is a calcium channel antagonist and an ABCB1 inhibitor. It can inhibit ABCB1 expression and P-glycoprotein synthesis, thereby increasing the concentration of chemotherapeutic drugs in tumor cells to overcome drug resistance[22,23]. Nevertheless, Chiu

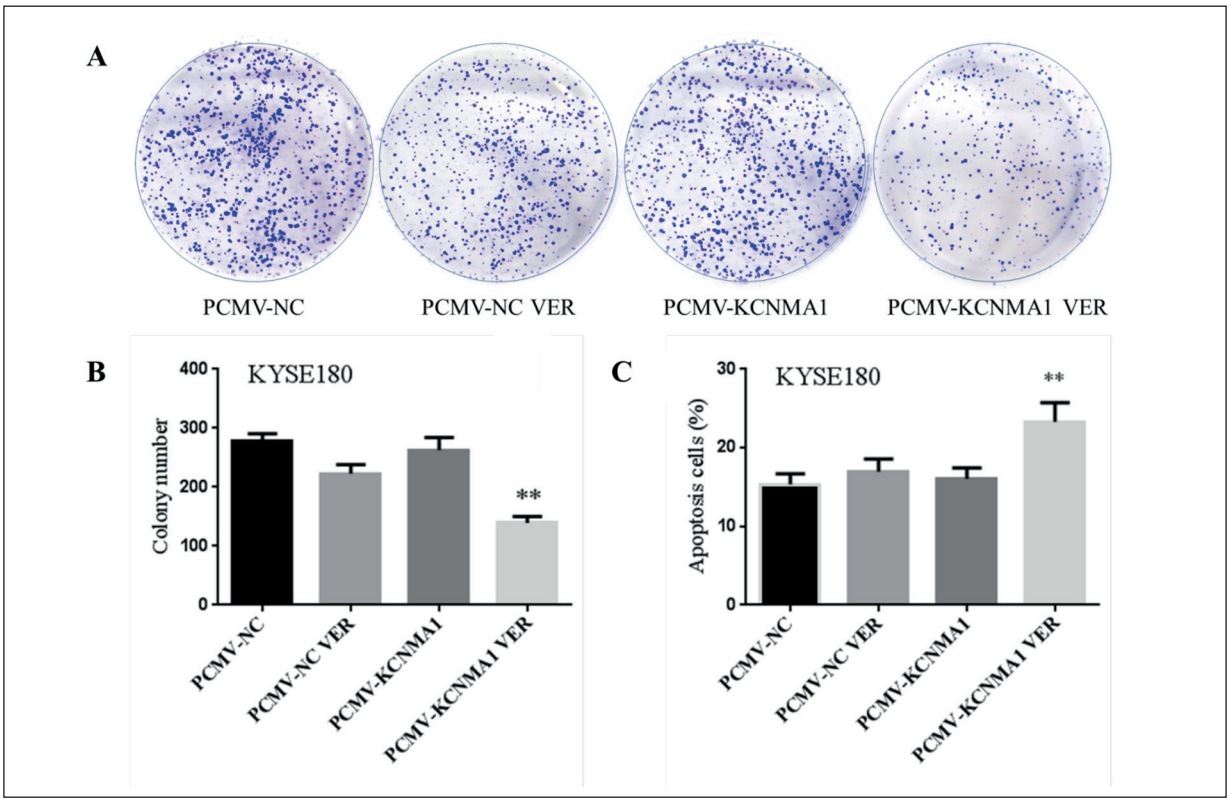

Figure 6. KCNMA1 enhances the antiproliferation and pro-apoptotic effects of VER on ESCC cells. **A,** Colonies were stained with crystal violet, and images were obtained. KYSE180 cells were transfected with PCMV-CN or PCMV-KCNMA1 and then treated with or without VER. The images were obtained at 200× magnification. **B,** The number of colonies was counted and shown as the mean ± SD. $^{**}p<0.01$ versus the PCMV-NC VER group. **C,** Cell apoptosis was detected after transfection of the KCNMA1 overexpression vector in KYSE180 ESCC cells. Apoptosis rate was calculated and shown as the mean ± SD. $p<0.05$, $^{**}p<0.01$ versus the PCMV-NC VER group.

et al[24] confirmed that VER is capable of reversing the resistance in lung carcinoma drug-resistant sublines independent of ABCB1 expression. Furthermore, Zhang et al[18] verified that P-glycoprotein expression level and the effect of VER have no direct correlation on the reversal of MDR in hepatocellular carcinoma cell lines. Moreover, VER has a diverse reversal ability in different cancer cells, but the underlying mechanisms remain unclear.

To explore new targets that can mediate the reversal effect of VER on drug resistance in ESCC cells, we investigated this effect on cisplatin resistance in three ESCC cell lines. Reversal effects were observed in the three cell lines, but the best reversal effect was noted in KYSE150 cells, and only a mild reversal effect was observed in KYSE180 cells. Subsequently, high-throughput transcriptome sequencing was conducted in VER reversal-sensitive KYSE150 cells and VER reversal-resistant KYSE180 cells to screen critical genes that can potentially mediate this effect. Several genes, including SLIT3, KCNMA1, NID1, KLK1, LPAR1, and CHRDL1, were significantly differentially expressed in ESCC cells with or without VER treatment. qRT-PCR and Western blot assays confirmed that KCNMA1 was downregulated in KYSE180 cells but upregulated in KYSE150 cells. Immunohistochemical staining of the tissues of patients with ESCC revealed that the expression level of KCNMA1 protein in the VER-sensitive group was higher than that in the VER-insensitive group. These results strongly suggested that KCNMA1 may play an important role in the reversal effect of VER on the chemoresistance to cisplatin of ESCC. To confirm further the role of KCNMA1 in mediating the drug resistance of ESCC, we used KCNMA1-targeting shRNA and overexpression vector transfection to manipulate KCNMA1 expression in the ESCC cells. Results showed that the reversal effect of VER significantly decreased after the KCNMA1

gene was silenced in KYSE150 cells. By contrast, KCNMA1 overexpression increased the reversal effect of VER on the chemoresistance to cisplatin of KYSE180 cells. Furthermore, KCNMA1 overexpression enhanced the antiproliferative and pro-apoptotic effects of VER on drugs in cisplatin-resistant cells.

KCNMA1, a subunit of calcium-regulated big potassium channels, which are usually activated by membrane depolarization and/or by intracellular Ca^{2+}, is associated with different malignancies[25]. Bloch et al[26] found that KCNMA1 gene amplification promotes prostate cancer cell proliferation. Khaitan et al[27] confirmed that metastatic breast cancer cells exhibit increased voltage-activated potassium channel activity, and blocking KCNMA1 can attenuate invasiveness and transendothelial migration in breast cancer cells. Oeggerli et al[28] reported that enhanced KCNMA1 expression correlates with, and contributes to, high proliferation rate and malignancy of breast cancer. Kuo et al[29] claimed that KCNMA1 is correlated with the drug-resistant state in residual ALDHbr cells. Samuel et al[30] observed that KCNMA1 is reduced in resistant ovarian cells and directly modulates cisplatin response, and they reported that loss of KCNMA1 leads to increased cisplatin resistance in ovarian cancer cells. Ma et al[31] established that KCNMA1 considerably inhibits the biological malignant behavior of gastric cancer cells by inducing cell apoptosis by suppressing PTK2 expression.

In the present study, we found that upregulating KCNMA1 expression via cisplatin combined with VER treatment enhanced the killing effect of cisplatin and increased the apoptosis rate in ESCC cells. By contrast, downregulating KCNMA1 expression weakened such promotion.

Conclusions

In summary, this study was the first to confirm that KCNMA1 mediates the reversal effect of VER on the chemoresistance to cisplatin of ESCC cells. However, given that only three cell lines were examined this study, more cell lines should be tested and additional *in vivo* experiments must be performed. The mechanism by which VER exerts this reversal effect warrants further investigation. Nevertheless, this study indicated that VER exhibits a reversal effect on drug resistance and thus offers a potential treatment for ESCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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