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Inhibition of Heat-shock Protein 27 Reduces 5-Fluorouracil-acquired Resistance in Human Colon Cancer Cells

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Abstract. *Background/Aim: In previous work we showed that expression of heat-shock protein 27 (HSP27; encoded by HSPB1) was associated with inherent resistance to 5-fluorouracil (5-FU). However, the relationship between HSP27 and acquired resistance remains unknown. Materials and Methods: We generated an acquired resistance model (WiDr-R) of a colon cancer cell line by exposing WiDr cells to 5-FU. Cell viability assays under treatment with 5-FU, as well as down-regulation of HSP27 using small interfering HSP27 RNA, were performed. HSP27 mRNA and protein expression was analyzed using real-time polymerase chain reaction and western blotting. Results: 5-FU-acquired resistance induced overexpression of HSP27 mRNA and protein levels in WiDr-R cells. Furthermore, siRNA knockdown of HSP27 in WiDr-R cells reduced 5-FU-acquired resistance. Conclusion: These findings demonstrate that HSP27 is associated with 5-FU resistance in human colon cancer cell cells and suggest that HSP27 regulation represents a novel approach to overcoming chemoresistance in colorectal cancer.*

Colorectal cancer (CRC) is a very common disease worldwide. In advanced cancer, systemic chemotherapy and surgery are key treatment approaches. Currently, systemic chemotherapy for CRC has progressed remarkably. 5-Fluorouracil (5-FU) is a classic and very important cytotoxic anticancer drug; this treatment is commonly combined with other cytotoxic drugs such as oxaliplatin, and irinotecan. Furthermore, a paradigm shift has occurred in the use of molecular target drugs. Administration of vascular endothelial growth factor inhibitors (bevacizumab/ramucirumab) and epidermal growth factor receptor (EGFR) inhibitors (cetuximab/panitumumab) has improved the prognosis of advanced unresectable or recurrent CRC by up to approximately 30 months (1-3).

However, resistance to 5-FU-based chemotherapy, which is widely used to treat CRC, remains an insurmountable clinical challenge. Therefore, novel approaches to overcoming 5-FU resistance are required, for which several strategies and drugs have been identified (4-6). Heat-shock proteins, including heat-shock protein 27 (HSP27; encoded by *HSPB1*), act as molecular chaperones in protein–protein interactions under physiological conditions. Additionally, HSP27 expression is increased in various types of malignant diseases (7-9). Furthermore, a relationship between HSP27 overexpression and drug resistance has been reported (9-11). We have previously suggested that HSP27 is associated with 5-FU resistance in certain human colon cancer cell lines. Cell lines with higher levels of HSP27 expression showed a higher degree of 5-FU resistance compared to those with lower expression levels (12). Additionally, in experiments using cell lines with high HSP27 expression (inherently resistant to 5-FU according to our hypothesis), down-regulation of HSP27 using small interfering RNA (siRNA) or short hairpin RNA reduced 5-FU resistance (12, 13). Furthermore, in the same setting, down-regulation of *HSP27* using apatorsen, an antisense oligonucleotide that targets HSP27, reduced 5-FU resistance *in vitro* and *in vivo* (14). Thus, HSP27-targeting gene therapy is promising for overcoming the inherent resistance to 5-FU-based chemotherapy in CRC.

The response rate to 5-FU-based chemotherapy in treating CRC is relatively high in a clinical setting, particularly in the

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first-line regimen. The response rate was reported to reach up to approximately 60% in a clinical trial (15). In most cases, although CRC initially responds to chemotherapy, resistance is generated with repeated treatment, causing chemotherapy failure. This implies that acquired resistance is often a more serious problem than inherent resistance to 5-FU-based chemotherapy for CRC. Although the relationship between HSP27 and acquired resistance to 5-FU in CRC remains unknown; Our previous research suggested that HSP27 is associated with both inherent and acquired resistance. Thus, in this study, we generated a model of acquired resistance by exposing the human colon cancer cell line WiDr to a high dose of 5-FU. We then evaluated the relationship between HSP27 and acquired resistance using this model.

Materials and Methods

Cells and reagents. The human colon cancer cell line WiDr was chosen as the parent cell line. WiDr cells (ATCC CCL-218) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) at 37˚C. The cells were maintained in medium containing 10% fetal bovine serum (CSL Ltd., Melbourne, Australia) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). 5-FU was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

Acquired resistance model. In summary, parental cells (5×10^6) growing in 100-mm tissue-culture treated culture dishes (Corning, Inc., Corning, NY, USA) were incubated for 24 h in DMEM and then exposed to a high dose of 5-FU (150 μg/ml) for 24 h. Surviving cells maintained in 5-FU-free DMEM for 1 month were considered as resistant (WiDr-R). The generated resistance was confirmed by performing cell proliferation assays as described below.

Cell proliferation assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed as described previously (16). Passaged cells were cultured in 100 mm dishes for 48 h in DMEM. Well-conditioned cells (5,000 cells/well) were then passaged and cultured in a 96-well plate (Corning, Inc.) for 24 h in DMEM followed by incubation with different concentrations of 5-FU (1.25, 2.5, 5, 10, 20, 40, 80, 160 μg/ml) for an additional 72 h. Viable cells were immediately stained using the Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan), and identified by measuring the absorbance at 450 and 600 nm with a spectrophotometer (Sunrise Basic Tecan Microplate Reader, Tecan, Männedorf, Switzerland) according to the manufacturer's instructions. The following formula was used to calculate cell viability:

Cell viability (%)=(mean absorbance of 5-FU-treated cells/mean absorbance of control cells) × 100%

The extent of drug resistance was calculated as the IC_{50}, which represents the 5-FU concentration required to reduce cell viability to 50%.

Quantitative real-time polymerase chain reaction (qPCR). HSPB1 was amplified by qPCR using the Fast SYBR Green Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. mRNA expression was measured using the comparative threshold cycle method (ΔΔCT method), and HSP27 expression (from *HSPB1*) was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primer sequences used were: *HSPB1* (5'-3'): GACGAGCATGGCTACATCT; *GAPDH (5'-3')*: ATCATCCCTGCCTCTACTGG.

Western blotting. Total cell lysates were collected as previously described (17). Cell lysates (20 μl) containing equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred to Immun-Blot polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes to which proteins had been transferred were blocked in phosphate buffered saline (Sigma-Aldrich) containing non-fat milk (5%) for 1 h and then incubated with primary antibody for 24 h, at 4˚C. The dilutions of antibodies used were 1:1,000 for anti-HSP27 (mouse monoclonal; Thermo Fisher Scientific) and 1:2,500 for anti-β-actin (mouse monoclonal; BD Biosciences, San Jose, CA, USA). The membranes were then exposed to a horseradish peroxidase-conjugated anti-mouse IgG (1:3,000 dilution; Promega Corp., Fitchburg, WI, USA) for 10 min. Finally, the targeted proteins were labeled with the Luminata Forte Western HRP substrate (Merck Millipore Co., Darmstadt, Germany) and immediately detected using the FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA). Protein bands were quantitatively measured by densitometry according to the manufacturer's instructions with AlphaView software (ProteinSimple, San Jose, CA, USA), and HSP27 expression was evaluated relative to that of β-actin.

Transfection of HSP27 siRNA. We transfected HSP27 siRNA (Thermo Fisher Scientific) and scrambled siRNA (Thermo Fisher Scientific) as a negative control into the cells using Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, WiDr-R or WiDr cells were plated at a density of 4.0×10^5 cells in 2.5 ml of DMEM per well in 6-well plates (Corning, Inc.). After 24 h of incubation, 500 μl of Opti-MEM (Gibco, Grand Island, NY, USA) containing 7.5 μl of Lipofectamine RNAiMAX and 15 pmol of HSP27 siRNA or scrambled siRNA was added (5 nM siRNA concentration). The transfectants were incubated for 24 h and then evaluated for HSP27 expression and 5-FU resistance by qPCR, western blotting, and MTT assays as described above.

Statistical analysis. Values are expressed as the mean±standard error. Each experiment was performed in triplicate. Statistical analysis was performed using the Mann-Whitney *U*-test using STATA version 11.2 software (StataCorp LP, College Station, TX, USA). A value of $p<0.05$ was considered statistically significant.

Results

Generation of a model of acquired resistance. We used the WiDr colon cancer cell line because it is known to express low levels of HSP27 (low inherent resistance to 5-FU) (12). We successfully generated an acquired resistance model of WiDr by exposing parental WiDr cells to a high dose of 5-FU, and named the resulting cell line WiDr-R. To analyze

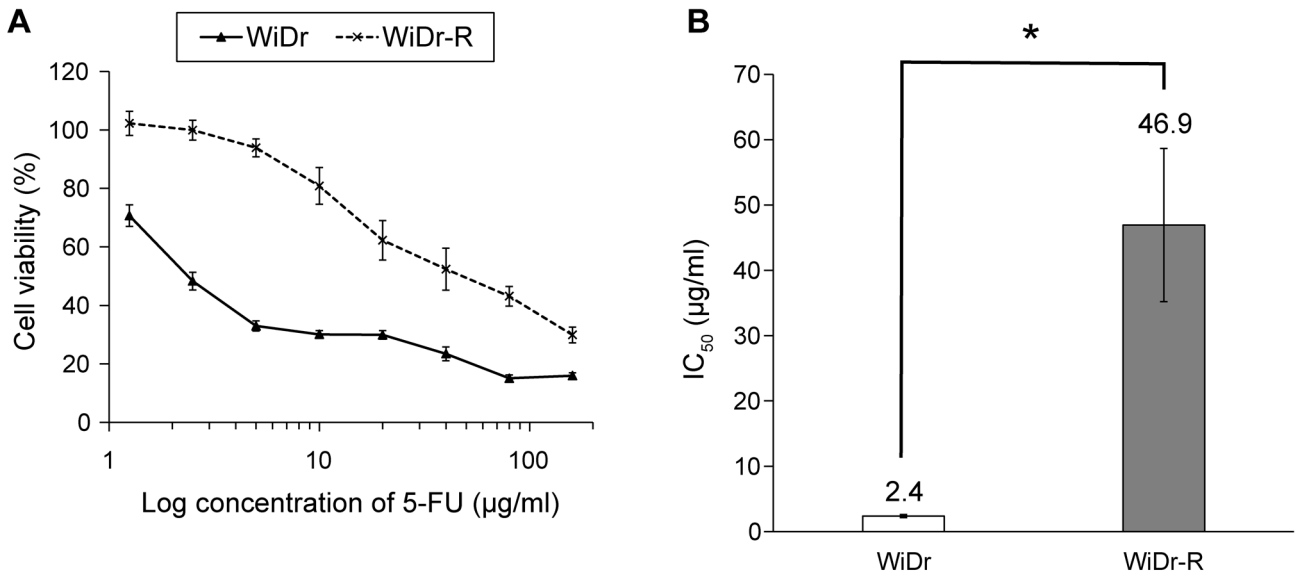

Figure 1. *A model of acquired resistance to 5-fluorouracil (5-FU) in WiDr cells was generated (WiDr-R). A: Cells were exposed to different concentrations of 5-fluorouracil (5-FU) for 72 h and the cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. B: The half-maximal inhibitory concentration (IC_{50}) for WiDr-R was significantly higher than that for WiDr when cells were treated with 5-FU. Data are shown as mean values±standard error of an experiment performed in triplicate. *p<0.05 (Mann–Whitney U-test).*

the 5-FU resistance of the cells, cell viability assays using 5-FU treatment were performed. As shown in Figure 1, the IC_{50} of WiDr-R cells was significantly higher than that of WiDr cells (46.9 *vs*. 2.4 μg/ml, $p<0.05$) following treatment with 5-FU.

HSP27 overexpression in WiDr-R. We examined the HSP27 mRNA and protein expression levels of WiDr-R compared to WiDr cells using qPCR and western blotting. HSP27 mRNA and protein were overexpressed in WiDr-R cells (Figure 2). Thus, the mRNA (0.43 *vs*. 0.13, $p<0.05$) and protein (1.49 *vs*. 0.70, $p<0.05$) levels of HSP27 were significantly higher in WiDr-R cells than in WiDr cells.

Down-regulation of HSP27 in WiDr-R. We examined whether down-regulation of HSP27 affected acquired resistance to 5-FU in WiDr-R cells treated with 5-FU. HSP27 siRNA was transfected into WiDr-R cells showing acquired 5-FU resistance. As shown in Figure 3A and B, transfection of WiDr-R with HSP27 siRNA successfully reduced both the mRNA and protein levels of HSP27 compared to transfection with scrambled siRNA (0.03 *vs*. 0.11, $p<0.05$; and 0.54 *vs*. 1.09, $p<0.05$, respectively). As shown in Figure 3C, the IC_{50} of HSP27 siRNA transfectants was significantly lower than that of scrambled siRNA transfectants (11.1 *vs*. 75.6 μg/ml, $p<0.05$) following treatment with 5-FU.

Down-regulation of HSP27 in WiDr. We also examined whether down-regulation of HSP27 affected the inherent resistance to 5-FU in WiDr cells treated with 5-FU. Transfection of HSP27 siRNA successfully reduced both the mRNA and protein levels of HSP27 in WiDr cells, as shown in Figure 4A and B (0.03 *vs*. 0.08, $p<0.05$; and 0.54 *vs*. 1.02, $p<0.05$, respectively). However, as shown in Figure 4C, no significant difference in the IC_{50} was observed between HSP27 siRNA and scrambled siRNA transfectants (5.2 *vs*. 4.9 μg/ml, $p=0.51$) when the cells were treated with 5-FU.

Discussion

Despite improvements in systemic chemotherapy for CRC, drug resistance to 5-FU, a key agent in CRC chemotherapy, remains a serious clinical challenge. We have previously reported that HSP27 is a novel and promising target for overcoming inherent resistance to 5-FU in CRC (12, 13). Similarly, Shimada *et al*. showed that apatorsen, an antisense oligonucleotide targeting HSP27, reduces inherent 5-FU resistance in small rodents (14). In contrast, clinical observations (particularly, a relatively high response rate in 5-FU-based chemotherapy as a first-line regimen in CRC) suggest that acquired resistance to 5-FU is rather critical. However, the relationship between HSP27 and acquired resistance to 5-FU in CRC remains unknown. In this study, we first showed that down-regulation of HSP27 using siRNA reduced the acquired resistance in the resistance model.

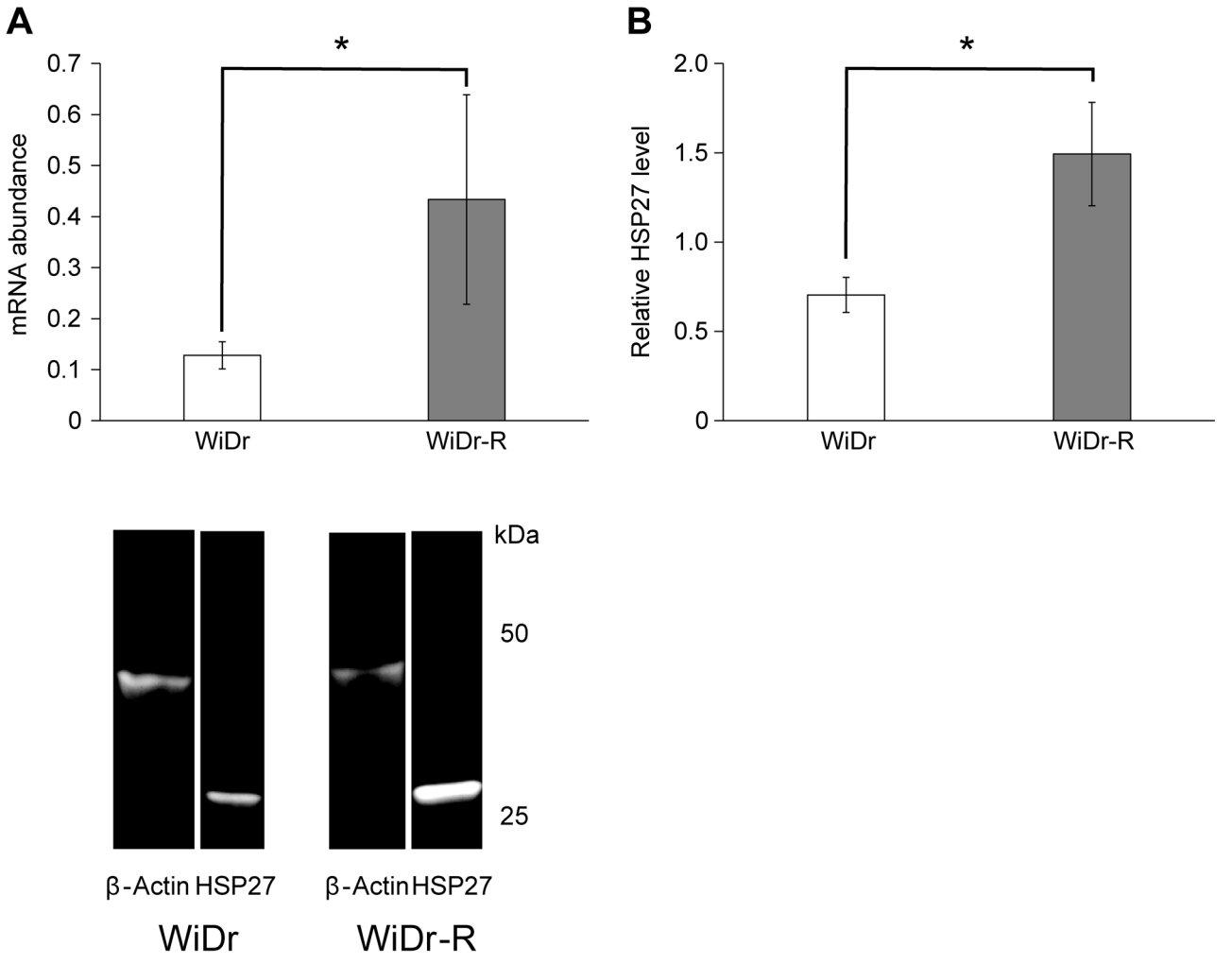

Figure 2. *Heat-shock protein 27 (HSP27) overexpression in WiDr-R cells. A: Compared to that of WiDr cells quantitative polymerase chain reaction results showed significantly higher HSP27 mRNA expression in WiDr-R cells. B: Western blots and graph from densitometric analysis showed significantly higher HSP27 protein expression in WiDr-R cells. Data are shown as mean values±standard error of an experiment performed in triplicate. *p<0.05 (Mann–Whitney U-test).*

Therefore, HSP27 is a promising target for reducing acquired resistance to 5-FU in CRC, which may lead to improvement in the prognosis of CRC.

A previous study similarly proposed another protein as a novel and promising target in the same setting. Wang *et al.* reported that down-regulation of transient receptor potential canonical 5 protein (TRPC5) using siRNA reduced acquired resistance to 5-FU in a resistance model (18). In comparison, use of HSP27 should be more practical due to the available antisense nucleotide, apatorsen, which can down-regulate HSP27 and has already been developed and used in a clinical trial (19). Numerous basic and clinical studies have explored the underlying mechanisms of HSP27-mediated chemoresistance (8, 19, 20). Thus, compared to other proteins, targeting of HSP27 may be easier in clinical applications.

Furthermore, we demonstrated that suppressing HSP27 using siRNA was not effective against the parental WiDr cells, which initially expressed a low level of HSP27. However, this approach would be effective after HSP27 levels are increased by exposure to 5-FU and with the development of 5-FU resistance. These results suggest a new chemotherapeutic strategy for CRC based on HSP27 expression level. For example, measuring the HSP27 level before chemotherapy may help detect inherent 5-FU resistance. Patients with higher HSP27 levels might benefit from HSP27-targeting therapy combined with 5-FU-based chemotherapy, whereas those with lower HSP27 expression

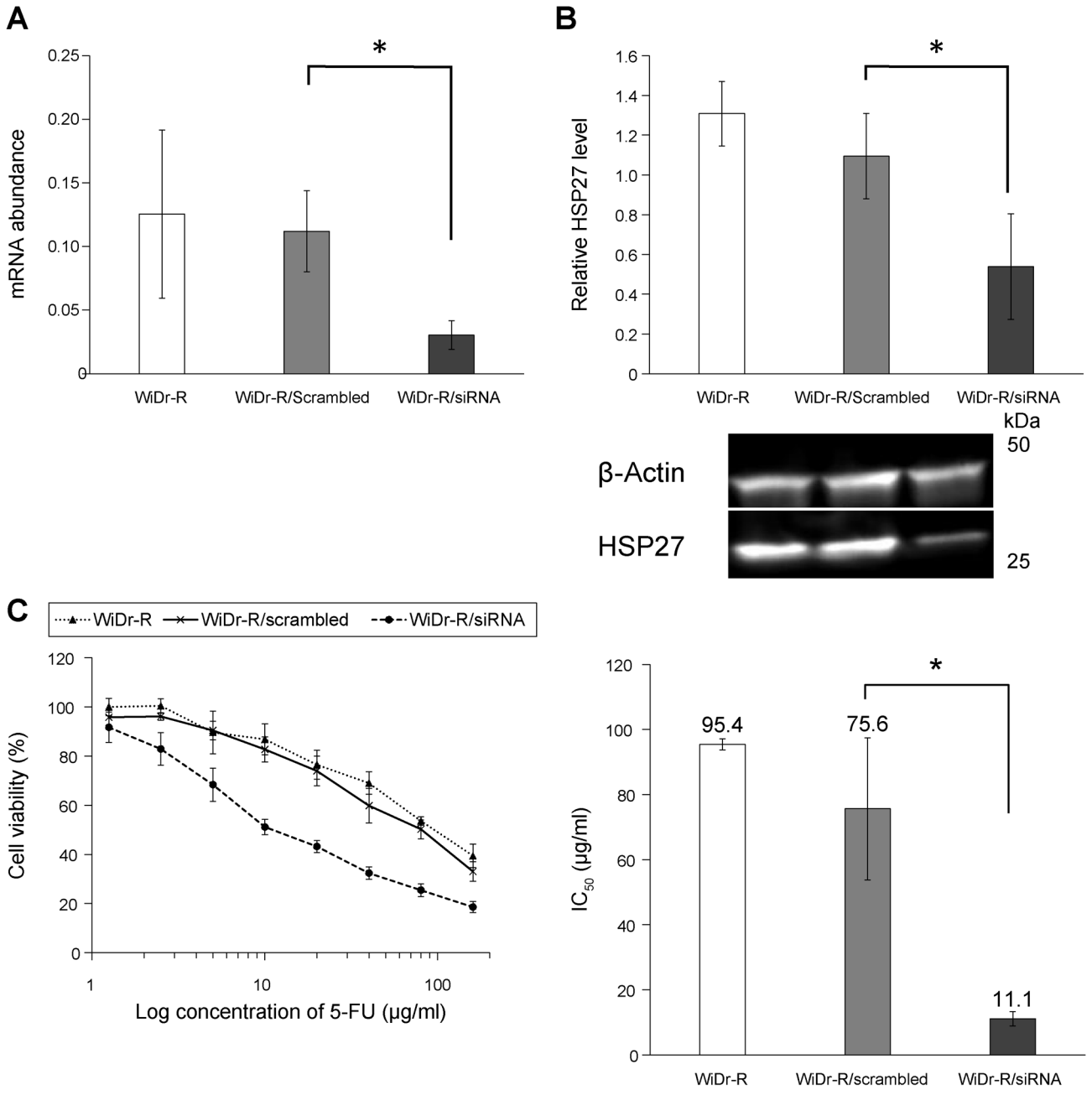

Figure 3. *Down-regulation of heat-shock protein 27 (HSP27) in WiDr-R cells. Treatment of WiDr-R cells with HSP27 small interfering RNA (siRNA) significantly reduced both mRNA (A) and protein (B) expression levels compared to treatment with scrambled siRNA as negative control. C: siRNA transfection resulted in a reduction in cell viability, and the half-maximal inhibitory concentration (IC_{50}) of HSP27 siRNA transfectants was significantly lower than that of scrambled siRNA transfectants when cells were treated with 5-fluorouracil (5-FU). Data are shown as mean values±standard error of an experiment performed in triplicate. *p<0.05 (Mann–Whitney U-test).*

should not be recommended HSP27-targeting therapy. However, even if the HSP27 level is low before treatment, it should be regularly monitored. Additional HSP27-targeting therapy should be considered when the HSP27 level is increased, as down-regulation of HSP27 may contribute to an increase or restoration of the effects of 5-FU.

Similarly, genetic changes during chemotherapy associated with acquired resistance are related to v-Ki-ras2 Kirsten rat

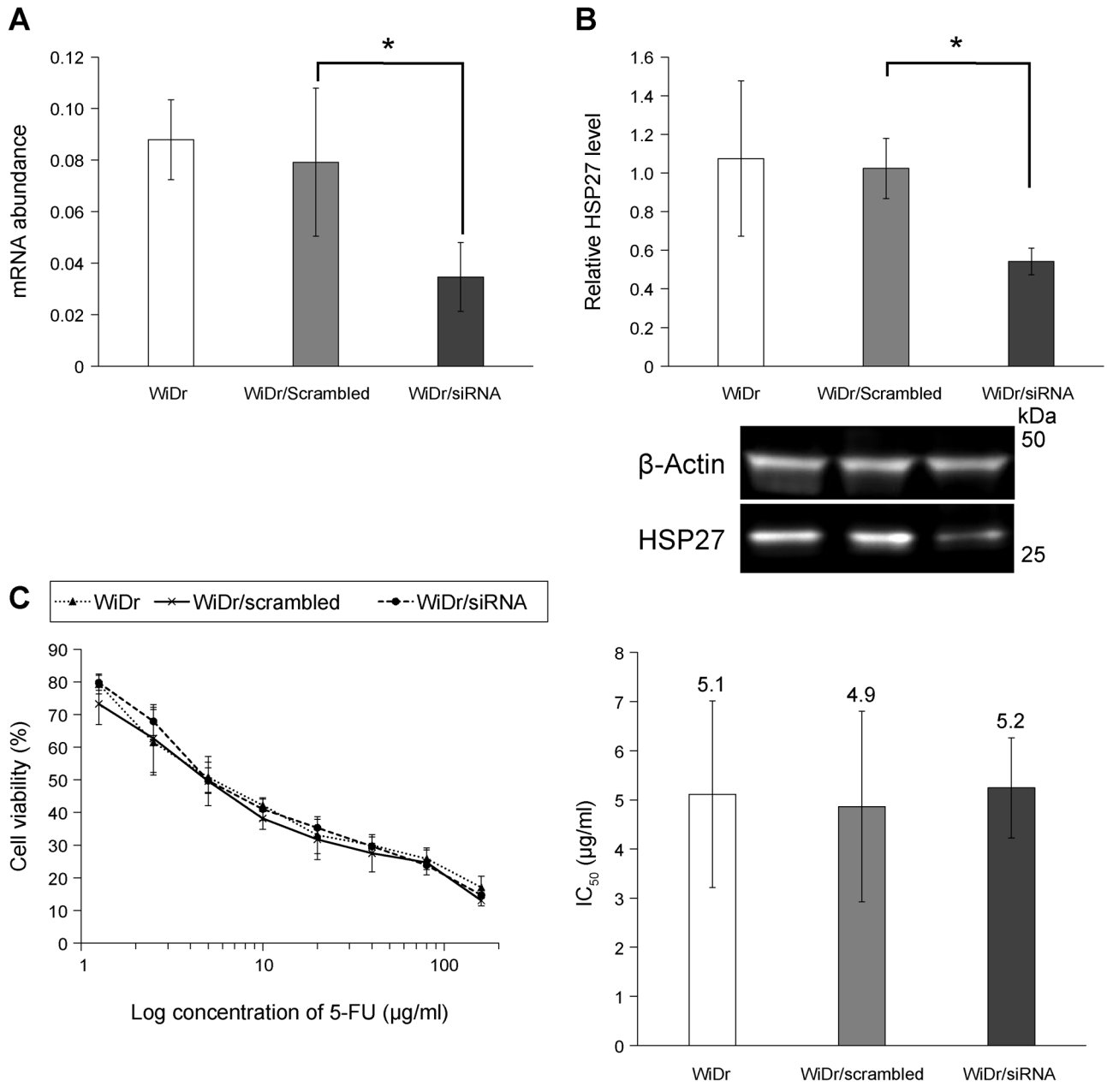

Figure 4. *Down-regulation of heat-shock protein 27 (HSP27) in WiDr cells. Treatment of WiDr cells with HSP27 small interfering RNA (siRNA) significantly reduced both mRNA (A) and protein (B) expression levels compared to treatment with scrambled siRNA as negative control. C: When HSP27 siRNA and scrambled siRNA transfectants cells were treated with 5-fluorouracil (5-FU), no significant changes in cell viability and half-maximal inhibitory concentration (IC_{50}) were observed. Data are shown as mean values±standard error of an experiment performed in triplicate. *p<0.05 (Mann–Whitney U-test).*

sarcoma viral oncogene homolog (*KRAS*), the status of which is a predictor of the effectiveness of EGFR inhibitors in CRC. Misale *et al*. showed that secondary *KRAS* mutations are not rare after EGFR-inhibitor treatment, and that this mutation is associated with the onset of acquired resistance to EGFR inhibitors used in CRC treatment (21). The authors concluded that in those cases, initiation of a mitogen-activated protein kinase inhibitor may be useful for reducing resistance; however, this strategy remains controversial at present. Additionally, the association between the HSP family and

KRAS has been previously reported. Bar *et al*. reported that HSP90 expression is associated with *KRAS* mutation and worse clinical parameters in CRC (22). The HSP family, including HSP27, may play certain roles in association with KRAS, which warrants further investigation. These findings suggest that monitoring of the molecular changes during treatment is useful for overcoming acquired resistance to chemotherapeutic drugs used in CRC treatment.

At the molecular level, our results may be explained by the suppression of apoptotic signals, which are generally recognized as important mechanisms for acquired resistance to cytotoxic agents. The main mechanism of 5-FU action that induces apoptosis in CRC involves the mitochondrial apoptotic pathway, which is related to the release of cytochrome *c* (23-25); HSP27 is a known negative effector of this cytochrome *c*-dependent apoptotic pathway (26). In our resistance model, induction of HSP27 as a self-defense function against 5-FU exposure may inhibit this apoptotic pathway, resulting in acquired resistance to 5-FU. Therefore, *HSP27* down-regulation may re-activate the apoptotic pathway to overcome acquired resistance. Lee *et al*. reported that down-regulation of the glucose-regulated protein, GRP78, in 5-FU-resistant CRC cells activated caspase-3, which is the downstream effector of cytochrome *c*, thereby inducing apoptosis (27). HSP27 may play a similar role as that of GRP78, hence further investigations are warranted.

Only a single cancer cell line was used in this study. In clinical practice, cells are heterogeneous, and thus, promising drugs in basic research require rigorous testing before clinical use. Studies using animal experiments are required, however it may be difficult to create an animal model of CRC with acquired resistance.

In conclusion, our study revealed the relationship between HSP27 and 5-FU-acquired resistance *in vitro*. These results suggest that HSP27 is a promising and novel determinant of 5-FU-acquired resistance in CRC.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Yusuke Asada and Masashi Tsuruta contributed equally to all aspects of this study, including contributing to the study design, coordination, and drafting of the article. Takehiro Shimada, Hirofumi Suzumura, Kaoru Koishikawa, and Shingo Akimoto performed the experiments and contributed to data acquisition. Koji Okabayashi, Kohei Shigeta, and Takashi Ishida contributed to the design of the study and performed the statistical analysis. Hirotoshi Hasegawa and Yuko Kitagawa contributed to the conception of the study, design, and coordination, and helped draft the article. All Authors read and approved the article and agreed to be held accountable for all aspects of the research.

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