Overexpression of suppressor of IKBKE 1 is associated with vincristine resistance in colon cancer cells

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Received April 15, 2016; Accepted July 28, 2016

DOI: 10.3892/br.2016.759

Abstract. In a previous study, the suppressor of IKBKE 1 expression level was confirmed to be higher in vincristine (VCR)-resistant HCT-8 (HCT-8/V) colon cancer cells than in non-VCR-resistant HCT-8 cells. In the current study, IKBKE 1 expression in VCR-resistant colon cancer cells was investigated further. HCT-8 and HCT-8/V human colon cancer cells were used, and polymerase chain reaction (PCR) primers were designed to amplify the *IKBKE 1* gene. Fluorescence reverse transcription-quantitative PCR (RT-qPCR) was performed to detect differences in *IKBKE 1* expression between sensitive and drug-resistant colon cancer cell lines. Western blotting was performed to further observe IKBKE 1 expression. Based on the RT-qPCR and western blot results, IKBKE 1 expression was observed to be markedly higher in the HCT-8/V cells, and this difference was significant ($P<0.05$). Thus, *IKBKE 1* expression was identified to be associated with the resistance of colon cancer cells to VCR.

Introduction

As a result of economic development and lifestyle changes, the incidence of colorectal cancer (CRC) is increasing annually, causing serious harm to human life and health. CRC is the third most common malignancy worldwide (1) and chemotherapy is an important component of comprehensive CRC treatment (2). Drug-resistant cells have become an issue for the treatment of malignant tumor cells, such as CRC cells; 90% of cancer patient mortalities are associated with the drug resistance of tumors (2,3). Therefore, understanding the occurrence and developmental mechanism of drug resistance is a key issue in the treatment of malignant tumors. Tumor resistance mechanisms act at the molecular and cell biology levels, and include changes in drug targets, the repair of damaged cells, activation or inhibition of cell death signaling pathways, genetic mutations, deletions, gene amplification, abnormal DNA methylation and other epigenetic changes, and post-transcriptional regulation by microRNAs (4-6).

Vincristine (VCR) is the most commonly administered chemotherapeutic agent to treat CRC in clinical practice. VCR is a cell cycle-specific medication that binds to tubulin; it inhibits the assembly of microtubule structures and arrests mitosis at metaphase (7). Suppressor of IKBKE 1 suppresses inhibitor-κB kinase ε (IKKε), a necrosis factor (NF)-κB modulator, via a non-canonical pathway (8). IKBKE 1 is known to interact with IKKε and TANK binding kinase 1 to inhibit virus-triggered and toll-like receptor 3-triggered activation (9). Hsieh *et al* (10) demonstrated that miR-146a-5p is a novel chemokine (C-X-C motif) ligand 12 and IKBKE 1 inhibitor. Therefore, regulating miR-146a-5p expression in mesenchymal stem cells may improve the engraftment of transplanted mesenchymal stem cells that are homing to injured tissues (10).

In the previous study, IKBKE 1 demonstrated decreasing expression in HCT-8 VCR-resistant (HCT-8/V) colon cancer cells using next-generation sequencing (11); however, the function and mechanism of IKBKE 1 require further investigation. In the current study, the expression of IKBKE 1 was further verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, and investigated their role in modulating VCR resistance. *IKBKE 1* may present as a novel candidate target for gene therapy in VCR-resistant ovarian cancer.

Materials and methods

Cell lines and culture. The human colon cancer cell line, HCT-8 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and HCT-8/V cells were generated according to our previous study (11). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, China), 100 μg/ml penicillin and 100 μg/ml streptomycin at

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Key words: colon cancer, drug resistance, HCT-8, suppressor of IKBKE 1, vincristine

37˚C in a CO_2 incubator. The cells were subcultured every 2-3 days following treatment with 0.02% EDTA acid and 0.1% trypsin (Hangzhou Genom Biological Pharmaceutical Technology Co., Ltd., Hangzhou, China).

RNA extraction. A total of 50-100 mg sample was grinded with liquid nitrogen, and 1 ml lysate (SinoGene Scientific Co., Ltd., Beijing, China) was added after samples were transferred to 1.5 ml RNase-free centrifuge tubes. The blended mixture was supplemented with 200 μl chloroform and was vigorously shaken for 30 sec. Then, the mixture was centrifuged for 15 min at 12,000 x g and 4˚C. The supernatant was separated in a 1.5 ml RNase-free centrifuge tube and an equal volume of isopropanol was added. After centrifugation for 15 min at 12,000 x g and 4˚C, the supernatant was removed and the remaining solution was supplemented with 750 μl 75% ethanol, and the mixture was centrifuged for 5 min at 12,000 x g and 4˚C. The supernatant was removed and 45 μl diethylpyrocarbonate (DEPC) water was added to dissolve the RNA after drying with ethanol. The extracted RNA was used or stored at -80˚C.

RT-qPCR. RT-qPCR was performed using a Custom RT-qPCR Gene Expression Assay kit (SinoGene Scientific Co., Ltd.) according to the manufacturer's instructions. The primers are presented in Table I (GAPDH served as an internal reference) and were synthesized by Shanghai Sangon Biological Engineering Technology Service Co., Ltd. (Shanghai, China). The volume of the reverse transcription (RT) system was 20 μl, including 10 μl total RNA, 1 μl Oligo(dT)18, 1 μl RT enzyme, 1 μl 10 mmol/l deoxynucleotides, 4 μl 5X Reaction Buffer, 0.5 μl ribonuclease inhibitor and DEPC water up to a volume of 20 μl. After centrifugation for 5 min at 12,000 x g and 4˚C, the mixture was incubated for 60 min at 37˚C and for 10 min at 85˚C to inactivate the reverse transcriptase. The qPCR system included 7.5 μl 2X SG Green qPCR Mix, 10 μM primers (0.25 μl), 1 μl cDNA, and DEPC water up to a volume of 20 μl. The PCR reaction conditions were as follows: Predegeneration for 10 min at 95˚C, followed by 45 cycles of 95˚C (15 sec), 60˚C (15 sec), and 72˚C (30 sec). The Cq value method ($2^{-\Delta\Delta Cq}$) was used to perform quantitative analysis (12).

Western blot analysis. Cellular proteins were extracted with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride. Equal quantities of protein (0.05 μg) were fractionated by 7% sodium dodecyl sulphate polyacrylamide gel electrophoresis (100 V for 30 min for concentration gel and 150 V for 80 min for separation gel) (13), transferred to a polyvinylidene difluoride membrane, and reacted with antibodies against IKBKE 1 (1;1,000 dilution; cat. no. B1310; Sigma-Aldrich), and β-actin (1;1,000 dilution cat. no. AC-74; Sigma-Aldrich). The band intensity was quantified using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA) after normalization to the corresponding loading control.

Statistical analysis. All data were analyzed with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± standard deviation. One-way analysis of variance and Student's t-tests were used for statistical analyses, and $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used in the present study.

Primer	Sequence (5'-3')	Product length (bp)
Suppressor of IKBKE 1	F: TGTCTTTCAAATCTGCCTCC R: GGCTTGGCAACAACTTTC	85
GAPDH	F: ACCCAGAAGACTGTGGATGG R: TTCAGCTCAGGGATGACCTT	125

F, forward; R, reverse; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table II. RNA extraction from HCT-8 and HCT-8/V cells.

Sample	RNA concentration (ng/μl)	$OD_{260/280}$
HCT-8	763	1.96
HCT-8/V	245	2.10

HCT-8/V, vincristine-resistant HCT-8; $OD_{260/280}$, ratio of the optical density at 260 and 280 nm.

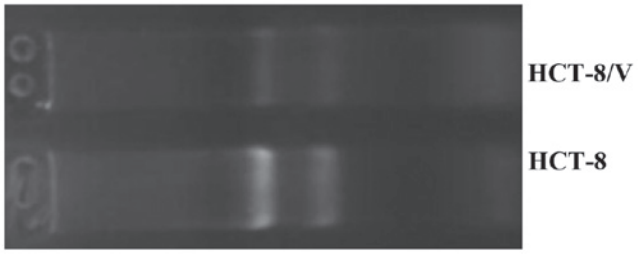

Figure 1. RNA extraction from HCT-8 and HCT-8/V cells. HCT-8/V, vincristine-resistant HCT-8.

Results

RNA extraction. RNA extraction is an important step in RT-PCR, and the quality of extracted RNA indicates the success of PCR. RNA extracted in the current study exhibited complete bands using electrophoresis (Fig. 1). The concentrations of RNA for HCT-8 and HCT-8/V were 763 and 245 ng/μl, respectively, and the ratios of the optical density at 260 and 280 nm were 1.96 and 2.10, respectively (Table II). These findings demonstrate that the extracted RNA was high quality and could be used to perform RT-qPCR.

RT-qPCR. Expression of *IKBKE 1* in the HCT-8/V colon cancer cells was detected using RT-qPCR using the extracted RNA and specific PCR primers. The amplification curves of *IKBKE 1* and *GAPDH* were observed to be 'S' type and the melting curves were characterized by a single curve, indicating effective amplification (Fig. 2A and B). Relative expression of *IKBKE 1* in HCT-8 sensitive cells (1.985±0.1050) was significantly higher than that in the HCT-8/V cells (1.000±0.0500) [1.985-fold ($P<0.05$; Fig. 2C and D)].

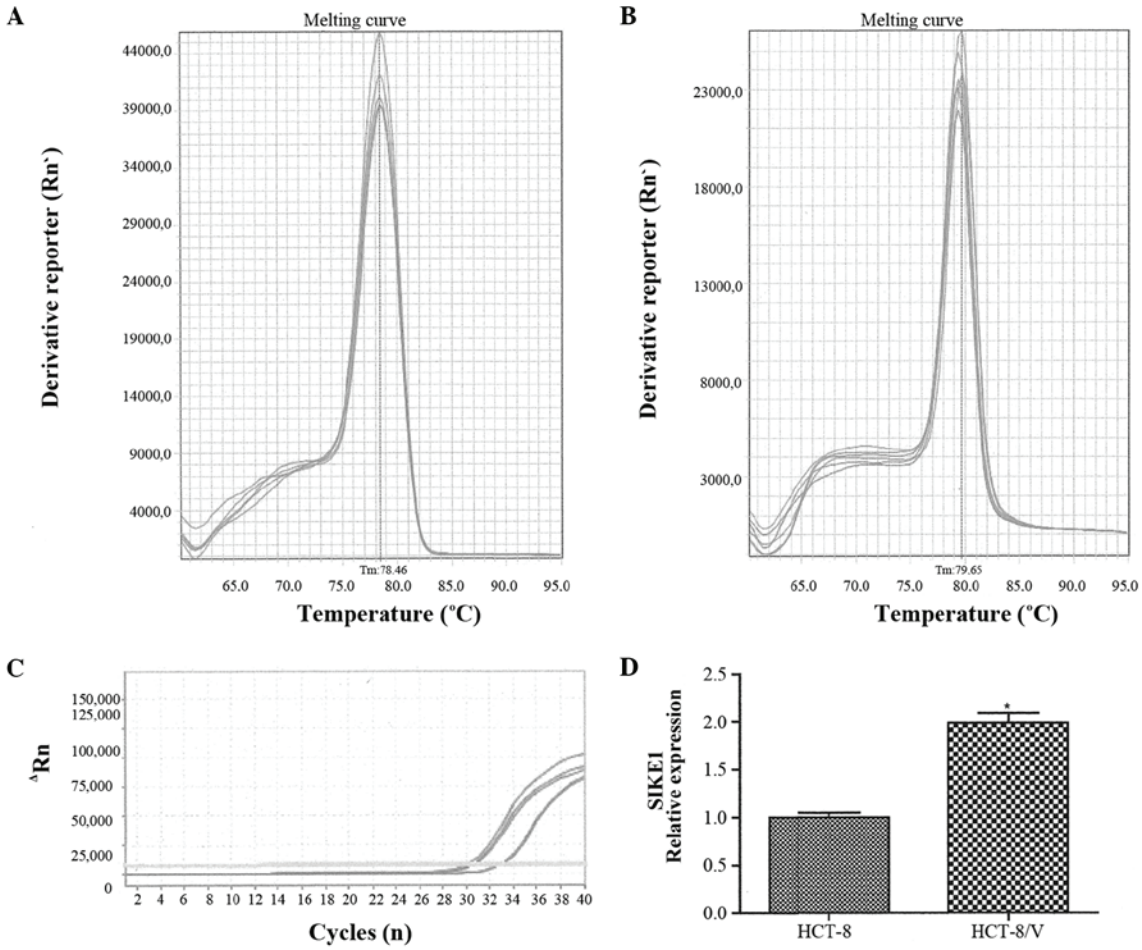

Figure 2. IKBKE 1 expression in HCT-8 and HCT-8/V cells was detected by reverse transcription-quantitative polymerase chain reaction. β-actin served as a loading control. Melting curves for (A) IKBKE 1 and (B) glyceraldehyde-3-phosphate dehydrogenase. (C) Amplification plot IKBKE 1. (D) Analysis of IKBKE 1 expression. *P<0.05 vs. HCT-8. IKBKE 1, suppressor of IKBKE 1; HCT-8/V, vincristine-resistant HCT-8; Rn, fluorescence of the SYBR-Green reporter dye; ΔRn, baseline-corrected Rn; Rn', first derivative of Rn; Tm, melting temperature.

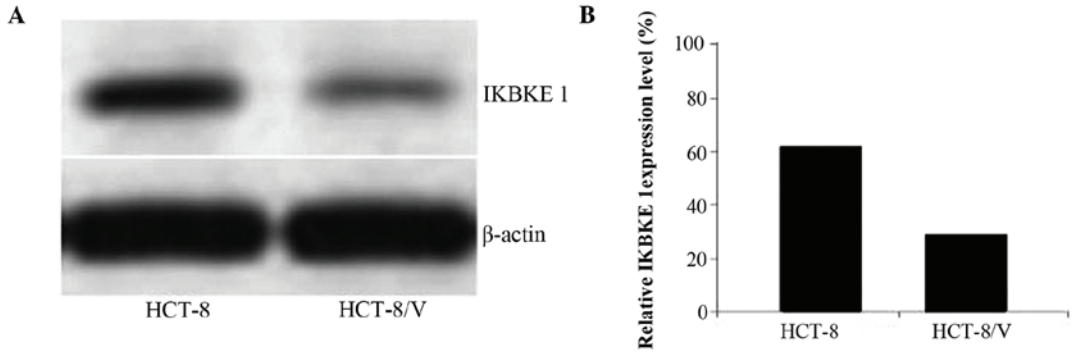

Figure 3. IKBKE 1 expression from HCT-8 and HCT-8/V was detected by (A) western blotting and β-actin served as a loading control. (B) Relative IKBKE 1 expression level of HCT-8 and HCT-8/V. IKBKE 1, suppressor of IKBKE 1; HCT-8/V, vincristine-resistant HCT-8.

Western blot analysis. The total protein extracted from HCT-8 and HCT-8/V cells was used to estimate relative expression levels after correction using the internal reference (β-actin). The expression level of IKBKE 1 in the HCT-8/V cells was significantly higher than that in the HCT-8 cells (P<0.05; Fig. 3). The mean expression level was 0.6233±0.3654 for HCT cells, and was 2.13-fold higher in the HCT-8/V cells than in the HCT-8 cells.

Discussion

VCR is widely administered to clinically treat certain cancers, including leukemia and lung cancer; however, tumor cells may develop drug resistance (14-17). The resistance mechanism of VCR is complex and involves numerou molecules, such as insulin like growth factor binding protein 7, multidrug resistance protein 1, miRNAs, and long non-coding RNA (11,18-23).

A previous study demonstrated that IKBKE 1 in VCR-resistant colon cancer cell lines was significantly increased by >14-fold compared with in non-resistant cells (17). Consistent with this result, the present study identified that IKBKE 1 expression in the VCR-resistant cells was significantly higher than in the non-resistant cells using RT-qPCR and western blotting. *IKBKE 1* is a target of miR-146a, which contributes to the proliferation of stem cells (10).

In conclusion, gene transcription and translation are two processes involved in gene expression, and certain genes are transcribed to mRNA, but are not translated to proteins (24). The results of the current study prove that the expression of IKBKE 1 is increased in VCR-resistant cells in colon cancer at the mRNA and protein levels. Therefore, the expression of IKBKE 1 has been demonstrated to be associated with VCR drug resistance; however, its function and underlying mechanisms require further investigation.

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