Heat shock protein 27 knockdown using nucleotide-based therapies enhances sensitivity to 5-FU chemotherapy in SW480 human colon cancer cells

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Abstract. Heat shock protein 27 (Hsp27) is a chaperone protein of low molecular weight that is produced in response to various stresses and has a cytoprotective function. In the present study we found that there is a strong correlation between sensitivity to 5-fluorouracil (5-FU) and the expression of Hsp27 in colorectal cancer. Apatorsen is an antisense oligonucleotide that targets Hsp27 and has various antitumor effects in some types of cancer, such as bladder and prostate. Although several clinical studies are currently studying apatorsen in many malignancies, to date no promising results have been reported for colorectal cancer. In the present study, we examined the impact of Hsp27 downregulation (via apatorsen) on 5-FU sensitivity in colon cancer both *in vitro* and *in vivo*. *In vitro*, apatorsen significantly decreased the levels of Hsp27 in a dose-dependent manner in human colon cancer SW480 cells. A cell proliferation assay revealed that although apatorsen did not inhibit tumor growth, it resulted in greater 5-FU sensitivity in comparison with treatment with OGX-411 (control). *In vivo*, intraperitoneal injection of apatorsen decreased the levels of Hsp27 in subcutaneous tumors in a xenograft mouse model using SW480 cells and enhanced 5-FU sensitivity, compared to controls. Although further research is warranted, the present study confirmed that concurrent treatment with Hsp27 knockdown using apatorsen and 5-FU could be a promising therapy for colon cancer.

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Introduction

Colorectal cancer (CRC) accounts for approximately 13% of all malignancies, and with 447,000 new cases diagnosed in Europe each year it is classified as the second most frequent cancer (1). The addition of other cytotoxic agents, such as oxaliplatin or irinotecan to 5-FU with folic acid has improved prognosis in patients with advanced CRC. In addition, the combination with molecular-targeted agents, such as vascular endothelial growth factor inhibitor (bevacizumab) or epidermal growth factor inhibitor (cetuximab/panitumumab) has now achieved over 30 months in median survival in patients with metastatic colon cancer. An oral multi-kinase inhibitor (regorafenib) and TAS-102 are also important in the survival time of patients in palliative care settings. Combination therapies are currently considered as first-line treatment regimens in an advanced stage of cancer (2,3). As aforementioned, 5-FU is a key drug for CRC chemotherapy, although the response rates in various trials are approximately 60% (4). Furthermore, chemotherapy alone can hardly provide a complete cure due to chemoresistance, which remains a critical problem.

Heat shock protein27 (Hsp27) is a member of the human small heat shock protein family characterized by a highly conserved α-crystalline domain. Hsp27 counteracts apoptotic cell death led by different inducers. Higher expression of Hsp27 correlates with worse clinical outcomes (5) and is also related to chemoresistance with negative cell modulation of cell death induced by cytotoxic agents (6). Several lines of evidence indicate that Hsp27 overexpression induces chemoresistance in various cancer cells and is considered as a promising target for cancer treatment (7-9). In previous studies, using human colon cancer cells (10) and a xenograft mouse model (11), we demonstrated that Hsp27 promoted resistance to 5-FU.

Antisense oligonucleotides (ASOs) are single-stranded stretches of nucleotides that are specifically hybridized with complementary mRNA regions and inhibit protein expression by forming RNA/DNA duplexes. To date, evidence from several studies have identified ASOs as potential therapeutic agents (12,13). Recently, a second-generation ASO targeting Hsp27 mRNA (apatorsen; OncoGenex Technologies, Vancouver, BC, Canada) is reported to enhance the effects of

chemotherapy in several cancers (14,15). Phase II trials are currently in progress for prostate, bladder, ovarian, breast and lung cancer (16). However, no detailed investigation of the effects of apatorsen in CRC has been performed.

In the present study, we examined the impact of Hsp27 downregulation via apatorsen on 5-FU sensitivity in colon cancer both *in vitro* and *in vivo*.

Materials and methods

Cells and reagents. Human colon cancer SW480 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA). The cells were supplemented with 10% fetal bovine serum (FBS; CSL Ltd., Melbourne, Australia) and 1% penicillin/streptomycin. The cells were cultured at 37˚C with 5% CO_2. SW480 cells are human colon cancer cells that overexpress Hsp27. In addition, 5-FU was purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). Stock solutions of 5-FU were prepared with phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA) to the required concentrations before each experiment.

Apatorsen and OGX-411. Apatorsen, 2'-O-(2-methoxyethyl) ASO, was provided by OncoGenex Technologies. A sequence of apatorsen corresponds to the human Hsp27 translation initiation site (5'-GGGACGCGGCGCTCGGTCAT-3'). OGX-411 (ODN; 5'-CAGCAGCAGAGTATTTATCAT-3'), a mismatch oligodeoxynucleotide, was used as a control oligonucleotide.

In vitro study. Transfection with ASO in SW480 cells. Transfection was performed according to previously reported methods (17). The SW480 cells were plated at a density of $1.0x10^6$ cells/10-cm dish for 24 h. Subsequently, the cells were treated for 48 h with various concentrations of apatorsen and OGX-411. Lipofectamine 3000 (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA), a cationic lipid, was used as a transfection agent for both apatorsen and OGX-411. After 24-h incubation, 1.2 ml Opti-MEM (Gibco; Life Technologies Corp., Grand Island, NY, USA) containing 18 μl Lipofectamine 3000 and 24 μl P3000 reagent (Invitrogen Life Technologies) was added with 30 μl apatorsen or OGX-411. The expression of Hsp27 was evaluated following additional incubation for 48 h.

Western blot analysis. To evaluate the effects of Hsp27 downregulation in parental SW480 cells, which constantly express Hsp27, the cells were transfected with apatorsen or OGX-411 as previously described and Hsp27 levels were evaluated via western blot analysis. SW480 cell lysates were extracted with a lysis buffer as previously described (18). The amount of cell lysates was assessed with a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of each sample, containing equal amounts of protein, were loaded onto an SDS-polyacrylamide gel before undergoing electrophoresis and were transferred to an ImmunoBlot™ polyvinylidene fluoride membrane (PVDF; Bio-Rad Laboratories). The membrane was blocked in PBS containing 5% non-fat milk powder for 1 h at room temperature and then incubated at 4˚C overnight at 1:1,000 (anti-human Hsp27 mouse monoclonal antibody; cat. no. MS-101-P0; Thermo Fisher Scientific, Carlsbad, CA, USA) or at 1:5,000 (anti-human, β-actin mouse monoclonal antibody; cat. no. 612656; BD Biosciences, San Jose, CA, USA). The membranes were incubated for 30 min at 1:5,000 (horseradish peroxidase-conjugated anti-mouse IgG; cat. no. W4021; Promega Corp., Fitchburg, WI, USA). Specific proteins were detected using the Luminata Forte Western HRP substrate (Merck Millipore Co., Darmstadt, Germany) according to the manufacturer's instructions. The density of the Hsp27 band was normalized to β-actin by densitometry using FluorChem FC2 with AlphaView software (Alpha Innotech, San Leandro, CA, USA). Each analysis was performed in triplicate.

Cell proliferation assay. To evaluate the effects of ASO alone, the cells were transfected with apatorsen or OGX-411 and cell viability was determined using a 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide inner salt (MTT) assay at 48 h as previously reported (13). The transfection method was similar to the aforementioned method: $2x10^3$ cells were seeded in 96-well microtiter plates (Corning Inc., Corning, NY, USA). After 24-h incubation in 100 μl DMEM supplemented with 10% FBS without any antibiotics, 100 μl Opti-MEM containing 8 μl Lipofectamine 3000 was added with various concentrations of apatorsen or OGX-411. Transfectants were further incubated for 48 h and then evaluated for cell viability.

To assess the effects of the combination treatment of ASO plus 5-FU, the cells were transfected with 50 nM apatorsen or the same concentration of OGX-411 as described above and then treated with the indicated concentrations of 5-FU for 48 h. Subsequently, Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan) was added to the plates and the cell viability was determined using an MTT assay. Absorbance in the wells was assessed with a microplate spectrophotometer at 450 and 630 nm (Tecan Japan Co., Ltd., Kawasaki, Japan). The cell viability was calculated using the following equation: (mean absorbance of drug well/mean absorbance of control wells) x 100%. The absorbance of each experimental well was adjusted to the mean absorbance of blank wells; 5-FU resistance was assessed based on the concentration of the drug required to inhibit cell growth by 50% relative to the untreated cells (half maximal inhibitory concentration, IC_{50}).

In vivo study

Xenograft model. To assess the effects of apatorsen on 5-FU sensitivity, a mouse xenograft model was used. Approximately $1x10^7$ SW480 cells in 100 μl PBS were inoculated subcutaneously at the bilateral dorsum using a 27-gauge needle (Terumo Co., Tokyo, Japan), under ether anesthesia, in 6-week-old SCID mice that were purchased from CLEA Japan, Inc. (Tokyo, Japan). The tumor volume was assessed once a week by the same observer using a sliding caliper. The estimated volume (EV) of tumors was calculated using the following equation: EV = length x width^2 x 1/2 (11). When the EV reached 500 mm^3, 30 mice were randomly divided into two groups: treatment with 5-FU plus apatorsen or OGX-411 (10 mg/kg). For treatment, 5-FU (30 mg/kg) was administered intraperitoneally three times a week for 3 weeks, accompanied by apatorsen or OGX411. EV was plotted against days from the

initiation of the 5-FU treatment in order to derive a xenograft growth curve. All of the mice were sacrificed 3 weeks after the initial treatment. The tumors were collected and fixed in 4% paraformaldehyde (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) at room temperature for 24 h before being processed for sectioning and immunohistochemical staining or TUNEL apoptosis assay. All animal procedures were performed with the appropriate institutional certification by the Keio University Institutional Animal Care and Use Committee (Tokyo, Japan).

Immunohistochemistry. Collectively, 51 SW480 xenograft cases were used for the present study. Methanol-fixed, paraffin-embedded sections containing the maximum diameter of each xenograft were examined immunohistochemically, as previously described (11). Briefly, 4-μm thick sections of methanol-fixed, paraffin-embedded xenograft tissue were deparaffinized with xylene and treated with 0.3% (v/v) hydrogen peroxide in methanol. A mouse monoclonal antibody to Hsp27 (Ab-1, dilution 1:200; Thermo Fisher Scientific) was used for immunohistochemical staining. Visualization was performed using 3,3'-diaminobenzidine tetrahydrochloride (Histofine Mousestain kit; Nichirei Biosciences Inc., Tokyo, Japan), followed by counterstaining with Mayer's hematoxylin (Muto Pure Chemicals). As a negative control, the same class of mouse immunoglobulin was used instead of the primary antibody. To evaluate the expression levels of Hsp27 in both groups, the tumors were categorized according to the percentage of immunopositive tumor cells as follows: <20%, negative (-); 20-40%, diffuse (+); 40-60%, uniform (++); and >60%, strong (+++). Two observers (A.S. and T.S.), who had no previous information about the groups, independently reviewed the immunohistochemically stained slides and all discrepancies were resolved by joint review of the slides.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling apoptosis (TUNEL) assay. The effects of various treatments on the proliferation of tumor cells were determined based on immunohistochemical staining of tissue sections with anti-proliferating-cell nuclear-antigen antibodies and a TUNEL assay (19). This experiment evaluated two mice of each treatment group and the largest cross-sectional slides of the tumor were assessed. The staining was performed according to the manufacturer's methods. The tissue was recorded using a cooled EVOS FL Cell Imaging System (Invitrogen Life Technologies). The number of positive cells was determined in randomly selected 10 rectangular fields of view at x20 magnification in each slide and their average was determined as the number of apoptotic cells per 3,000 cells of the tumor (19).

Statistical analysis. Statistical analyses were performed using the χ^2 test, Student's t-test or Mann-Whitney U test using STATA version 12.0 (StataCorp LP, College Station, TX, USA). P<0.05 was considered to indicate a statistically significant difference. Values are expressed as the medians ± standard errors.

Results

Dose-dependent inhibition of the expression of Hsp27 by apatorsen. Apatorsen significantly decreased Hsp27 levels in a dose-dependent manner in human colon cancer SW480 cells, as depicted in Fig. 1. Compared to OGX-411, apatorsen (1, 10 and 100 nM) reduced relative levels of Hsp27 1.29- (80.6%), 0.76- (47.7%), and 0.57-fold (35.7%), respectively. There was a significant difference in Hsp27 levels between OGX-411 and 100 nM apatorsen (p=0.03).

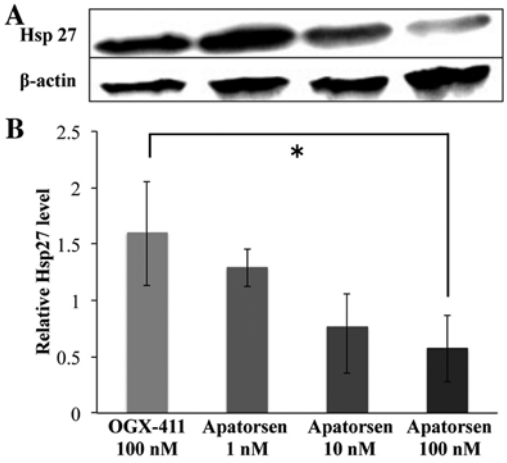


Figure 1. Dose-dependent suppression of the expression of Hsp27 by apatorsen in SW480 cells. (A) The SW480 cells were treated with various concentrations of apatorsen and OGX-411 control ODN for 48 h. After treatment, cellular proteins were extracted from cultured cells and Hsp27 and β-actin levels were analyzed by western blotting. (B) Quantitative analysis of Hsp27 levels after normalization to β-actin by densitometric analysis. Columns, means; error bars, SD. *p=0.03 (Student's t-test).

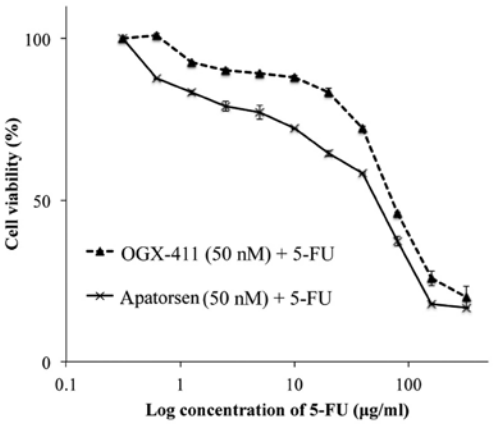


Figure 2. Cytotoxic effects of 5-FU treatment or combined treatment with apatorsen and 5-FU. SW480 cells were initially treated with 50 nmol/l apatorsen or OGX-411 for two days. Chemotherapy at increasing concentrations was added after two days of treatment with the respective ODN. After 24-h incubation, the cell viability was determined using an MTT assay. Apatorsen treatment resulted in an upper shift in cell viability compared to OGX-411. Points, medians; error bars, SE.

Apatorsen enhances 5-FU sensitivity in vitro. To determine whether the reduction of Hsp27 levels affects cell viability or 5-FU chemosensitivity in colon cancer, a cell proliferation assay was performed on cells, following 48-h exposure to apatorsen or OGX-411 with and without 5-FU. Monotherapy of apatorsen or OGX-411 had no influence on cell growth (data not shown). Apatorsen treatment resulted in reduced cell viability than OGX-411 (Fig. 2). Apatorsen demonstrated a

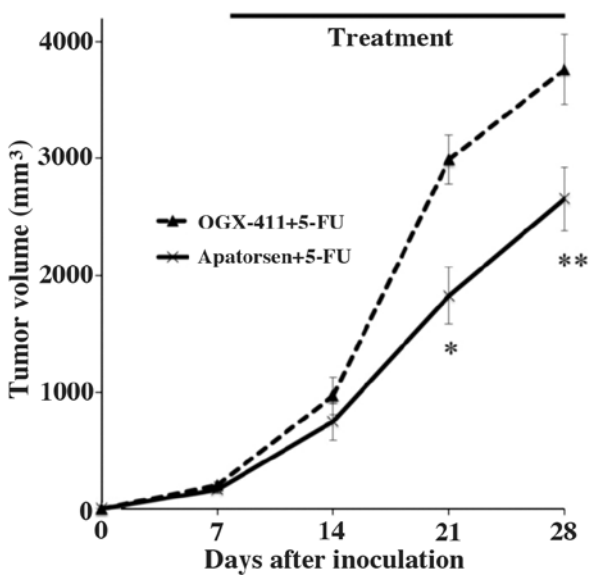
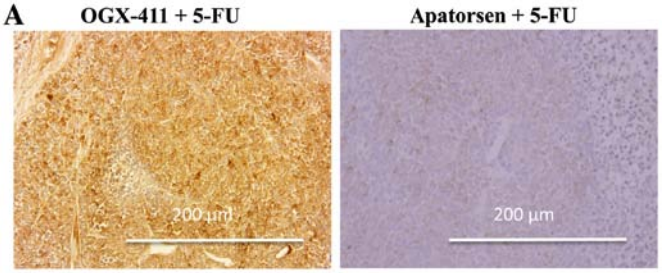


Figure 3. Effects of combination therapy (*in vivo*). Combination treatment was initiated when tumor volumes reached 500 mm^3. 5-FU (30 mg/kg) was administered intraperitoneally three times a week for 3 weeks with apatorsen or OGX411 (10 mg/kg, respectively). Points, medians; error bars, SE. *p=0.012, **p=0.001 (Mann-Whitney U test).



B

Hsp27 expression

	5-FU+OGX411	5-FU+apatorsen
<40%	3	18
>40%	18	3

Figure 4. Immunohistochemical staining. Low-power field (magnification x10). (A) SW480 tumors were harvested from each treatment group to detect Hsp27 expression by immunohistochemical staining. Sections of paraffin-embedded SW480 tumors were stained with mouse monoclonal antibody and imaged at x20, magnification. (B) After immunohistochemical staining, the number of immunopositive tumor cells was counted and expressed as a proportion of the total number of cells in each microscopic field at x20 magnification. Columns, means. *p<0.001 (χ^2 test).

relatively low IC_{50} (55.9 vs. 73.7 μg/ml) and an enhanced 5-FU sensitivity compared to OGX-411 in colon cancer cells.

Effects of apatorsen on 5-FU treatment in SW480 xenograft growth. Subsequently, we evaluated the effects of apatorsen on 5-FU treatment for colon cancer in a xenograft mouse model. After the EV of the SW480 cells reached 500 mm^3, apatorsen or OGX-411 with 5-FU were injected intraperitoneally three times a week for 3 weeks. Each group included 15 mice and tumor volumes were compared between groups (Fig. 3). Changes in EV were similar between groups before treatment (data not shown). Apatorsen treatment significantly enhanced tumor growth inhibition due to 5-FU, compared to OGX-411 (control) on days 14, 21 and 28, with measurements of 746 vs. 968 mm^3 (p=0.106), 1826 vs. 2992 mm^3 (p=0.012) and 2655 vs. 3764 mm^3 (p=0.001), respectively (Fig. 3).

Immunohistochemical staining of tumors revealed that apatorsen suppressed the expression of Hsp27 (Fig. 4A). The probability of a tumor having more than 40% of cells expressing Hsp27 was significantly lower in the apatorsen group than the OGX-411 group (14.3 vs. 85.7%, p<0.001) (Fig. 4B). A TUNEL assay revealed that a larger number of cells underwent apoptosis in the apatorsen group than in the OGX-411 group (26 vs. 75, p<0.001) (Fig. 5).

Discussion

In the present study, we investigated whether apatorsen could serve as a therapeutic strategy for CRC. We found, for the first time, that apatorsen affects 5-FU sensitivity in CRC. We revealed that SW480 cells, in which Hsp27 is expressed at higher levels, had greater resistance to 5-FU compared with previous studies. Treatment with apatorsen suppressed Hsp27 levels and consequently mitigated 5-FU resistance *in vitro*. In addition, apatorsen contributed to the effectiveness of 5-FU *in vivo* and 5-FU in combination with apatorsen inhibited the growth of human colon cancer xenografts and enhanced apoptosis significantly more than 5-FU plus control ODN.

The present study further supported existing data revealing that Hsp27 overexpression enhanced 5-FU resistance and that its suppression mitigates 5-FU resistance in colon cancer. We have previously demonstrated (10,11) that Hsp27 overexpression enhanced 5-FU resistance and that Hsp27 suppression mitigated 5-FU resistance in colon cancer cells *in vitro* and *in vivo* using siRNA and shRNA. Collectively, the results of the present study indicated that apatorsen downregulated Hsp27 and enhanced the chemoresponse in CRC as well as in other malignant diseases. *In vitro* the effect of apatorsen was less impressive than *in vivo* which may be due to critical reasons such as the differences at the duration of the treatment and the dose intensity. The duration of the cell culture is limited by the size of the dish *in vitro*, whereas the dose of apatorsen is much more in the *in vivo* study. In addition, the immunology of the living body may modify the effects of apatorsen *in vivo*.

It is known that the *in vitro* efficacy of siRNAs seems to surpass that of ASOs whereas *in vivo*, unmodified siRNA is rapidly cleared and the drug retention rate is low. siRNAs still present many problems (i.e. drug delivery system and unanticipated vascular or immune effects), although many studies are in progress (20). In the present study, we used apatorsen, which is a commercially-produced, second-generation antisense oligonucleotide, because we anticipated that it should be more affordable and effective in *in vivo* experiments or clinical practice compared to siRNA. However, there are some obstacles to be considered in the transfection of apatorsen into cancer cells, especially for clinical use, which are the affinity and efficacy of apatorsen. In the clinical setting, the heterogeneity of cancer cells could reduce the rate of affinity and efficacy. Even in *in vitro* experiments, Lipofectamine is suitable for transfection of apatorsen, while Oligofectamine (Invitrogen Life Technologies) is not. Although the drug delivery system, such as ligand or polymer conjugates and nanoparticles has

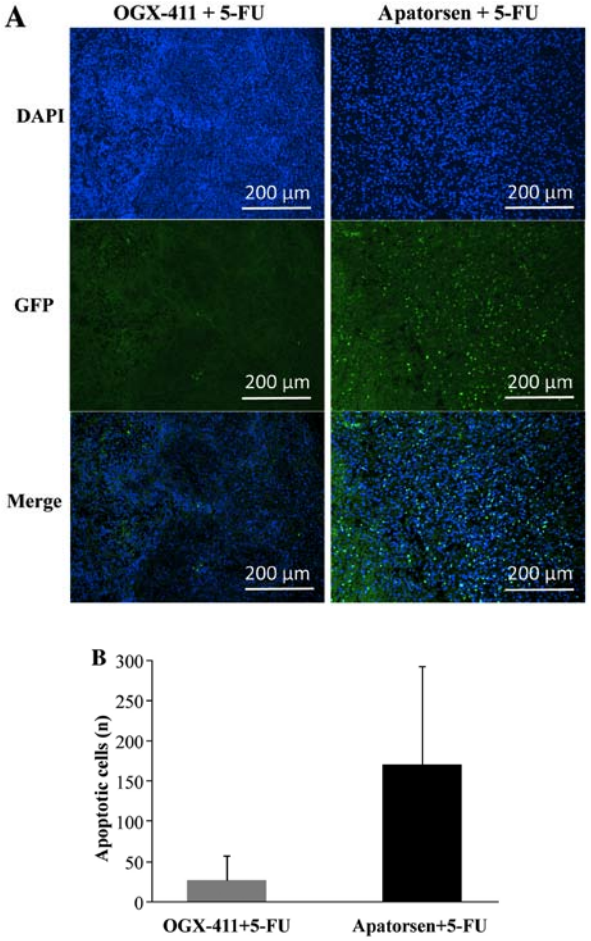


Figure 5. (A) TUNEL apoptosis assay. Low-power field (magnification, x10). SW480 tumors were harvested from each treatment group to detect apoptosis by TUNEL staining. Sections of paraffin-embedded SW480 tumors were stained with digoxigenin dUTP antibody to detect apoptotic cells and imaged at x10 magnification. The expression of DAPI (DNA, blue), GFP (apoptotic cells, green) and merged images is shown. (B) After TUNEL staining, the number of apoptotic cells was counted and expressed as a proportion of the total number of cells per 3,000 cells in each microscopic field at magnification, x20. Columns, medians; error bars, SE. $^{*}p<0.001$ (Mann-Whitney U test).

improved the delivery of oligonucleotides to targeted organs and cells (21), future research should concentrate on the investigation of the appropriate dosage and dosing period of apatorsen in clinical use.

In contrast to previous research (14) however, no evidence of apatorsen monotherapy efficacy was observed in the present study. Apatorsen monotherapy did not inhibit tumor growth *in vitro* or *in vivo* (data not shown), probably due to the unique function of Hsp27 in facilitating the transcriptional activity of the androgen receptor. In hormone-refractory prostate cancer, upregulated Hsp27 facilitates the genomic activity of the androgen receptor, which ultimately leads to the proliferation and progression of the tumor cells through the activation of Stat-3. Therefore, CRC which does not express the androgen receptor, would not be affected by treatment with apatorsen alone (22-24).

One of the functions of Hsp27 is to suppress apoptosis via many pathways: by inhibiting Bax, or Daxx and mainly by affecting caspase-3 (25), as well as via two other pathways such as decreasing reactive oxygen species and enhancing NF-κB activity by increasing degradation of its main inhibitor, I-κBα (26-29). These broad functions on cancer cell signaling, proliferation and survival identify Hsp27 as a potential therapeutic target and failure of cancer cells to undergo apoptosis may contribute to resistance to chemotherapy.

5-FU is well known to induce cancer cell apoptosis mainly through the mitochondrial pathway, which involves cytochrome *c* and subsequent activation of the upstream initiator caspase-9 and the downstream effector caspase-3 (30). In the present study, enhanced caspase-3 expression was not observed in the apatorsen group (data not shown), which is different from certain previously published studies that have demonstrated that Hsp27 may function as a negative regulator of the cytochrome *c*-dependent activation of procaspase-3. Nevertheless, apatorsen accelerated apoptosis by suppressing Hsp27. This indicated that another mechanism of apoptosis is active during 5-FU treatment of CRC and the biopharmaceutical properties of apatorsen. Kamada *et al* (14) reported that apatorsen is predisposed to induce p53-independent apoptic triggers. SW480 is also p53-mutant, so this is one of the possible reasons for the impact of apatorsen on SW480 cells. Further studies that take into account these pathways or pharmacodynamic effects are needed.

The present study raised the possibility that apatorsen accelerated apoptosis of CRC cells due to 5-FU. Hsp27-knockdown using apatorsen also enhanced sensitivity to 5-FU chemotherapy in SW480 cells, which is in accordance with our previous study. These findings have important implications for developing clinical applications for apatorsen in CRC (10,11,31).

The present study was limited by a lack of information on other human CRC cells. Due to the heterogeneity of CRC, the effects of apatorsen may vary between different cancer cells and be limited to Hsp27-expressing cells. Combination chemotherapy is currently the gold standard for CRC treatment. The results of the present study indicated that combining apatorsen with 5-FU was beneficial. Therefore, the combination of apatorsen with other chemotherapeutic agents (i.e., irinotecan and oxaliplatin) or molecular-targeting agents should be further evaluated. Furthermore, the rate of colon cancer patients that overexpress Hsp27 is unclear, although this treatment offers benefits only to such patients. Currently, phase II or III clinical trials are running and the use of apatorsen in third-line chemotherapy is expected in the future.

Despite these limitations, this is the first study to establish evidence that apatorsen could be a novel weapon to enhance the effectiveness of 5-FU targeting Hsp27 for the treatment of CRC. Although several hurdles still exist before these findings can be applied to clinical practice, this could be the first step in further improving CRC prognosis.

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