Siva-1 regulates multidrug resistance of gastric cancer by targeting MDR1 and MRP1 via the NF-κB pathway

FAN-BIAO KONG[1*], QIAO-MING DENG[2*], HONG-QIANG DENG[1*], CHEN-CHENG DONG[1], LEI LI[3], CHUN-GANG HE[1], XIAO-TONG WANG[3], SHENG XU[1] and WEI MAI[3]

[1]Department of Surgery, People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi Zhuang Autonomous Region 530021; [2]Department of Surgery, Guangxi Traditional Chinese Medical University Affiliated First Hospital, Nanning, Guangxi Zhuang Autonomous Region 530023; [3]Department of Gastrointestinal and Peripheral Vascular Surgery, People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China

Received December 17, 2019; Accepted May 15, 2020

DOI: 10.3892/mmr.2020.11211

Abstract. Siva-1 is a well-known anti-apoptosis protein that serves a role in multiple types of cancer cells. However, whether Siva-1 affects multidrug resistance via the NF-κB pathway in gastric cancer is currently unknown. The present study aimed to determine the possible involvement of Siva-1 in gastric cancer anticancer drug resistance *in vitro*. A vincristine (VCR)-resistant KATO III/VCR gastric cancer cell line with stable Siva-1 overexpression was established. The protein expression levels of Siva-1, NF-κB, multidrug resistance 1 (MDR1) and multidrug resistance protein 1 (MRP1) were detected via western blotting. The effect of Siva-1 overexpression on anticancer drug resistance was assessed by measuring the 50% inhibitory concentration of KATO III/VCR cells to VCR, 5-fluorouracil and doxorubicin. The rate of doxorubicin efflux and apoptosis were detected by flow cytometry. Additionally, colony formation, wound healing and Transwell assays were used to detect the proliferation, migration and invasion of cells, respectively. The results of the current study revealed that the Siva-1-overexpressed KATO III/VCR gastric cancer cells exhibited a significantly decreased sensitivity to VCR, 5-fluorouracil and doxorubicin. The results of flow cytometry revealed that the percentage of apoptotic cells decreased following overexpression of Siva-1. The colony formation assay demonstrated that cell growth and proliferation were significantly promoted by Siva-1 overexpression. Additionally, Siva-1 overexpression increased the migration and invasion of KATO III/VCR cells *in vitro*. Western blot analysis determined that Siva-1 overexpression increased NF-κB, MDR1 and MRP1 levels. The current study demonstrated that overexpression of Siva-1, which functions as a regulator of MDR1 and MRP1 gene expression in gastric cancer cells via promotion of NF-κB expression, inhibited the sensitivity of gastric cancer cells to certain chemotherapies. These data provided novel insight into the molecular mechanisms of gastric cancer, and may be of significance for the clinical diagnosis and therapy of patients with gastric cancer.

Correspondence to: Professor Sheng Xu, Department of Surgery, People's Hospital of Guangxi Zhuang Autonomous Region, 6 Taoyuan Road, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China
E-mail: xvsheng@hotmail.com

Dr Xiao-Tong Wang, Department of Gastrointestinal and Peripheral Vascular Surgery, People's Hospital of Guangxi Zhuang Autonomous Region, 6 Taoyuan Road, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China
E-mail: 008.wxt@163.com

*Contributed equally

Key words: Siva-1, multidrug resistance, gastric cancer

Introduction

Advanced gastric cancer often recurs and metastasizes subsequent to surgery, and eventually the metastatic cancer cells develop resistance to the chemotherapeutic drugs (1-3). Multidrug resistance (MDR) accounts for poor prognosis in gastric cancer (4), the development of multidrug resistance is a key issue for tumor recurrence and metastasis, leading to treatment failure of gastric cancer. Cancer cells may become unresponsive to chemotherapeutics via multidrug resistance, which interrupts apoptosis signaling. Multidrug resistance involves the overexpression of energy-dependent ATP-binding cassette transporter protein, which detoxifies cancer cells and lowers intracellular concentrations under the therapeutic threshold by pumping drugs out at the expense of ATP hydrolysis (5,6). Therefore, it is necessary to identify multidrug resistant molecules in gastric cancer cells, and to develop more effective diagnostic and therapeutic clinical strategies in order to treat advanced gastric cancer.

Siva-1 exists in a wide variety of tissues and cells, and serves as a proapoptotic protein (7). Siva-1 was elucidated by Prasad *et al* (8) from a HeLa cell library using yeast two-hybrid screening with a tumor necrosis factor receptor. Although numerous studies (9-11) have demonstrated that Siva-1 functions in the cytoplasm, studies have also determined that Siva-1 can relocate into the nucleus (12,13). The human Siva

gene is located on chromosome 14 (14), a region which is often targeted for chromosomal translocation. Previous studies have demonstrated that Siva-1 arrests apoptosis and facilitates cancer development in osteosarcoma, hepatocellular carcinoma and non-small cell lung cancer (15-18). However, the molecular function of Siva-1 regulating multidrug resistant in gastric cancer currently remains uncertain as previous studies have presented confusing and ambiguous results (16), which has prompted further investigation into both its function and associated signaling pathway. Whether Siva-1 acts as a determinant for gastric cancer development therefore requires further examination.

With the aim of gaining further knowledge regarding the specific mechanisms of Siva-1 in gastric cancer and elucidating molecular determinants for multidrug resistance, the current study overexpressed Siva-1 in gastric cancer cells using a lentiviral vector. Subsequently, effects on chemotherapeutic compound 50% inhibitory concentration (IC_{50}) values, apoptosis, colony formation, metastasis and invasion were observed. Preliminary experiments revealed that drug-sensitive (native) gastric cancer cells were sensitive to chemotherapeutic drugs at very low doses. Thus, it is appropriate to choose a chemotherapeutic drug resistance gastric cancer cell line as a research tool. The five commonly used chemotherapeutic drugs, including vincristine (VCR), doxorubicin (DOX), platinum drugs, 5-fluorouracil (5-FU) and paclitaxel (PTX), in gastric cancer is of great clinical interest (19). Therefore, the vincristine (VCR)-resistant KATO III/VCR gastric cancer cell line was selected for further experimentation. DOX is a common substrate for P-glycoprotein (P-gp), which is one of the major energy-dependent efflux transporters that contribute to MDR. The ability to pump DOX was analyzed by flow cytometry to reveal potential molecular determinants for multidrug resistance. Additionally, the possible underlying mechanisms of multidrug resistance were also investigated in the current study.

Materials and methods

Reagents. VCR, trypsin, penicillin and streptomycin were obtained from Sigma-Aldrich (Merck KGaA). DOX (0.4 μg/ml) was purchased from Sigma-Aldrich (Merck KGaA). Cells were cultured in RPMI-1640 medium, which was purchased from Invitrogen (Thermo Fisher Scientific, Inc.) along with fetal bovine serum (FBS). Siva-1 (cat. no. 12532), NF-κB (cat. no. 8242), multidrug resistance 1 (MDR1; cat. no. 13342), multidrug resistance protein (MRP1; cat. no. 72202), Lamin B1 (cat. no. 13435) and GAPDH (cat. no. 5174) antibodies were purchased from Cell Signaling Technology, Inc. Horseradish peroxide (HRP)-conjugated goat anti-rabbit IgG H&L (cat. no. ab205718) was purchased from Abcam. VCR (1.8 μg/ml), and 5-FU (20 μM) were purchased from SHRbio.

Cell culture. KATO III gastric cancer cells (obtained from the Experimental Center of the People's Hospital of Guangxi Zhuang Autonomous Region) and 293T cells (obtained from the Xiangya Central Laboratory at Central South University, Changsha Hunan, China) were cultured in RPMI-1640 supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin), and incubated at 37˚C in a fully-humidified atmosphere containing 5% CO_2. KATO III/VCR cells were maintained culture medium was supplemented with 0.6 μg/ml VCR to maintain drug-resistant phenotypes.

Gene transfection. The DNA sequence of SIVA-1 was obtained from the Gene Bank (ID No. NM_6427) and the cDNA, which included the entire coding sequence (CDS) of SIVA-1 was obtained from Shanghai Cancer Institute. pGV358-GFP (Shanghai Cancer Institute), which express green fluorescent protein, were used to construct the Siva-1-overexpression lentivirus. The Siva-1-overexpression lentiviral vector (pGV358-GFP-SIVA-1) and negative control vector (pGV358-GFP) was stored in the laboratory of Guangxi People's Hospital. Lentiviruses were generated by co-transfecting pGV358-GFP-SIVA-1 with pHelper1.0 and pHelper2.0 plasmids (Shanghai Genechem Co., Ltd) into 293T cells according to the manufacturer's protocol (20). Recombinant pGV358-GFP-SIVA-1 plasmid was transfected into 293T cells to determine LV titers using the end-point dilution method, which involved counting the number of infected green cells under fluorescence microscopy (magnification, x100). The lentiviral titer was calculated by the formula: lentiviral titer (TU/ml) = number of positive cells x dilution times/volume of lentivirus used. KATO III/VCR cells were plated at a low density ($5x10^4$ cells/well) in 6-well plates. Following incubation for 24 h, KATO III/VCR cells were infected with polybrene (2 mg/ml; Shanghai Genechem Co., Ltd) combined with recombinant lentiviruses at a multiplicity of infection (MOI) value of 12 PFU/cell (MOI, 12), as previously described (21). Transfected cells were subsequently cultured in the presence of 600 mg/ml G418 (Invitrogen; Thermo Fisher Scientific) for 4 weeks, after which stably overexpressed cell lines were generated. Cells were then divided into three groups: i) KATO III/VCR + LV-Siva-1; ii) KATO III/VCR + LV-negative control (NC); and iii) KATO III/VCR.

Cytotoxicity assay. Cells ($5x10^4$ cells/ml) were cultured in 96-well tissue microplates (100 μl/well) and exposed to VCR (1.8 μg/ml). Following incubation for 48 h at 37˚C, the cytotoxicity of KATO III/VCR + LV-Siva-1, KATO III/VCR + LV-NC and KATO III/VCR cells was determined via an MTT assay (Biological Industries) in accordance with the manufacturer's protocol. MTT (0.1 mg/well) was added for 4 h at 37˚C before harvesting, and then DMSO (150 μl/well) was added to dissolve all the precipitation. Absorbance values were measured at 450 nm using a microplate reader (PR 3100 TSC; Bio-Rad Laboratories, Inc.). Relative drug resistance folds were analyzed and compared with IC_{50} values.

Measurement of DOX pump rate via flow cytometry. The fluorescence intensity of intracellular doxorubicin was determined using flow cytometry. KATO III gastric cancer cells were seeded into 6-well plates, after which doxorubicin was added to each well to a final concentration of 4 mg/ml. Samples were then cultured at 37˚C for 30 min. Cells were subsequently washed twice with fresh culture medium and incubated at 37˚C for 1 h. Doxorubicin levels were subsequently determined by measuring the fluorescence intensity of doxorubicin in cells with an excitation wavelength

of 488 nm and an emission wavelength of 575 nm (21,22). The cells were analyzed using an EPICS XL-MCL flow cytometry system (Beckman Coulter) and the data was analyzed using MultiCycle Software for Windows (version 3.0, Phoenix Flow Systems). The procedure was performed in triplicate and an average value was obtained to calculate the pump rate of doxorubicin using the following formula: Releasing index = (accumulation value - retention value)/accumulation value.

Quantification of apoptosis via flow cytometry. KATO III/VCR + LV-Siva-1, KATO III/VCR + LV-NC and KATO III/VCR cells were harvested using 0.25% trypsin. Cells ($1x10^6$ cells/ml) were subsequently washed twice with ice-cold (4˚C) PBS, treated with trypsin and fixed with 70% ethanol at 4˚C for 30 min. The cell suspension was incubated with Annexin V-PE (2 μl/ml, BD Biosciences) and 7-amino-actinomycin D (7-AAD; 2 μl/ml) apoptosis detection kits (BD Biosciences) at room temperature for 15 min according to the manufacturer's protocols. The percentage of apoptotic cells were analyzed using flow cytometry with an EPICS XL-MCL FACSCanto II cytometer (BD Biosciences). The percentage of apoptotic cells (early and late) in each quadrant was calculated using MultiCycle Software for Windows (Beckman Instruments, Inc.) with the following equation: Apoptotic index = the rate of Early apoptotic cells in lower right quadrant + the rate of late apoptosis or necrosis in the upper right quadrant.

Colony formation assay. Cells were seeded in 6-well plates (200 cells/well) and incubated in the presence of VCR (0.6 g/ml) in a humidified atmosphere of 95% air, 5% CO_2 at 37˚C for 14 days. Cells were then washed twice with PBS, fixed with glutaraldehyde (6.0% v/v) and stained with crystal violet (0.5% w/v). Colony numbers were counted manually under an Olympus CKX53 inverted microscope (Olympus Corporation) at x40 magnification.

Transwell invasion assay. Cell invasion was assessed using an 8-μm Matrigel invasion chamber (BD Bioscience) with 24-well plates. Cells in the upper chamber ($5x10^4$) were suspended in 100 μl RPMI-1640 containing Matrigel (BD Biosciences) without serum, and the lower chamber was seeded with 700 μl RPMI-1640 containing 10% FBS. Following 48 h incubation, cells that had invaded through the membranes were fixed using 4% polyoxymethylene for 5 min and stained with Giemsa dye for 20 min at room temperature. The number of visible cells was counted in five random fields of view under a light microscope (magnification, x200).

Wound healing assay. A total of ~$3x10^6$ cells were seeded in 6-well plates. When cell confluence reached between 90-100%, a straight central linear wound was created in confluent cells using a 200-μl sterile pipette tip. Subsequently, cells were rinsed twice with PBS to remove any debris prior to culture in serum-free growth medium. Wound healing was observed at different time points (0, 24, 48 and 72 h), and the wound size was imaged under an Olympus CKX53 inverted light microscope (magnification, x40; Olympus Corporation). The Digimizer software system (version no. 5.3.4; MedCalc Software) was used to measure the distance between the two edges of the scratch.

Western blotting. Cytoplasmic proteins were extracted using a cell lysate extraction kit (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's protocol. Nuclear proteins were extracted using a EpiQuik™ Nuclear Extraction kit (cat. no. OP-0002; EpiGentek Group, Inc.), $1x10^7$ cells were transferred to a 1.5-ml microcentrifuge tube and centrifuged at 500 x g at 4˚C for 3 min to harvest the cell pellet. Ice-cold cytoplasmic extraction reagent was added, and the microcentrifuge tubes were left to incubate on ice for 1 min. Following incubation, the tubes were centrifuged at 4˚C for 5 min in a microcentrifuge (16,000 x g at 4˚C) to get the insoluble fraction, and subsequently suspended in ice-cold nuclear extraction reagent. Then, the tubes were centrifuged at 16,000 x g at 4˚C for 10 min to get nuclear extract (the supernatant). The concentration of extracted protein was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 2 μg of protein was loaded per lane and separated via 12% SDS-PAGE, transferred to PVDF membranes and blocked with 5% skimmed milk at 37˚C for 60 min. Membranes were subsequently incubated with the following primary antibodies overnight at 4˚C: Anti-Siva-1 (1:100), anti-MDR1 (1:1,500), anti-MRP1 (1:1,000), anti-NF-κB (1:1,500), anti-Lamin B1 (1:1,000) and anti-GAPDH (1:1,000). After three washes in TBS with 0.1%[v/v] Tween-20 (Thermo Fisher Scientific, Inc.), membranes were incubated with the HRP-conjugated goat anti-rabbit IgG H&L secondary antibody (1:1,000) for 1 h at room temperature. The Odyssey Fc Imaging System (LI-COR Biosciences) was used to analyze the optical density of samples. The semi-quantitative analysis was performed using ImageJ software (v 1.8.0; National Institutes of Health). GAPDH and Lamin B1 served as loading controls.

Statistical analysis. All statistical analyses were performed using SPSS version 13.0 (SPSS, Inc.), using Student's t-test, χ^2 test or one-way ANOVA. Bonferroni post hoc analysis was employed to perform multiple comparison tests. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Construction and identification of LV-Siva-1-GFP lentiviral vectors. DNA sequence analysis demonstrated that the RNA coding frames and frame sequences, as well as the recombinant pGV-Siva-1-GFP and pGV-NC-GFP plasmids were constructed successfully. Siva-1 and NC lentiviral vectors, LV-Siva-1-GFP and LV-NC-GFP, were produced following co-transfection with a packaging vector (pHelper 1.0) and a vesicular stomatitis virus glycoprotein expression plasmid (pHelper 2.0) in 293T cells. As presented in Fig. 1, GFP fluorescence indicated that the lentiviral vector was successfully generated for use in the present study. The viral titer was $5x10^8$ TU/ml medium.

Overexpression of Siva-1 in gastric cancer cells with recombinant lentivirus. To determine the effects of Siva-1

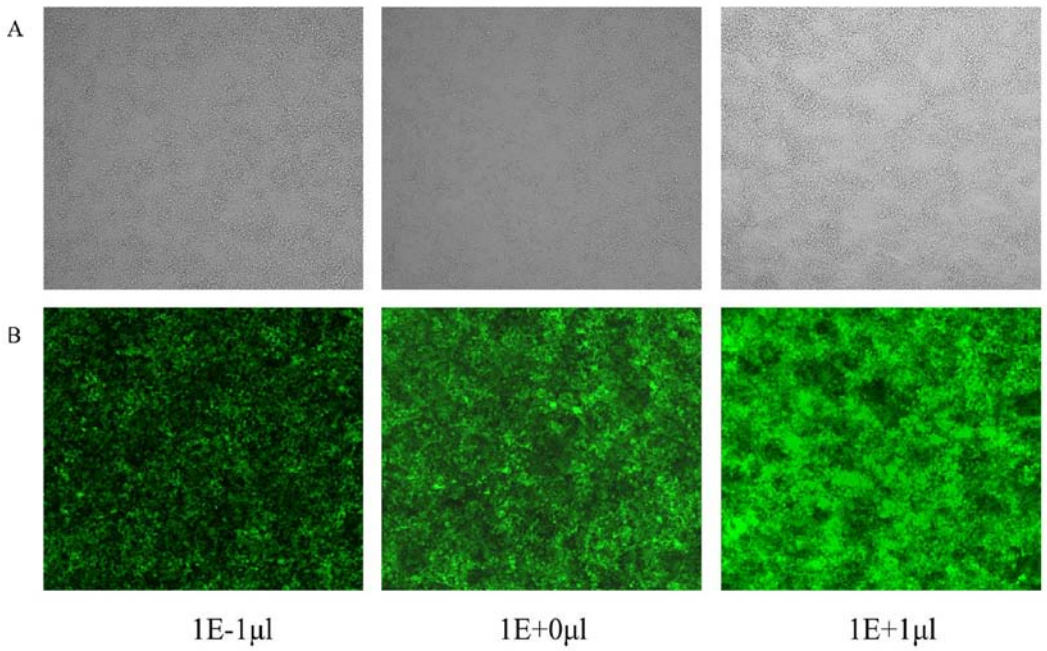

Figure 1. Recombinant pGV358-GFP-SIVA-1 plasmid was transfected into 293T cells to determine lentivirus titers using the end-point dilution method, which involved counting the number of infected green cells under fluorescence microscopy (magnification, x100). The viral dilution factor was 1:1,000. LV, lentivirus; NC, negative control; MOI, multiplicity of infection. (A) In light microscope; (B) in fluorescence microscopy.

overexpression in gastric cancer cells transfected with recombinant lentivirus, proteins were extracted from KATO III/VCR + LV-Siva-1, KATO III/VCR + LV-NC and KATO III/VCR cells, after which Siva-1 expression was assessed by western blotting. The results indicated significantly increased (1.6-fold) Siva-1 protein expression in KATO III/VCR + LV-Siva-1 cells compared with KATO III/VCR + LV-NC and KATO III/VCR cells ($P<0.05$; Fig. 2 and Table I). No significant differences were identified between the KATO III/VCR + LV-NC and KATO III/VCR groups. The results indicated that KATO III/VCR cells transfected with LV-Siva-1-GFP effectively translated more Siva-1 protein.

Siva-1 overexpression promotes multidrug resistance. To elucidate the role of Siva-1 overexpression in anticancer drug resistance, the IC_{50} values of KATO III/VCR + LV-Siva-1 cells exposed to three clinical chemotherapeutic drugs (VCR, 5-fluorouracil and doxorubicin) at 37˚C were determined. The diluted VCR (1.8 μg/ml), 5-FU (20 μM) and DOX (0.4 μg/ml) were added to each well for 48 h. The results revealed that compared with the KATO III/VCR + LV-NC group and the KATO III/VCR group, the KATO III/VCR + LV-Siva-1 group exhibited significantly increased IC_{50} values for VCR, 5-fluorouracil and doxorubicin ($P<0.05$; Fig. 3 and Table II).

Siva-1 overexpression increases the pump rate of doxorubicin. MRP1 is best known for its contributions to chemoresistance, serving a role in anticancer drug efflux (23). Intracellular drug accumulation and retention were evaluated using doxorubicin as a probe in gastric cancer cells. As indicated in Fig. 4, the pump rates of doxorubicin in KATO III/VCR + LV-Siva-1 vs. KATO III/VCR + LV-NC cells and KATO III/VCR cells were 44.12±1.54% vs. 27.66±2.12% and 32.72±1.36%, respectively ($P<0.05$). The results indicated that the KATO III/VCR + LV-Siva-1 group exhibited significantly decreased doxorubicin accumulation and retention, as well as higher release indices of doxorubicin, which suggested that Siva-1 overexpression increased drug efflux in gastric cancer cells and promoted drug resistances.

Siva-1 overexpression prevents cellular apoptosis and promotes KATO III/VCR cell proliferation. To verify the hypothesis that Siva-1 overexpression suppressed gastric cancer cell apoptosis and promoted vincristine-resistant human gastric cancer cell proliferation, the effect of LV-Siva-1-GFP on vincristine-induced gastric cancer cell apoptosis was determined by calculating the apoptosis index. Cells were stained with Annexin V PE and 7-AAD, and analyzed by flow cytometry. The results revealed that the apoptotic rate of the KATO III/VCR + LV-Siva-1 group was 8.03±0.2%, which was significantly lower than that of the KATO III/VCR + LV-NC (18.99±0.34%) and KATO III/VCR groups (17.93±0.29%; $P<0.05$; Fig. 5A and B). Furthermore, the results of the colony formation assay indicated that Siva-1-overexpressing KATO III cells increased colony formation (21.00±2.00) compared with control cells (11.33±2.52 and 10.67±3.06, respectively; $P<0.05$; Fig. 5C and D).

Siva-1 promotes migration and invasion in vitro. Stable Siva-1 overexpression was induced in KATO III/VCR, KATO III/VCR + LV-Siva-1 and KATO III/VCR + LV-NC cells to ascertain the role of Siva-1 in cell migration and invasion. The results of the wound healing assay revealed that Siva-1 overexpression in KATO III/VCR cells significantly enhanced wound healing by increasing wound closure and cell migration ($P<0.05$; Fig. 6A and B). In addition, the results of Transwell

Table I. Relative expression of various proteins determined by western blotting.

Group	Siva-1	MDR1	MRP1	NF-κB
KATO III/VCR + LV-Siva-1	1.38±0.04	0.48±0.02	0.89±0.03	2.30±0.07
KATO III/VCR + LV-NC	0.56±0.04	0.21±0.03	0.30±0.03	0.75±0.03
KATO III/VCR	0.70±0.06	0.18±0.02	0.19±0.07	0.91±0.03

The relative expression rate is based on the expression levels of target protein vs. the internal reference. LV, lentivirus; MDR1, multidrug resistance 1; MRP1, multidrug resistance protein 1; NC, negative control; VCR, vincristine.

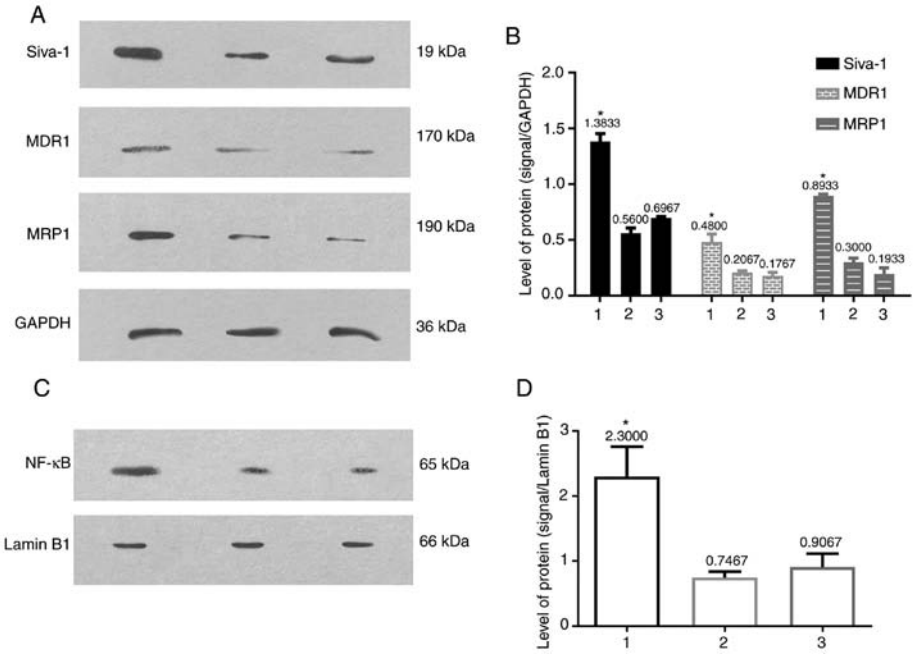

Figure 2. Siva-1, NF-κB, MDR1 and MRP1 protein expression is determined via western blotting. MDR1 and MRP1 protein levels were increased following Siva-1 overexpression and NF-κB was active following its rapid translocation into the nucleus after the same treatment. (A) Western blot analysis and (B) subsequent semi-quantification of Siva-1, MDR1 and MRP1 protein levels in the three groups. (C) Western blot analysis and (D) subsequent semi-quantification of NF-κB protein levels in the three groups. Expression was normalized to that of GAPDH or Lamin B1, and presented as the mean ± standard deviation. *P<0.05 vs. group 2 and 3. 1, KATO III/VCR + LV-Siva-1; 2, KATO III/VCR + LV-NC; 3, KATO III/VCR. MDR1, multidrug resistance 1; MRP1, multidrug resistance protein 1; VCR, vincristine; LV, lentivirus; NC, negative control.

assays indicated that cells with higher Siva-1 expression demonstrated significantly increased invasion (P<0.05; Fig. 6C and D). These data suggested that Siva-1 overexpression increased the invasive abilities of VCR-resistant human gastric cancer cells *in vitro*.

Siva-1 overexpression increases NF-κB, MDR1 and MRP1 expression. To investigate the mechanism by which LV-Siva-1-GFP induces MDR in KATO III/VCR cells, levels of several well-known multidrug resistance-associated proteins, including MDR1 and MRP1, were determined by western blotting. A cell fractionation kit was used to obtain nuclear lysates, after which western blotting was performed using antibodies against NF-κB in the nucleus. The results revealed that MDR1, MRP1 and nuclear NF-κB levels were higher in the KATO III/VCR + LV-Siva-1 group compared with the KATO III/VCR + LV-NC and KATO III/VCR groups (P<0.05). However, no significant differences were identified between the latter two groups (Fig. 2).

Discussion

Gastric cancer is one of the most common types of digestive tract malignancy worldwide with the highest incidence and mortality rates (24) Although the surgical removal of lesions is currently the main treatment for patients with gastric cancer, chemotherapy still serves a key role post-surgery in eradicating malignant cells as the majority of patients are diagnosed at an advanced stage. Multidrug resistance is usually associated with the poor prognosis of patients with gastric cancer (25,26). Therefore, preventing multidrug resistance to improve the efficacy of chemotherapy is imperative. Chemoresistance represents an event whereby cancer cells exhibit tolerance to a specific chemotherapeutic agent or class of pharmaceutical drug. The development of chemoresistance is a major obstacle for successful anticancer therapy. Understanding the molecular mechanisms underlying chemoresistance is therefore necessary to improve the therapeutic efficacy of cytotoxic drugs.

Table II. IC_{50} values were determined for anticancer drugs applied to KATO III/VCR cells by a MTT assay.

Group	Vincristine (μg/ml)	5-fluorouracil (μg/ml)	Doxorubicin (μg/ml)
KATO III/VCR + LV-Siva-1	873.27±29.31	645.91±20.37	217.55±21.12
KATO III/VCR + LV-NC	563.47±35.25	502.14±19.57	177.01±25.91
KATO III/VCR	582.63±37.25	512.62±13.72	167.35±16.52

LV, lentivirus; NC, negative control; VCR, vincristine.

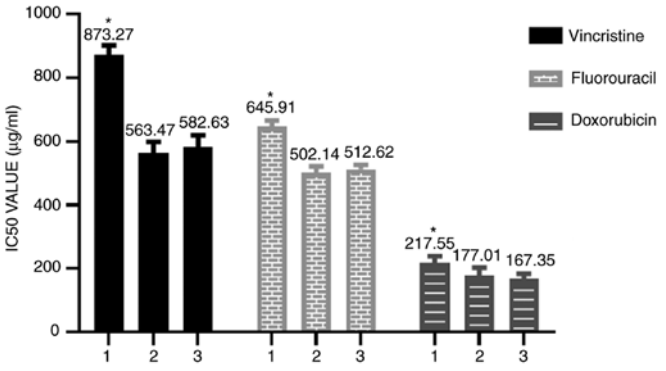

Figure 3. IC_{50} values for anticancer drugs applied to KATO III/VCR cells were evaluated using a MTT assay. Data were presented as the mean ± standard deviation of four independent experiments. *P<0.05 vs. group 2 and 3. 1, KATO III/VCR + LV-Siva-1; 2, KATO III/VCR + LV-NC; 3, KATO III/VCR. VCR, vincristine; LV, lentivirus; NC, negative control; IC_{50}, 50% inhibitory concentration.

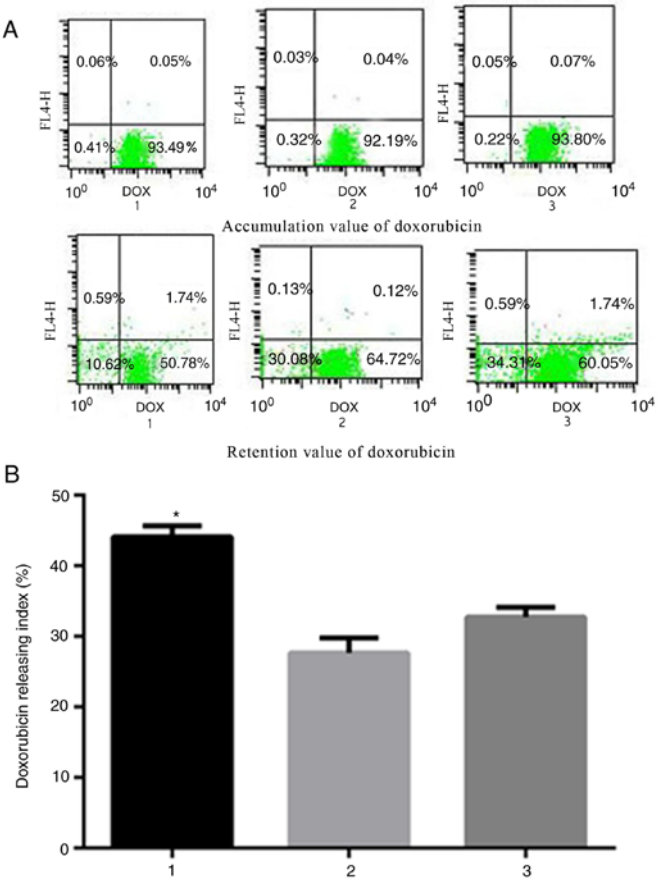

Figure 4. Pump rate of doxorubicin in KATO III/VCR cells after Siva-1 gene transfection. (A) Pump rate was analyzed by flow cytometry and (B) plotted. Data are presented as the mean ± standard deviation. *P<0.05 vs. group 2 and 3. 1, KATO III/VCR + LV-Siva-1; 2, KATO III/VCR + LV-NC; 3, KATO III/VCR. VCR, vincristine; LV, lentivirus; NC, negative control; DOX, doxorubicin.

The Siva-1 protein serves a crucial role in certain extrinsic and intrinsic apoptosis signaling pathways (16). However, Siva-1 has been demonstrated to serve contradictory roles in previous studies. For example, Siva-1 is downregulated in colorectal cancer and breast cancer (27-29), but as an important regulator of apoptosis and metastasis, Siva-1 is also highly expressed and facilitates tumorigenesis in a number of malignant tumors, including ovarian cancer (30), osteosarcoma (18), non-small cell lung cancer (17) and gastric cancer (29). Although Siva-1 was initially identified as a promoter of apoptosis (7), the underlying molecular mechanism requires further investigation. The results of the present study indicated that Siva-1 overexpression inhibited apoptosis and enhanced multidrug resistance. It was also revealed in this study that Siva-1 increased the colony formation and invasion of cells, potentially by acting to decrease the expression of NF-κB. NF-κB is a transcription factor that regulates the expression of a wide variety of genes involved in various cellular events, including inflammation, immune response, proliferation, apoptosis and multidrug resistance (31-33). Additionally, NF-κB serves a key role in cancer development and metastasis (34). NF-κB is inactive in the cytoplasm when bound to IκB. When IκB is ubiquitinated and subsequently degraded, NF-κB is exposed to a nuclear localization sequence on the NF-κB subunit RelA (p65), transferring the molecule to the nucleus (35,36). The results of the current study indicated that Siva-1 functioned as a regulator of MDR1 and MRP1 gene expression in gastric cancer cells via promotion of NF-κB expression. Overexpression of Siva-1 in VCR-resistant cell lines decreased the sensitivity of KATO III/VCR cells towards VCR by enhancing the activity of NF-κB and thereby increasing the expression of MDR1 and MRP1 to enhance chemoresistance. This result is consistent with a previous report in which NF-κB activated the overexpression of antiapoptotic genes (37).

The findings of the present study indicated that Siva-1 may serve as a regulator for drug-related signal proteins, MDR1 and MRP1, *in vitro*. These results are consistent with additional *in vivo* analyses performed in a separate study: KATO III/VCR cells were implanted subcutaneously into the flanks

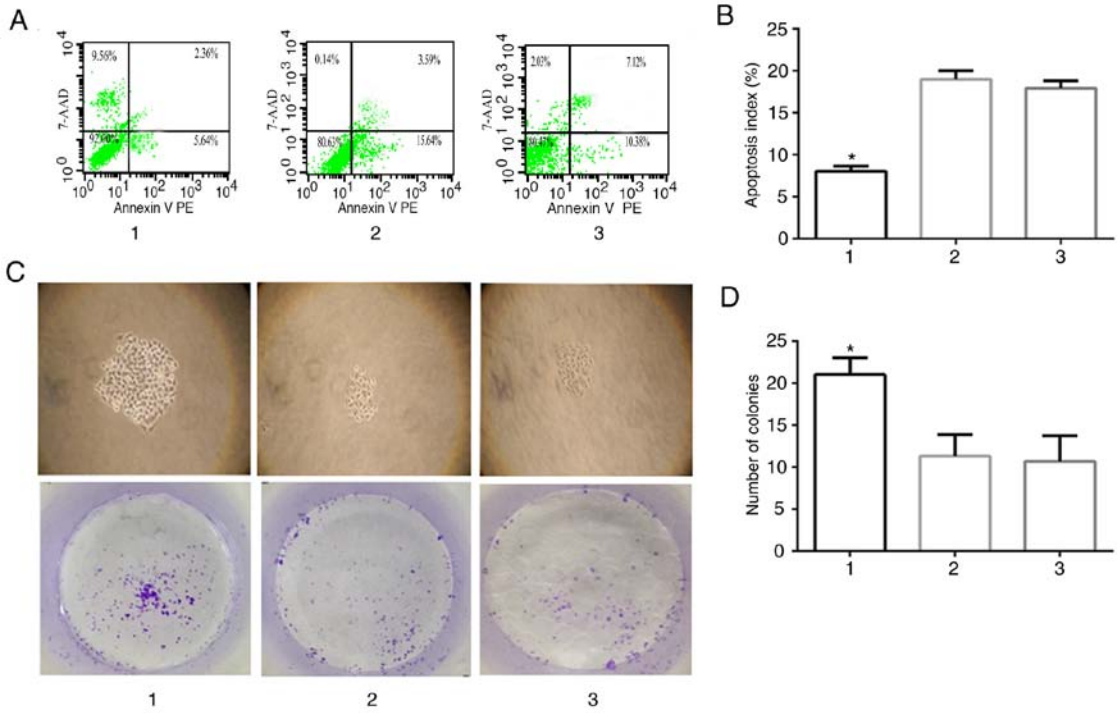

Figure 5. Effect of Siva-1 overexpression on KATO III/VCR cell growth. (A and B) Apoptotic rate in Siva-1 overexpressed-KATO III/VCR cells was analyzed by flow cytometry. (C and D) KATO III/VCR + LV-Siva-1 cells, KATO III/VCR + LV-NC cells and KATO III/VCR cells were plated in 6-well plates at a density of 200 cells/well, after which colony growth was observed under an optical microscope following 14 days (magnification, x40). The surviving fraction of cells (visible colonies) was stained with gentian violet and counted manually. Data are presented as the mean ± standard deviation from 3 independent experiments. *P<0.05 vs. group 2 and 3. 1, KATO III/VCR + LV-Siva-1; 2, KATO III/VCR + LV-NC; 3, KATO III/VCR; VCR, vincristine; LV, lentivirus; NC, negative control; 7-AAD, 7-amino-actinomyosin D.

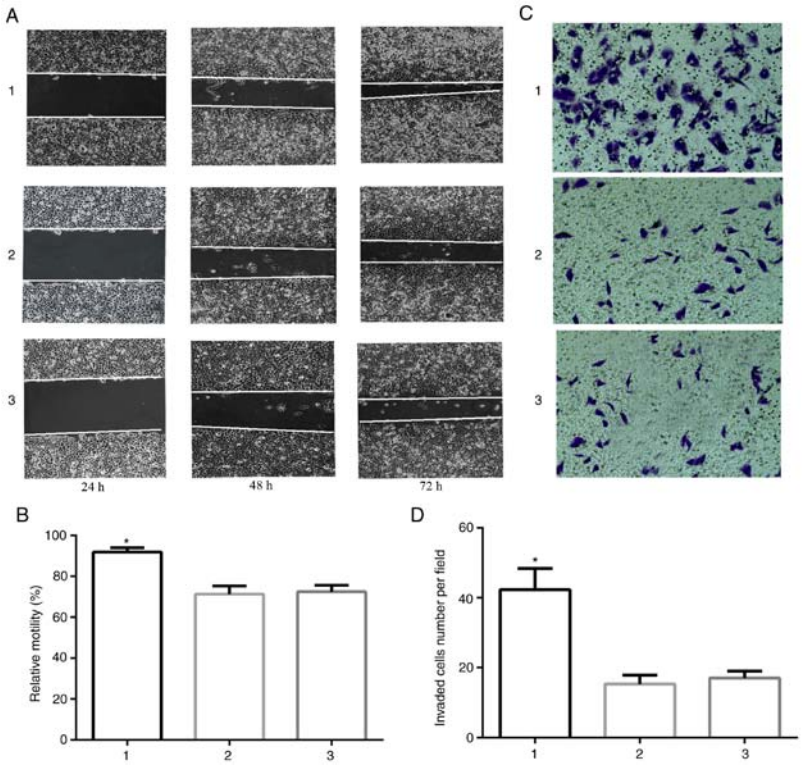

Figure 6. Siva-1 overexpression increases KATO III/VCR cell migration and invasion. (A and B) KATO III/VCR + LV-Siva-1 cells, KATO III/VCR + LV-NC cells and KATO III/VCR cells were cultured to confluence on 6-well plates, after which a central linear wound was created with a 200-μl sterile pipette tip. The wound was imaged over a 4-day interval (magnification, x40). (C and D) KATO III/VCR + LV-Siva-1 cells, KATO III/VCR + LV-NC cells and KATO III/VCR cells were loaded into the upper chambers of a Matrigel-coated Transwell plate. Filtrated cells on the undersurface of the polycarbonate membranes were stained and counted under an optical microscope after 48 h (magnification, x200). *P<0.05 vs. group 2 and 3. 1, KATO III/VCR + LV-Siva-1; 2, KATO III/VCR + LV-NC; 3, KATO III/VCR. VCR, vincristine; LV, lentivirus; NC, negative control.

of BALB/c nude mice. When the resulting tumor measured 5 mm in diameter, animals were administered an intratumoral injection of LV-Siva-1-GFP or LV-GFP. VCR was administered via intraperitoneal injection. The tumor volumes were monitored and analyzed (38,39). The above results (unpublished data) of the procedure *in vivo* was consistent with the results of the current study. This may also indicate the role served by Siva-1 in overcoming drug resistance in gastric cancer.

MDR1 and MRP1 have been widely investigated as multidrug resistance proteins and are associated with cancer therapeutic resistance. MDR1 and MRP1 have also been identified as the major drug efflux pumps responsible for multidrug resistance (40). The MDR1 gene sequence was examined by Bentires-Alj *et al* (41) and a putative NF-κB binding site (CCTTTCGGGG) was identified in the first intron of the MDR1 gene promoter. The expression of MDR1 (also termed P-glycoprotein) RNA can be reduced by promoting the expression of NF-κB so that sensitivity to chemotherapy can be enhanced in digestive malignant cells (42). Drug efflux transporters, including MRP1, can significantly influence the transfer of drugs. The major roles of MRP1 include the efflux of endogenous metabolites, the transport of inflammatory mediators and the development of drug resistance in a variety of diseases (43). MRP1, an ATP-dependent transmembrane glycoprotein, is ubiquitously expressed and participates in the multidrug resistance of various types of tumor cell (44,45).

The current study hypothesized that suppressed NF-κB levels mediated by Siva-1 could be used to treat patients with gastric cancer and multidrug resistance. The study indicated that Siva-1 acts as a cancer-promoting factor and a antiapoptotic protein. MDR1 and MRP1 gene regulation were analyzed by overexpressing Siva-1 and subsequently upregulating NF-κB in the KATO III gastric cancer cell lines. Given the crucial role of Siva-1 in the regulation of apoptosis and tumor metastasis, it may represent a potential target to address the major challenges of therapeutic intervention in patients with cancer, including cancer relapse and chemotherapy resistance.

Acknowledgements

The authors would like to thank Mrs. Chang Wang (Guangxi Medical University, Taiyuan Langdong Hospital) for her assistance and contribution to the team.

Funding

The current study was supported by grants from the Scientific Research Project of Guangxi Health Commission (grant no. Z20180739), the Youth Foundation of People's Hospital of Guangxi Zhuang Autonomous Region (grant no. QN2018-22) and the Natural Science Foundation of China (grant no. 81660416).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FBK performed the western blot experiments and made substantial contributions to writing the manuscript. QMD established the gastric cancer cell line with stable overexpression of Siva-1. HQD measured the IC_{50} of gastric cells to vincristine, 5-fluorouracil and doxorubicin. CCD performed the flow cytometry experiments. LL performed the colony formation assay and wound healing assay. CGH performed the Transwell assay. XTW acquired funding for the project and performed the western blot experiments. SX drafted the manuscript and contributed to the conception and design. WM analyzed the data generated during the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Emmings E, Mullany S, Chang Z, Landen CN Jr, Linder S and Bazzaro M: Targeting mitochondria for treatment of chemoresistant ovarian cancer. Int J Mol Sci 20: 229, 2019.
2. Kongsema M, Wongkhieo S, Khongkow M, Lam EW, Boonnoy P, Vongsangnak W and Wong Ekkabut J: Molecular mechanism of Forkhead box M1 inhibition by thiostrepton in breast cancer cells. Oncol Rep 42: 953-962, 2019.
3. Zeng L, Liao Q, Zou Z, Wen Y, Wang J, Liu C, He Q, Weng N, Zeng J, Tang H, *et al*: Long non-coding RNA XLOC_006753 promotes the development of multidrug resistance in gastric cancer cells through the PI3K/AKT/mTOR signaling pathway. Cell Physiol Biochem 51: 1221-1236, 2018.
4. Chen S, Wu J, Jiao K, Wu Q, Ma J, Chen D, Kang J, Zhao G, Shi Y, Fan D, *et al*: MicroRNA-495-3p inhibits multidrug resistance by modulating autophagy through GRP78/mTOR axis in gastric cancer. Cell Death Dis 9: 1070, 2018.
5. Kozovska Z, Gabrisova V and Kucerova L: Colon cancer: cancer stem cells markers, drug resistance and treatment. Biomed Pharmacother 68: 911-916, 2014.
6. Alamolhodaei NS, Tsatsakis AM, Ramezani M, Hayes AW and Karimi G: Resveratrol as MDR reversion molecule in breast cancer: An overview. Food Chem Toxicol 103: 223-232, 2017.
7. Liu T, Ma Y, Wang Z, Zhang W and Yang X: Siva 1 inhibits cervical cancer progression and its clinical prognosis significance. Cancer Manag Res 12: 303-311, 2020.
8. Prasad KV, Ao Z, Yoon Y, Wu MX, Rizk M, Jacquot S and Schlossman SF: CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. Proc Natl Acad Sci USA 94: 6346-6351, 1997.
9. Chen JY, Yang LX and Huang ZF: The N terminal 33 amino acid domain of Siva 1 is sufficient for nuclear localization. Braz J Med Biol Res 46: 1021-1027, 2013.
10. Cottle DL, McGrath MJ, Wilding BR, Cowling BS, Kane JM, D'Arcy CE, Holdsworth M, Hatzinisiriou I, Prescott M, Brown S, *et al*: SLIMMER (FHL1B/KyoT3) interacts with the proapoptotic protein Siva-1 (CD27BP) and delays skeletal myoblast apoptosis. J Biol Chem 284: 26964-26977, 2009.
11. Chu F, Borthakur A, Sun X, Barkinge J, Gudi R, Hawkins S and Prasad KV: The Siva-1 putative amphipathic helical region (SAH) is sufficient to bind to BCL-XL and sensitize cells to UV radiation induced apoptosis. Apoptosis 9: 83-95, 2004.

12. Balci-Peynircioglu B, Waite AL, Hu C, Richards N, Staubach-Grosse A, Yilmaz E and Gumucio DL: Pyrin, product of the MEFV locus, interacts with the proapoptotic protein, Siva. J Cell Physiol 216: 595-602, 2008.
13. Lin FT, Lai YJ, Makarova N, Tigyi G and Lin WC: The lysophosphatidic acid 2 receptor mediates down-regulation of Siva-1 to promote cell survival. J Biol Chem 282: 37759-37769, 2007.
14. Rapisarda V, Ledda C, Matera S, Fago L, Arrabito G, Falzone L, Marconi A, Libra M and Loreto C: Absence of t(14;18) chromosome translocation in agricultural workers after short term exposure to pesticides. Mol Med Rep 15: 3379-3382, 2107.
15. Li T, Lv M, Chen X, Yu Y, Zang G and Tang Z: Plumbagin inhibits proliferation and induces apoptosis of hepatocellular carcinoma by downregulating the expression of SIVA. Drug Des Devel Ther 13: 1289-1300, 2019.
16. Vachtenheim J Jr, Lischke R and Vachtenheim J: Siva 1 emerges as a tissue specific oncogene beyond its classic role of a proapoptotic gene. Onco Targets Ther 11: 6361-6367, 2018.
17. Van Nostrand JL, Brisac A, Mello SS, Jacobs SB, Luong R and Attardi LD: The p53 target gene SIVA enables non-small cell lung cancer development. Cancer Discov 5: 622-635, 2015.
18. Du W, Jiang P, Li N, Mei Y, Wang X, Wen L, Yang X and Wu M: Suppression of p53 activity by Siva1. Cell Death Differ 6: 1493-1504, 2009.
19. Wei L, Sun J, Zhang N, Zheng Y, Wang X, Lv L, Liu J, Xu Y, Shen Y and Yang M: Noncoding RNAs in gastric cancer: Implications for drug resistance. Mol Cancer 19: 62, 2020.
20. Wang XT, Xie YB and Xiao Q: Lentivirus mediated RNA interference targeting E2F 1 inhibits human gastric cancer MGC 803 cell growth in vivo. Exp Mol Med 43: 638-645, 2011.
21. Yan LH, Wang XT, Yang J, Lian C, Kong FB, Wei WY, Luo W, Xiao Q and Xie YB: Reversal of multidrug resistance in gastric cancer cells by CDX2 downregulation. World J Gastroenterol 19: 4155-4165, 2013.
22. Yan LH, Wei WY, Cao WL, Zhang XS, Xie YB and Xiao Q: Overexpression of CDX2 in gastric cancer cells promotes the development of multidrug resistance. Am J Cancer Res 5: 321-332, 2014.
23. Lorendeau D, Dury L, Genoux Bastide E, Lecerf Schmidt F, Simões Pires C, Carrupt PA, Terreux R, Magnard S, Di Pietro A, Boumendjel A, *et al*: Collateral sensitivity of resistant MRP1 overexpressing cells to flavonoids and derivatives through GSH efflux. Biochem Pharmacol 90: 235-245, 2014.
24. Karimi P, Islami F, Anandasabapathy S, Freedman ND and Kamangar F: Gastric cancer: Descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev 23: 700-713, 2014.
25. Zhang ZZ, Yu WX, Zheng M, Liao XH, Wang JC, Yang DY, Lu WX, Wang L, Zhang S, Liu HK, *et al*: PIN1 Inhibition Sensitizes Chemotherapy in Gastric Cancer Cells by Targeting Stem Cell-like Traits and Multiple Biomarkers. Mol Cancer Ther 19: 906-919, 2020.
26. Peng L, Li Y, Wei S, Li X, Dang Y, Zhang W and Zhang G: LAMA4 activated by Androgen receptor induces the cisplatin resistance in gastric cancer. Biomed Pharmacother 124: 109667, 2020.
27. Barkinge JL, Gudi R, Sarah H, Chu F, Borthakur A, Prabhakar BS and Prasad KV: The p53-induced Siva-1 plays a significant role in cisplatin-mediated apoptosis. J Carcinog 8: 2, 2009.
28. Okuno K, Yasutomi M, Nishimura N, Arakawa T, Shiomi M, Hida J, Ueda K and Minami K: Gene expression analysis in colorectal cancer using practical DNA array filter. Dis Colon Rectum 44: 295-299, 2001.
29. Li N, Jiang P, Du W, Wu Z, Li C, Qiao M, Yang X and Wu M: Siva1 suppresses epithelial-mesenchymal transition and metastasis of tumor cells by inhibiting stathmin and stabilizing microtubules. Proc Natl Acad Sci USA 108: 12851-12856, 2011.
30. Wei SH, Lin F, Wang X, Gao P and Zhang HZ: Prognostic significance of stathmin expression in correlation with metastasis and clinicopathological characteristics in human ovarian carcinoma. Acta Histochem 110: 59-65, 2008.
31. Wang X, Huang S, Jiang Y, Liu Y, Song T, Li D and Yang L: Reactive astrocytes increase the expression of P gp and Mrp1 via TNF α and NF κB signaling. Mol Med Rep 17: 1198-1204, 2017.
32. Cui X, Shen D, Kong C, Zhang Z, Zeng Y, Lin X and Liu X: NF-κB suppresses apoptosis and promotes bladder cancer cell proliferation by upregulating survivin expression in vitro and in vivo. Sci Rep 7: 40723, 2017.
33. Shen MY, Wang Y, Cui SY, Wu XL, Guo Y and Xu RR: MicroRNA-125a regulates proliferation and apoptosis of acute myeloid leukemia through targeting NF-κB pathway. Eur Rev Med Pharmacol Sci 23: 3594-3601, 2019.
34. Huber MA, Azoitei N, Baumann B, Grünert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T: NF κB is essential for epithelial mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Investig 114: 569-581, 2004.
35. Nakanishi C and Toi M: Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 5: 297-309, 2005.
36. Liu T, Wei R, Zhang Y, Chen W and Liu H: Association between NF-κB expression and drug resistance of liver cancer. Oncol Lett 17: 1030-1034, 2018,
37. Tanaka T, Hozumi Y, Iino M and Goto K: NAP1L1 regulates NF-κB signaling pathway acting on anti-apoptotic Mcl-1 gene expression. Biochim Biophys Acta Mol Cell Res 1864: 1759-1768, 2017.
38. Wei W, Cao W, Zhan Z, Yan L, Xie Y and Xiao Q: miR-1284 suppresses gastric cancer progression by targeting EIF4A1. OncoTargets Ther 12: 3965-3976, 2019.
39. Cao W, Wei W, Zhan Z, Xie D, Xie Y and Xiao Q: Regulation of drug resistance and metastasis of gastric cancer cells via the microRNA647 ANK2 axis. Int J Mol Med 41: 1958-1966, 2018.
40. Strouse JJ, Ivnitski Steele I, Njus HM, Foutz TD, Yao T, Weiner WS, Schroeder CE, Simpson DS, Maki BE, Li K, *et al*: Selective Efflux Inhibition of ATP binding Cassette Sub family G Member 2. In: Probe Reports from the NIH Molecular Libraries Program (Internet). Bethesda (MD): National Center for Biotechnology Information (US); Nov 30, 2010 9 (updated Feb 25, 2013).
41. Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N, Gielen J, Merville MP and Bours V: NF-kappaB transcription factor induces drug resistance through MDR1 expression in cancer cells. Oncogene 22: 90-97, 2003.
42. Yuan Z, Liang X, Zhan Y, Wang Z, Xu J, Qiu Y, Wang J, Cao Y, Le VM, Ly HT, *et al*: Targeting CD133 reverses drug resistance via the AKT/NF κB/MDR1 pathway in colorectal cancer. Br J Cancer 122: 1342-1353, 2020.
43. Yang AK, Zhou ZW, Wei MQ, Liu JP and Zhou SF: Modulators of multidrug resistance associated proteins in the management of anticancer and antimicrobial drug resistance and the treatment of inflammatory diseases. Curr Top Med Chem 10: 1732-1756, 2010.
44. Sisodiya SM, Lin WR, Harding BN, Squier MV and Thom M: Drug resistance in epilepsy: Expression of drug resistance proteins in common causes of refractory epilepsy. Brain 125: 22-31, 2002.
45. Han F, Liu G, Sun C and Wei J: Ailanthone reverses multidrug resistance by inhibiting the P glycoprotein mediated efflux in resistant K562/A02 cells. Cell Mol Biol (Noisy le grand) 64: 55-61, 2018.