Role of HSP27 in the multidrug sensitivity and resistance of colon cancer cells

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Abstract. Multidrug resistance in cancer cells is a primary factor affecting therapeutic efficacy. Heat shock 27 kD protein 1 (HSP27) is associated with cell apoptosis and resistance to chemotherapy. However, the mechanisms underlying HSP27-associated pathways in colon cancer cells remain unclear. Therefore, the present study used short hairpin (sh) RNA to inhibit HSP27 expression in colon cancer cells in order to investigate the effects *in vitro* and *in vivo*. Flow cytometry was used to investigate cell apoptosis and a xenograft model was employed to examine the tumorigenesis. Protein expression was measured by Western blotting. The results revealed that suppression of HSP27 expression significantly increased cell apoptosis, inhibited tumor growth and enhanced sensitivity to the anti-cancer agents 5-fluorouracil (5-FU) and vincristine (VCR). shHSP27 significantly decreased the expression of notch receptor 1 and the phosphorylation level of Akt and mTOR, and enhanced the effect of 5-FU and VCR. In conclusion, HSP27 suppression enhanced the sensitivity of colon cancer cells to 5-FU and VCR, and increased colon cancer cell apoptosis with and without chemotherapy. Therefore, the development of novel therapeutic agents that inhibit the expression of HSP27 may offer a new treatment option for colon cancer.

Introduction

Colon cancer is one of the most prevalent cancers worldwide (1,2). Data from the United States of America revealed that >1.4 million people were diagnosed with colorectal cancer before 2016 (1). The morbidity and mortality of colon cancer are gradually increasing worldwide (2,3). Although adjuvant chemotherapy has been used to reduce the risk of tumor recurrence and improve survival (4,5), the 5-year survival rates of patients with colon cancer remain unfavorable due to the presence of multidrug resistance in colon cancer cells (6). Chemoresistance develops in the majority of patients with colon cancer, and leads to a decrease in the therapeutic efficacy and potential failure of anticancer agents (6,7).

A number of cellular and non-cellular mechanisms conferring chemoresistance have been described (8-10). Heat shock proteins (HSPs), which increase the migration and invasion of cancer cells and decrease apoptosis, are involved in both cellular and non-cellular resistance in cancer cells (11,12). Heat shock 27 kD protein 1 (HSP27), a member of the HSP family, has been revealed to influence both oncogenesis and chemotherapeutic resistance (13). Previous studies have shown that the upregulation of HSP27 is associated with cell apoptosis and resistance to chemotherapy and radiotherapy in breast (14), lung (15) and colon cancer (16,17). The downregulation of HSP27 may therefore increase the sensitivity of colon cancer cells to several chemotherapeutic agents, including 5-fluorouracil (5-FU) (18), cisplatin (19) and SN38 (20).

HSP27 was found to decrease apoptosis in cancer cells, an effect that contributes to chemoresistance (13,17). However, the underlying mechanisms in colon cancer cells remain unclear. Furthermore, cross-talk among the notch receptor 1 (NOTCH1), mTOR and PI3K/Akt signaling pathways is associated with apoptosis (21). The binding of NOTCH1 to its ligands allows DNA-binding proteins to regulate the expression of NOTCH1 target genes, which are involved in the proliferation, differentiation and apoptosis of various tumor cells (22-24). Additionally, PI3K and its effectors, including Akt and mTOR, play a key role in the proliferation and survival of tumor cells (25). The NOTCH1-Akt/mTOR signaling pathway has been recognized as a potential therapeutic target for the treatment of cancer (26). Therefore, the present study investigated the effect of suppressing HSP27 on multidrug sensitivity in colon cancer cells and explored the NOTCH1-Akt-mTOR signaling pathway as an underlying mechanism.

Materials and methods

Cell culture and treatment with chemotherapeutic agents. The human colon cancer cell line SW480 (27) was obtained from

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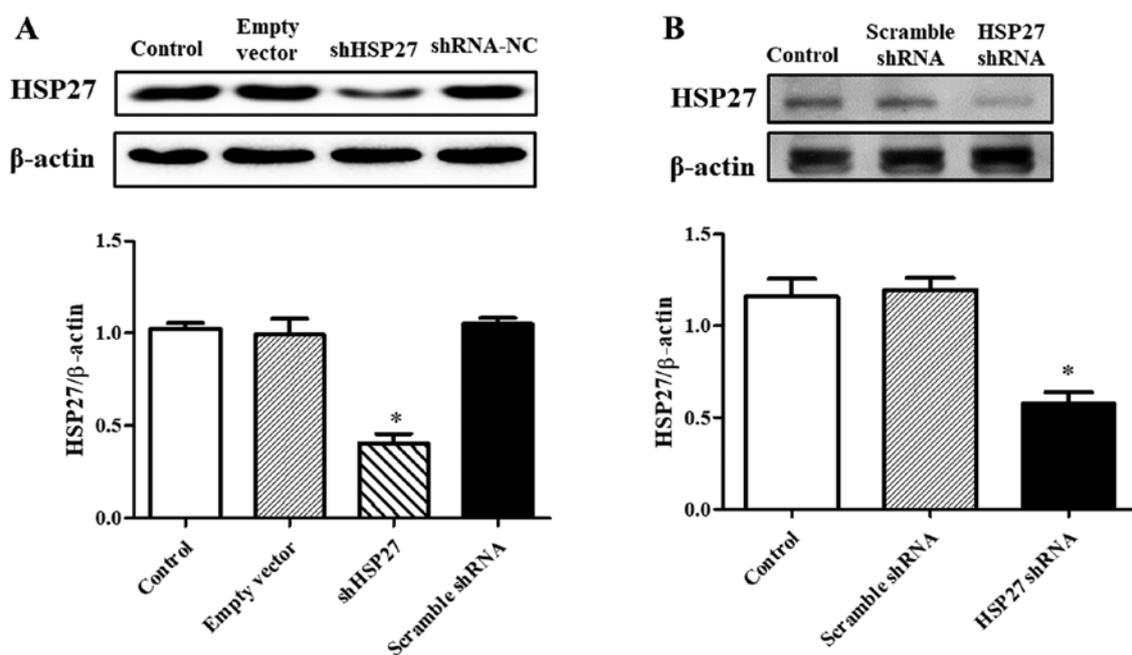E-mail: xiangfengwang@tmmu.edu.cn

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Figure 1. Effect of shHSP27 on HSP27 expression (A) *in vitro* and (B) *in vivo*. Results are expressed as the ratio of HSP27 and β-actin. *P<0.05 vs. the others. n=4 in each group. sh, short hairpin; HSP27, heat shock 27 kD protein 1; NC, negative control.

the American Type Culture Collection (ATCC) and cultured in ATCC-formulated Leiboviz's L-15 medium (Biowest) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO_2. The chemotherapeutic agents 5-FU and vincristine (VCR; both Sigma-Aldrich; Merck KGaA) were diluted with phosphate-buffered saline (PBS). SW480 cells were treated with 50 µg/ml 5-FU (16) and 16 µM VCR (28) at 37°C for 48 h.

Short hairpin (sh) RNA HSP27 transfection in vitro. SW480 cells were transfected with a lentivirus delivering shHSP27 as previously described (29,30). Briefly, SW480 cells (1x10⁶ per well) were plated in 12-well plates and incubated overnight at 37°C. The cells were subsequently transfected with the pLVX-shHSP27 lentivirus or an empty lentiviral vector as control (1x10¹⁰ pfu/ml; Shanghai Genechem, Co., Ltd.). The sequence of the shRNA targeting HSP27 used in the present study was as follows: 5'-AGCTGACGGTCAAGACC AA-3' (31). Scrambled shRNA (5'-TGACGATAAGAACAA TAAC-3') was used as the negative control. Cells were incubated with the lentiviruses at 37°C for 72 h. The transfection efficiency was determined by western blotting.

Flow cytometry. SW480 cells (1x10⁶ per well) were collected and suspended in PBS in a 1 ml eppendorf tube. The cells were subsequently stained with 10 µl Annexin V-FITC and 5 µl propidium iodide (PI; Roche Applied Science) according to the manufacturer's instructions. The percentages of apoptotic SW480 cells transfected with shHSP27 and treated with the chemotherapeutic agents were investigated using a flow cytometer and analyzed with FlowJo software (version 10.0; FlowJo LLC).

Xenograft model in nude mice. A total of 24 male nude mice (weight, 15-20 g) were purchased from Vital River Laboratory

Animal Technology Co., Ltd. and randomly divided into 6 groups as follows: i) Control; ii) shHSP27; iii) control + 5-FU; iv) control + VCR; v) shHSP27 + 5-FU; and vi) shHSP27 + VCR. A total of 1x10⁶ SW480 cells transfected with shHSP27 or scrambled shRNA, suspended in 100 µl PBS, were subcutaneously injected into the left axilla. At 3 days post-injection, the mice were treated with 50 mg/kg/week 5-FU, 80 mg/kg/day VCR or PBS intraperitoneally for 21 days. The mice were subsequently euthanized by the intraperitoneal injection of 200 mg/kg sodium pentobarbital. The tumor tissues were collected for further study and the tumor volume was calculated using the formula: (length x width²)/2 as previously described (32).

The present study was approved by the Research Council and Animal Care and Use Committee of Daping Hospital, Army Medical University. All experiments conformed to the guidelines of the American Association for the Accreditation of Laboratory Animal Care (33).

Western blotting. SW480 cells or tumor tissues were lysed in lysis buffer (Beyotime Institute of Biotechnology). The protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Hyclone Pierce). Protein homogenates (20 µg/lane) were separated by SDS-PAGE on a 8-10% gel and transferred onto polyvinylidene fluoride membranes. The membranes were washed with Tris-buffered saline (TBS) and blocked with 5% milk powder in TBS at room temperature for 1 h. The membranes were incubated with primary antibodies against phospho-Akt (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), Akt (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.), phospho-mTOR (1:1,000; cat. no. 2971; Cell Signaling Technology, Inc.), mTOR (1:1,000; cat. no. 2983; Cell Signaling Technology, Inc.), NOTCH1 (1:1,000; cat. no. 3608; Cell Signaling Technology, Inc.) and β-actin (1:1,000; cat. no. sc-376421; Santa Cruz Biotechnology, Inc.) at 4°C over
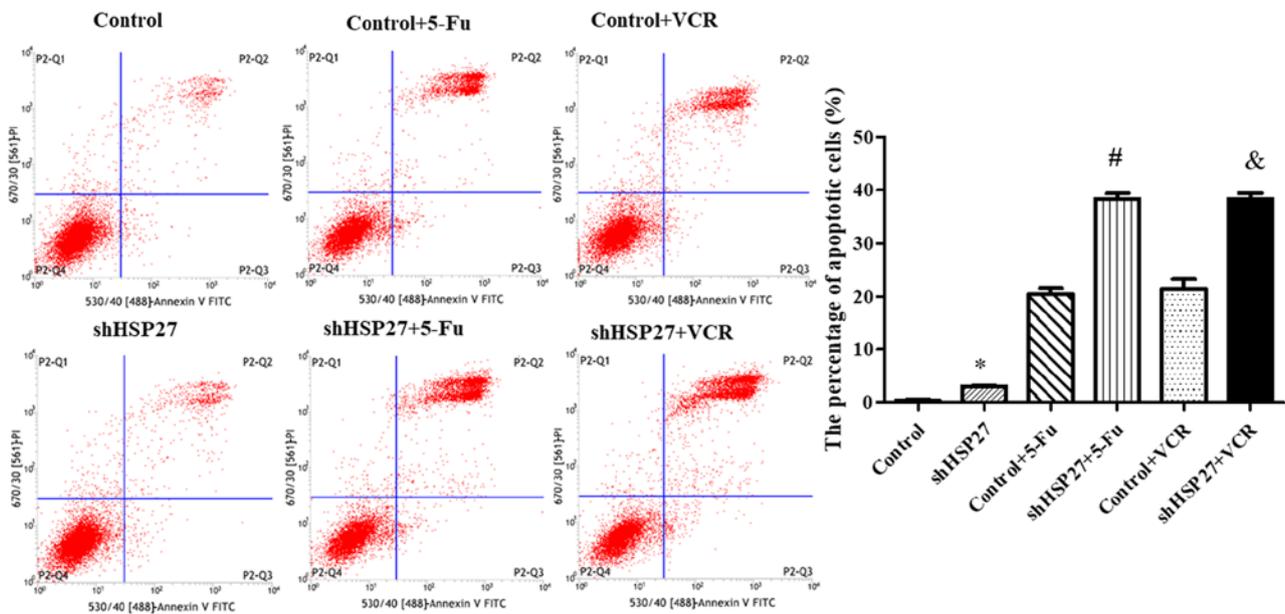
Figure 2. Annexin V-FITC/PI staining of SW480 cells with/without shHSP27 and chemotherapy. *P<0.05 vs. the other groups, #P<0.05 vs. control + 5-FU group, &P<0.05 vs. control + VCR group. n=5 in each group. sh, short hairpin; HSP27, heat shock 27 kD protein 1; 5-FU, 5-fluorouracil; VCR, vincristine.

for 12 h. Then, the primary antibodies were detected with goat anti-rabbit-IgG secondary antibodies (1:5,000; Abcam; cat. no. ab6721) conjugated to horseradish peroxidase, and the bands were visualized with enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). The optical density (OD) value was analyzed with Quantity One (Version 4.6.2; Bio-Rad Laboratories, Inc.). β-actin served as the loading control. Each western blotting was performed ≥3 times.

Statistical analysis. Statistical analyses were performed using SPSS software (version 22.0; IBM Corp.). Comparisons between ≥3 groups were conducted using one-way ANOVA, and comparisons among groups were conducted using one-way ANOVA followed by the Holm-Sidak post hoc test. The data are expressed as the mean ± SEM. P<0.05 was considered to indicate a statistically significant difference.

Results

Suppression of HSP27 expression in SW480 cells. In order to inhibit HSP27 expression, SW480 cells were transfected with a lentiviral vector delivering shHSP27. SW480 cells transfected with an empty lentiviral vector or a lentiviral delivering scrambled shRNA served as the controls. As shown in Fig. 1A, the expression of HSP27 in SW480 cells transfected with shHSP27 was significantly decreased compared with the controls. Further *in vivo* studies revealed that HSP27 expression in tumor tissue was decreased in mice injected with the shHSP27-transfected cells compared with the controls (Fig. 1B).

Role of HSP27 suppression in cancer cell growth and multidrug sensitivity. To further determine the role of HSP27 suppression in cancer cell growth, SW480 cells were transfected with shHSP27 and treated with two chemotherapeutic agents, 5-FU and VCR. Annexin V-FITC/PI staining and flow cytometry revealed that the percentage of apoptotic cells was
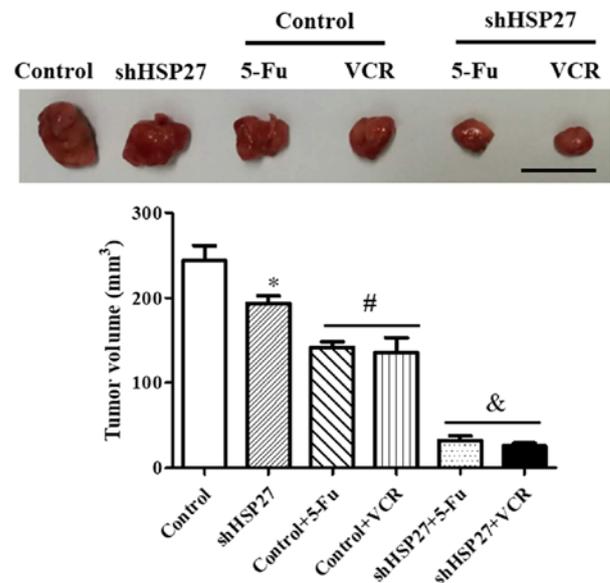

Figure 3. Tumor sizes in the xenograft model. *P<0.05 vs. the other groups, #P<0.05 vs. control, &P<0.05 vs. control + 5-FU or control + VCR. n=4 in each group. sh, short hairpin; HSP27, heat shock 27 kD protein 1; 5-FU, 5-fluorouracil; VCR, vincristine. Scale bar=100 mm.

increased in shHSP27-transfected cells compared with the controls (Fig. 2). Although treatment with 5-FU and VCR alone increased the percentage of apoptotic SW480 cells, the combination of shHSP27 and chemotherapeutic agents resulted in a greater increase in the percentage of apoptotic cells compared with the control groups (Fig. 2).

The volumes of the tumors obtained from the nude mice were subsequently calculated. The data revealed that mice injected with shHSP27-transfected SW480 cells exhibited smaller tumors compared with the control group. Furthermore, shHSP27-transfected SW480 cells increased the inhibitory
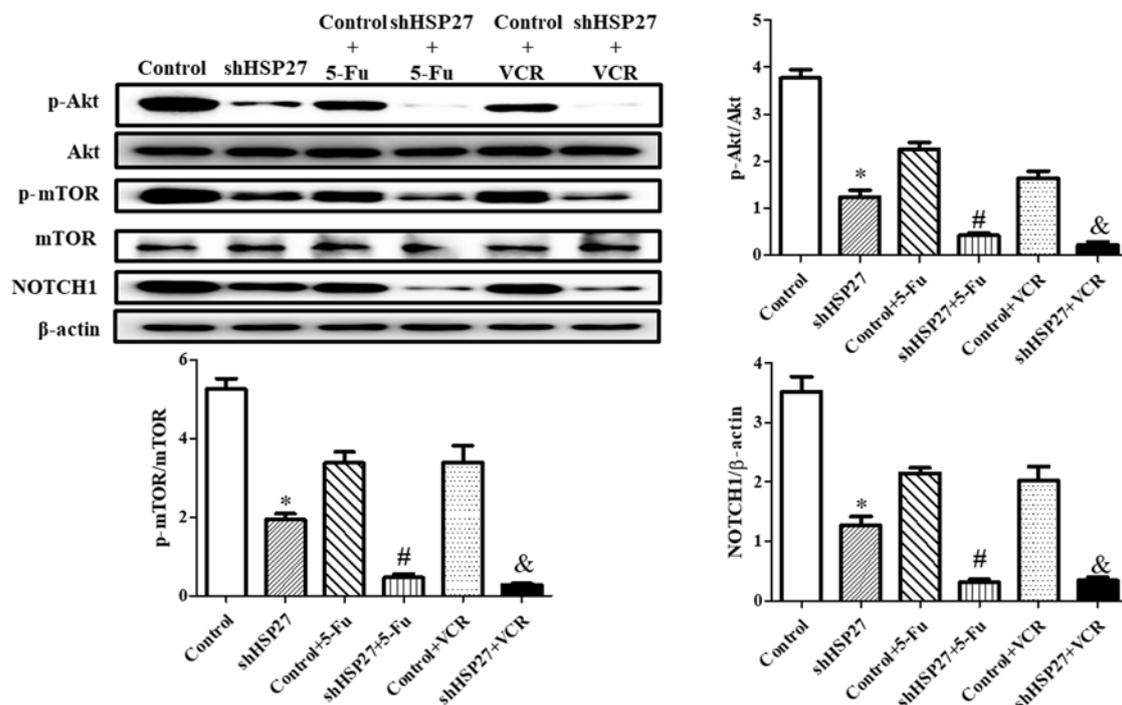
Figure 4. Effect of shHSP27 and chemotherapy on the phosphorylation and expression of the NOTCH1-Akt-mTOR pathway *in vitro*. *P<0.05 vs. the other groups, #P<0.05 vs. control + 5-FU group, &P<0.05 vs. control + VCR group. n=4 in each group. sh, short hairpin; HSP27, heat shock 27 kD protein 1; NOTCH1, notch receptor 1; 5-FU, 5-fluorouracil; VCR, vincristine; p, phosphorylated.
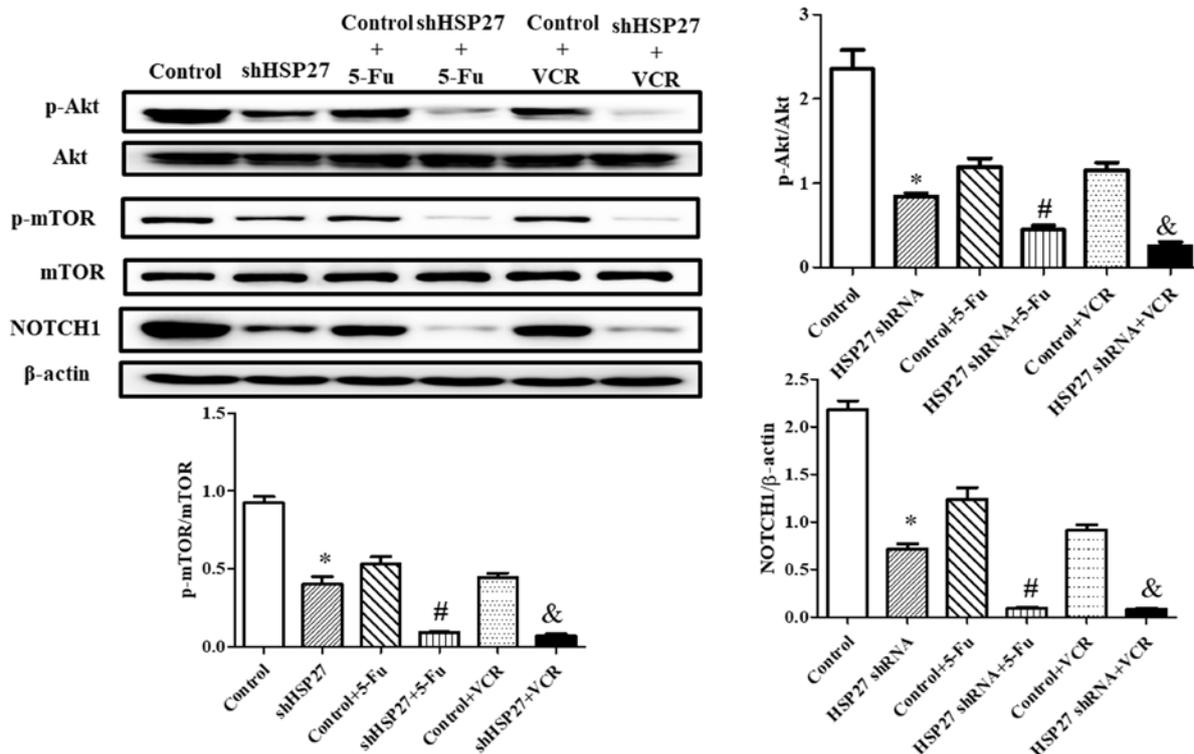

Figure 5. Effect of shHSP27 and chemotherapy on the phosphorylation and expression of the NOTCH1-Akt-mTOR pathway *in vivo*. *P<0.05 vs. the others, #P<0.05 vs. control + 5-FU group, &P<0.05 vs. control + VCR group. n=4 in each group. sh, short hairpin; HSP27, heat shock 27 kD protein 1; NOTCH1, notch receptor 1; 5-FU, 5-fluorouracil; VCR, vincristine.

effect of 5-FU and VCR on tumor growth (Fig. 3), indicating that the suppression of HSP27 might increase sensitivity to anti-cancer agents.

HSP27 suppression decreases chemoresistance via the NOTCH1-Akt-mTOR signaling pathway. The present study revealed that HSP27 suppression significantly decreased

NOTCH1 expression and the phosphorylation level of Akt and mTOR. shHSP27 enhanced the effect of 5-FU and VCR on the downstream Akt-mTOR signaling pathway (34). The decrease in the expression of NOTCH1 and the phosphorylation level of Akt and mTOR following 5-FU or VCR treatment was increased in shHSP27-transfected cells *in vitro* (Fig. 4) and *in vivo* (Fig. 5), compared with the controls.

Discussion

Previous studies have attempted to improve the treatment of colon cancer by combining 5-FU with other agents, such as VCR (2,4,5,35). However, due to multidrug resistance, chemotherapy sensitivity is decreased and chemotherapeutic efficacy is limited, and is a major clinical challenge in the treatment of colon cancer (36). As a chaperone protein that stabilizes proteins under stress, HSP27 is associated with chemoresistance in various cancer cells (19,37,38). Cell proliferation and anti-apoptotic mechanisms have been identified as the main causes of chemoresistance and poor patient prognosis (6-8). Therefore, the role of HSP27 in chemoresistance remains unclear.

In the present study, shRNA was used to decrease the expression of HSP27 in SW480 cells. The results revealed that suppression of HSP27 expression significantly increased cell apoptosis and inhibited tumor growth, and enhanced sensitivity to 5-FU and VCR, compared with the controls.

Increasing evidence has revealed the role of HSP27 in 5-FU resistance in colon cancer cells (15-20). Increased expression of HSP27 resulted in greater resistance to 5-FU in colon cancer cells (18,39), whereas suppression of HSP27 via gene knockdown (16,38) or pharmaceuticals (39,40), enhanced sensitivity to 5-FU. Moreover, HSP27 is associated with VCR resistance in cancer cells (41). Proteome analysis indicated that HSP27 is differentially expressed between VCR-sensitive and VCR-resistant human gastric carcinoma cells (41). Furthermore, suppression of HSP27 expression enhanced VCR chemosensitivity and induced the cancer cells to exhibit apoptotic morphological features following VCR treatment (41). The data in the present suggested that decreased expression of HSP27 contributed to decreased VCR resistance.

5-FU and VCR exert their anti-cancer effect by inducing apoptosis, mainly through the Akt-mTOR signaling pathway (42,43). Evidence shows that the Akt/mTOR signaling pathway is a major negative regulator of apoptosis (43,44). Decreased cell apoptosis, which is induced by increased Akt/mTOR activation, also plays a key role in drug resistance (45). HSP27, which acts as a scaffold protein, can directly bind to Akt, resulting in increased phosphorylation of Akt and mTOR. This activates the Akt/mTOR signaling pathway and leads to decreased apoptosis and increased chemoresistance in cancer cells (13,15). The results obtained in the present study suggested that the inhibition of HSP27 expression suppressed Akt and mTOR phosphorylation, and increased apoptosis associated with chemotherapeutic agents.

NOTCH1 is involved in the regulation of the Akt/mTOR signaling pathway (46,47), which is widely acknowledged to promote cancer development (22-26,42). The present study revealed that shHSP27 decreased NOTCH1 expression, indicating that HSP27 might regulate chemoresistance in colon cancer cells via NOTCH1 and that NOTCH1 might regulate of HSP27 in the Akt/mTOR signaling pathway. Future studies are required to investigate the interactions between HSP27 and the NOTCH1-Akt/mTOR signaling pathway.

In summary, the present study revealed that HSP27 suppression enhanced the sensitivity of SW480 cells to 5-FU and VCR, and increased the apoptosis of the cells with and without chemotherapy. Therefore, the development of pharmacological agents that inhibit HSP27 expression may offer a new treatment approach for colon cancer.

Availability of data and materials

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

Authors' contributions

ZL, BL and XW conceived and designed the study. ZL, YLi and YLo performed the experiments. ZL, YLo and XW wrote the paper. BL, YLi and XW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research.

Ethics approval and consent to participate

The present study was approved by the Research Council and Animal Care and Use Committee of Daping Hospital, Army Medical University (Chongqing, China). All experiments conformed to the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the tumor burden did not exceed the recommendations of the University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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