Knockdown of KLK11 inhibits cell proliferation and increases oxaliplatin sensitivity in human colorectal cancer

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Abstract. It has been reported that kallikrein 11 (KLK11) is crucially involved in the development and progression of various types of cancer. However, the molecular mechanisms that underlie the involvement of KLK11 in aberrant colorectal cancer (CRC) cell growth remain largely unclear. The aim of the present study was to investigate the role of KLK11 and the effects of KLK11 on oxaliplatin (L-OHP) chemosensitivity by knocking down KLK11 in LOVO and HCT-8 cells. Loss-of-function assays revealed KLK11 inhibition significantly inhibited growth and induced apoptosis of CRC cells *in vitro*. Notably, further experiments found that knockdown of KLK11 expression increased the L-OHP chemosensitivity of CRC cells. KLK11 inhibition of increased L-OHP-induced apoptosis may be associated with activation of caspase-3 cleavage and the apoptosis signaling pathway. The present results indicated that KLK11 may be an potential target of interest for future research into therapies for CRC.

Introduction

Colorectal cancer (CRC) is among the most common malignancies and the second leading cause of cancer-associated mortality, following lung cancer (1). The 5-year survival rate for CRC is still low because patients diagnosed with CRC have progressed to the advanced stage (2-5). Survival rates have increased with the introduction of irinotecan and oxaliplatin chemotherapy, as well as the use of targeted therapies in the past decade (6-8). Combined perioperative chemotherapy and surgery is a major therapeutic treatment for patients with initially resectable liver metastases from CRC (9-11). However, the development of drug resistance in cancer cells raises a major challenge to chemotherapy and restricts the anticancer efficacy of chemotherapeutic drugs (12-14). Therefore, improving the sensitivity to drug resistance remains an urgent requirement for chemoresistance.

Human kallikrein 11 (KLK11) is a member of the human *KLK* gene family and located at the chromosomal locus 19q13.3-q13.4 (15). Previous experiments have indicated that KLK11 is ubiquitously expressed in human brain, skin, stomach, breast, prostate, ovary and intestine tissue (16). Recent results demonstrated that KLKs were involved in human malignancies and that KLK11 may be a favorable prognostic biomarker for ovarian and prostate cancer due to the high serum levels of KLK11 in 70% of women with ovarian cancer and in 60% of men with prostate cancer (17). Alexopoulou *et al* have shown that KLK11 mRNA expression was upregulated in colorectal adenocarcinoma and could be considered as a new molecular prognostic biomarker (18). However, the value of KLK11 as a prognostic biomarker remains controversial and more evidence is needed for further clinical application. It has been reported that KLK11 mRNA expression could serve as a novel and independent biomarker for diagnosis and prognosis in laryngeal cancer (19). Unal *et al* have suggested that KLK11-positive patients had higher disease-free survival and overall survival compared to those with KLK11-negative expression (20). However, little is known concerning the possible involvement of KLK11 in human CRC.

The aim of the present study was to investigate the role of KLK11 in human CRC. Additionally, the potential use of shRNA-mediated KLK11 gene knockdown associated with apoptosis and drug resistance were further examined.

Materials and methods

Cell culture and reagents. Two human-derived CRC cell lines LOVO (CCL-229) and HCT-8 (CCL-244) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured with RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin

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and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 5% CO_2 at 37˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA from cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's directions. Then, 1 μg total RNA was used for reverse transcription reaction using SuperScript III reverse transcriptase (Invitrogen). qPCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA), and the mRNA expression of human KLK11 and β-actin was evaluated using a LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). PCR amplification was performed by denaturation at 95˚C for 10 min, annealing and extension at 60˚C for 60 sec for 40 cycles. RT-qPCR analysis was performed using the following primers: KLK11 forward: 5'-GTTCGAGAAGACGCGGCTAC-3'; KLK11 reverse: 5'-GGTGGGAGAGGTGAGTGAC-3'. β-actin forward: 5-CCAACCGCGAGAAGATGA-3'; β-actin reverse: 5'-CCA GAGGCGTACAGGGATAG-3'. The relative expression level of KLK11 was calculated using the ΔΔCq method (21) and normalized against that of β-actin. All PCR amplification was performed in triplicate and repeated in three independent experiments.

Gene silencing with the lentivirus encoding specific shRNA. In order to silencing KLK11, the short hairpin RNA (shRNA) were generated by ligating synthetic oligonucleotides (Invitrogen) against the target genes into the *Age*I and *Eco*RI sites of pLKO.1-TRC cloning vector (provided by Dr Xuchao Zhu; Tenth People's Hospital, Affiliated to Tongji University, Shanghai, China). The sequences of the KLK11 shRNA (shKLK11) and shRNA control (SCR) were as follows: KLK11-SH1 sense, 5'-CCGGCCAACAAAGACCACCGC AATGCTCGAGCATTGCGGTGGTCTTTGTTGGTTTTT G-3' and antisense, 5'-AATTCAAAAACCAACAAAGAC CACCGCAATGCTCGAGCATTGCGGTGGTCTTTG TTGG-3'; KLK11-SH2 sense, 5'-CCGGGAGATGCTGTC ACTTAATAATCTCGAGATTATTAAGTGACAGCATCT CTTTTTG-3' and antisense, 5'-AATTCAAAAAGAGATGCT GTCACTTAATAATCTCGAGATTATTAAGTGACAGCA TCTC-3'; KLK11-SH3 sense, 5'-CCGGCTGGTCTGTAA CCAGTCTCTTCTCGAGAAGAGACTGGTTACAGACCA GTTTTTG-3' and antisense, 5'-AATTCAAAAACTGGTCTG TAACCAGTCTCTTCTCGAGAAGAGACTGGTTACAGA CCAG-3'; control shRNA sense, 5'-CCGGAAACTACCGTT GTTATAGGTGTTCAAGAGACACCTATAACAACGGTA GTTTTTTTTG-3' and antisense, 5'-AATTCAAAAAAA ACTACCGTTGTTATAGGTGTCTCTTGAACACCTATA ACAACGGTAGTTT-3'. Lentiviral virions were produced by co-transfection of HEK293T cells with 5 μg pLKO.1-puro vector and 5 μg packaging and envelope vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Lentivirus was harvested 48 h after transfection. LOVO and HCT-8 cells were infected with lentivirus containing shKLK11 or SCR for 24 h. Two days later, the virus-infected cells were selected by 2 μg/ml puromycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 48 h and subjected to required assays.

Cell viability assay. Cell viability was quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (22). Briefly, 3×10^3 transiently transfected LOVO and HCT-8 cells (SCR or shKLK11) were seeded in 96-well plates and 20 μl MTT solution (5 mg/ml; Sigma-Aldrich) was added to each well 72 and 96 h later. The optical density was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 595 nm.

For drug sensitivity, cells were plated in 96-well plates at 5×10^4 cells per well, followed by treatment with 0, 5 or 10 μmol/lL-OHP for 24 h. The optical density was then measured and the cell viability was calculated.

Annexin V-FITC apoptosis detection. Apoptosis detection was performed using an Annexin V Apoptosis Detection kit I (BD Biosciences, Franklin Lakes, NJ, USA). In brief, cells were collected and washed with phosphate-buffered saline (PBS). Then, 5 μl annexin V and propidium iodide was added to the cell suspension and incubated at room temperature in the dark for 30 min. The volume was then made up to 500 μl and the cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Caspase-3 activity analysis. The activity of caspase-3 was measured using a Caspase-3 Assay kit (Abnova Corporation, Taipei, Taiwan) according to the manufacturer's instructions. In brief, 5×10^6 cells were harvested, resuspended in 50 μl chilled cell lysis buffer and incubated on ice for 10 min. Then, 50 μl 2.0X Reaction Buffer was added to each sample, along with 5 μl DEVD-pNA (4 mM) substrate and incubated for 2 h at 37˚C. The optical density was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Western blot analysis. Cell lysates were prepared in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% NP-40 (v/v) and 150 mM NaCl, supplemented with a mixture of complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). Equal quantities of protein (40 μg) were then separated on 10% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). Blocking was performed at room temperature using Tris-buffered saline with 0.1% Tween-20 (TBST; J&K Chemical Ltd., Shanghai, China) containing 5% non-fat milk for 1 h. The membrane was then incubated with primary mouse monoclonal KLK11 antibody (sc-20387; 1:500) and rabbit polyclonal β-actin (sc-47778; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bcl-2 (#2872; 1:1,000) and Bax (#2772; 1:1,000) antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) in TBST at 4˚C overnight and with the appropriate horseradish peroxidase-conjugated secondary antibody (CW0103M; 1:3000; CWbiotech, Beijing, China) for 1 h at room temperature. Specific antibody binding was detected using an ECL system (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was considered to be indicated by P<0.05.

Results

Stable knockdown of KLK11 specifically inhibits the expression of KLK11 mRNA and protein in CRC cells. A previous study has shown that the expression of KLK11 was upregulated in colorectal tumors (18). To determine whether KLK11 is involved in the progression of CRC, three different lentivirus-based shRNAs (KLK11 SH1, KLK11 SH2 and KLK11 SH3) were employed to downregulate KLK11 expression. The mRNA and protein levels of KLK11 in stably transfected LOVO and HCT-8 cells were confirmed using RT-qPCR and western blot analysis (Fig. 1). The mRNA expression levels of KLK11 were significantly decreased in KLK11 SH3 groups in both cell lines. The subsequent assays were performed with KLK11 SH3, which is furthermore referred to as shKLK11. These results suggested that the lentivirus-mediated shRNA targeting KLK11 could effectively knockdown KLK11 expression in CRC cells.

Downregulation of KLK11 inhibits growth and enhances apoptosis of CRC cells. To determine the biological function of KLK11 in CRC progression, MTT assays were used to examine the proliferative ability of CRC cells. As shown in Fig. 2A and B, the proliferation rates of shKLK11-infected cells started to decrease and were reduced compared with those of the SCR groups on days 3 and 4.

There is considerable evidence indicating that apoptosis has a close association with cell growth (23). In the present study, apoptosis assays were performed using CRC cells following KLK11 silencing. The results of flow cytometric analysis indicated that the percentages of apoptotic cells were significantly increased in shKLK11-infected LOVO and HCT-8 cells compared with the respective SCR groups (Fig. 2C and D). This finding indicated that KLK11 may serve a crucial function in the proliferation and tumorigenesis of CRC cells *in vitro*.

Downregulation of KLK11 expression inactivates the apoptosis signaling pathway in CRC cells. To examine the mechanism underlying the inhibition of cell growth, the expression of Bcl-2 and Bax, two important proteins of the apoptosis signaling pathway (24) was investigated. Western blot analysis showed that knockdown of KLK11 lead to a reduction of Bcl-2 and an increase of Bax in both cell lines (Fig. 3A). Caspase-3, a crucial mediator of apoptosis, is a frequently activated death protease, catalyzing the specific cleavage of various key cellular proteins (25). A significant increase in caspase-3 activity was detected in shKLK11-infected LOVO and HCT-8 cells compared with SCR groups (Fig. 3B). Collectively, these data demonstrated that the proliferative effect of KLK11 in CRC cells is regulated via the apoptosis pathway.

KLK11 silencing enhances sensitivity of CRC cells to L-OHP and L-OHP-induced apoptosis in vitro. Our previous study has shown that dysregulation of KLK11 expression had an association with FOLFOX4 chemotherapy in human CRC cells (26). However, whether KLK11 played a key role in affecting the sensitivity of CRC cells was not fully understood. To elaborate on this, LOVO and HCT-8 cells with stable KLK11 silencing were treated with 0, 5 or 10 μmol/l L-OHP for 24 h. The results of MTT assay suggested that knockdown of KLK11 led to a significant reduction in the viability of CRC cells in response to L-OHP in a dose-dependent manner compared with control (Fig. 4A and B). Furthermore, flow cytometric analysis showed that the apoptotic rate of cells with stable KLK11 silencing treated with L-OHP was significantly higher than that of cells treated with control (Fig. 4C and D). Collectively, these results suggest that knockdown of KLK11 could increase the chemosensitivity of CRC cells to L-OHP by inducing apoptosis enhancement *in vitro*.

Discussion

Overexpression of KLK11 is a general feature in numerous human malignancies including CRC, and the overexpression is often correlated with malignant behavior (27). In the present study, it was found that KLK11 silencing inhibited the growth and increased the apoptosis of CRC cells. Downregulation of KLK11 also increased caspase-3 activity by activating the apoptosis signaling pathway, which induced a reduction of the Bcl-2/Bax ratio.

KLK11 is a member of the KLK family, which are dysregulated in multiple tumors (17). Previous experiments have shown that KLK11 was upregulated in malignant CRC tissues in comparison with noncancerous tissues, and was associated with highly invasive and positive nodal status (18). Therefore, we hypothesized that KLK11 might be an oncogene in colorectal tumors. As expected, lentivirus-mediated KLK11 silencing was able to effectively suppress the proliferation of colon cancer cells *in vitro*. Furthermore, knocking down KLK11 resulted in a significant upregulation of apoptosis in CRC cells. From these data, we suggest that KLK11 has a positive impact on the progression of CRC cells *in vitro*.

In order to determine the underlying mechanisms by which KLK11 is involved in cell growth, the present study analyzed apoptosis signaling in CRC cells. Bcl-2 and Bax, two crucial regulatory proteins that play important roles in the induction of apoptosis have been reported to regulate cancer growth (28,29). The results of the present study indicated that KLK11 silencing may activate the apoptosis signaling pathway by increasing the expression level of Bax and decreasing the expression level of Bcl-2, which induced a reduction of the Bcl-2/Bax ratio. Furthermore, the downregulation of KLK11 promotes caspase-3 activity, which results in the death of tumor cells.

There is considerable evidence supporting the hypothesis that mechanisms involved in resistance to chemotherapy correlate with apoptosis (30). The present study next investigated whether the KLK11 silencing was associated with sensitivity to L-OHP. Consistent with the above data, KLK11 silencing resulted in a higher inhibition of cell growth and apoptosis following exposure to L-OHP.

Based on these data, we conclude that knockdown of KLK11 could inhibit cell proliferation, induce apoptosis and increase the sensitivity of CRC cells to L-OHP *in vitro*, which may offer a novel therapeutic approach for L-OHP-resistant CRC treatment. Further studies are required to determine whether these findings are present *in vivo*.
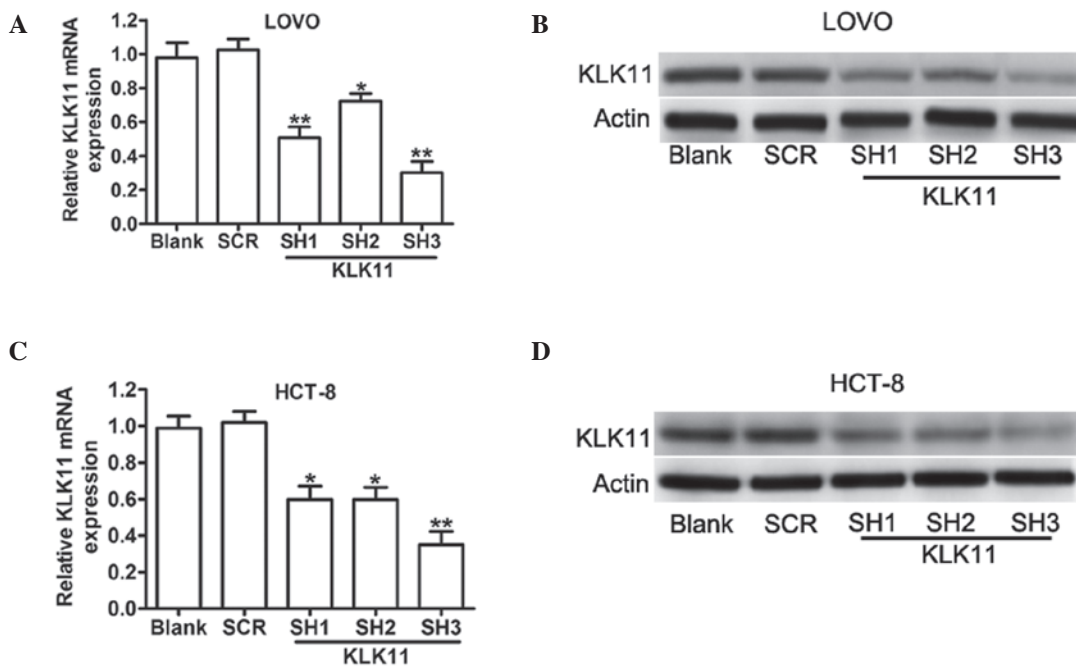
Figure 1. Establishment of cell lines that stably knockdown KLK11. Expression of (A) KLK11 mRNA and (B) KLK11 protein in LOVO cells and (C) KLK11 mRNA and (D) KLK11 protein in HCT-8 cells. mRNA levels were detected by reverse transcription-quantitative polymerase chain reaction, and protein levels by western blot analysis. β-actin was used as a loading control. *P<0.05, **P<0.01 vs. SCR. KLK11, kallikrein 11; SCR, short hairpin RNA control.
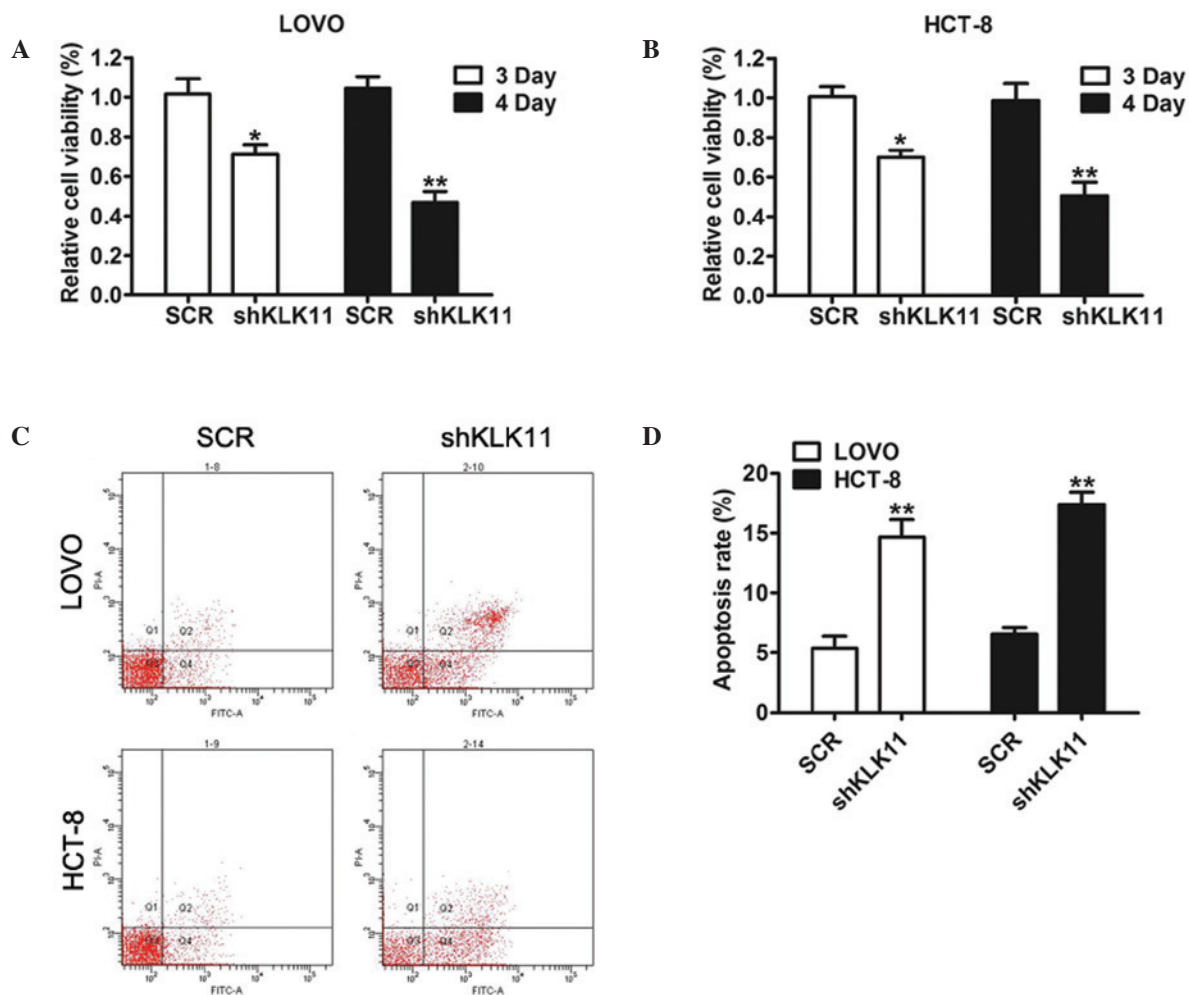

Figure 2. KLK11 shRNA inhibited cell proliferation and enhanced apoptosis. Proliferation levels of (A) LOVO and (B) HCT-8 cells after KLK11 silencing analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (C) Flow cytometric analysis of apoptosis in LOVO and HCT-8 cells transfected with SCR or shKLK11. (D) Quantification of the apoptotic cells. *P<0.05, **P<0.01 vs. SCR. SCR, short hairpin RNA control; KLK11, kallikrein 11.
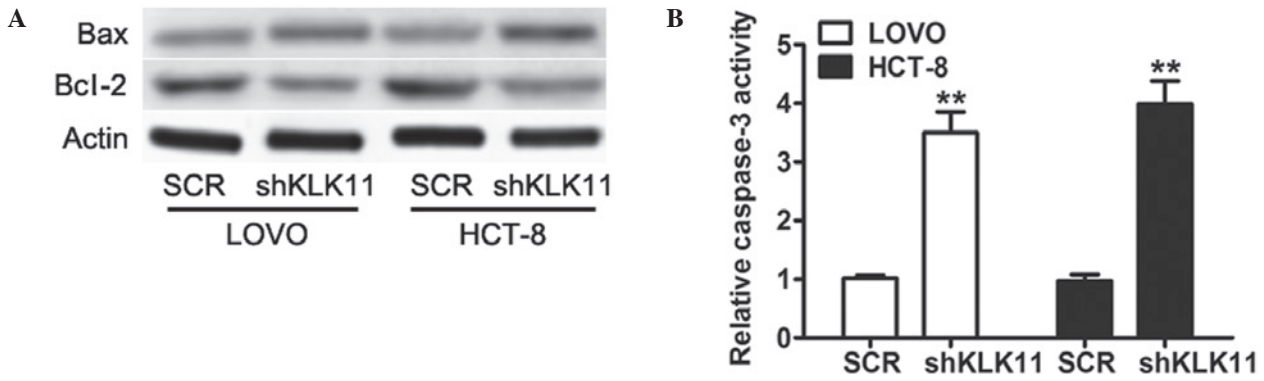
Figure 3. Effect of KLK11 silencing on the apoptosis signaling pathway. (A) Western blot analysis of Bcl-2 and Bax protein expression in LOVO and HCT-8 cells transfected with SCR or shKLK11. β-actin was used as a loading control. (B) Relative caspase-3 activity was determined in LOVO and HCT-8 cells transfected with SCR or shKLK11. **P<0.01 vs. SCR. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated protein X; SCR, short hairpin RNA control; KLK11, kallikrein 11.
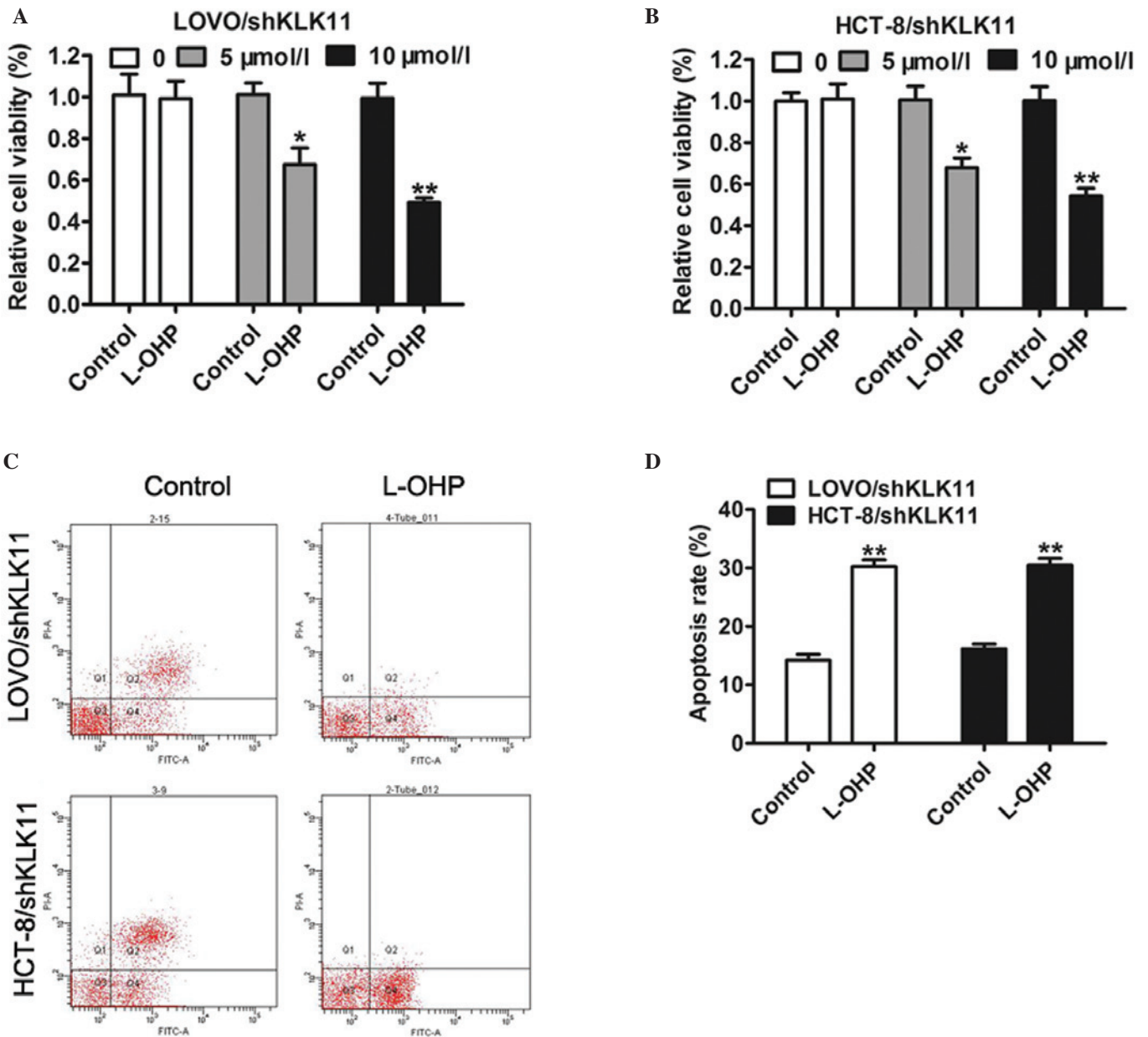

Figure 4. KLK11 silencing modulates the sensitivity to L-OHP. Viability of KLK11 knockdown (A) LOVO and (B) HCT-8 cells treated with the indicated concentrations of L-OHP for 24 h. (C) The effect of L-OHP on apoptotic levels of the indicated cells analyzed by flow cytometry. (D) Quantification of the apoptotic cells. *P<0.05, **P<0.01 vs. control. KLK11, kallikrein 11; L-OHP, oxaliplatin.

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