ANTICANCER RESEARCH *25*: 2737-2742 (2005)

Exogenous Expression of hRFI Induces Multidrug Resistance through Escape from Apoptosis in Colorectal Cancer Cells

TSUYOSHI KONISHI, SHIN SASAKI, TOSHIAKI WATANABE, JOJI KITAYAMA and HIROKAZU NAGAWA

Department of Surgical Oncology, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Abstract. *Background: hRFI is a newly discovered gene encoding a Ring Finger domain highly homologous to that of the X-chromosome-linked inhibitor of apoptosis protein, which is among the most potent inhibitors of apoptosis. The hRFI protein is preferentially expressed in colorectal cancers, although its actual function is unknown. The aim of this study was to determine whether hRFI possesses an anti-apoptotic function in colorectal cancer cells against chemotherapeutic agents. Materials and Methods: HCT116 colorectal cancer cells were exogenously transfected with hRFI or LacZ as a control. After exposure to either cisplatin, irinotecan or 5-fluorouracil, apoptosis and caspase-3 activity were evaluated by flow cytometry analysis. Results: The hRFI transfectant exhibited significant resistance to apoptosis induced by each of the three agents, along with inactivation of caspase-3. Growth in the normal medium was not altered. Conclusion: hRFI plays an important role in the resistance to chemotherapeutic agent-induced apoptosis in colorectal cancer cells.*

5-Fluorouracil (5-FU), cisplatin (CDDP) and irinotecan hydrochloride (CPT-11) are widely used agents for advanced colorectal cancer. However, the prognosis of patients with advanced colorectal cancer is still poor because of *de novo* or acquired chemoresistance against these chemotherapeutic agents. Inducing apoptosis is one of the main mechanisms by which many chemotherapeutic agents, such as 5-FU, CDDP and CPT-11, eliminate cancer cells. Therefore, it is a subject of considerable interest to elucidate the mechanisms of cancer cell resistance to the apoptosis induced by chemotherapeutic agents, in order to improve the efficacy of treatment and thus the survival rates in patients with advanced colorectal cancer.

hRFI (accession no. AB084914), or "human Ring Finger homologous to Inhibitor of Apoptosis Protein (IAP) type", is a newly discovered gene which has been isolated by means of a yeast two-hybrid screening method using hTID-1, an apoptosis regulator protein, as bait (1, 2). hRFI encodes a Ring Finger domain highly homologous to that of the X-chromosome-linked inhibitor of apoptosis protein (XIAP), which is the most potent inhibitor of apoptosis among the known IAPs (3). hRFI also encodes a specific cleavage site targeted by caspase-3 at the residues 230-233. This structural evidence suggests a possible role for hRFI in protecting cells against apoptosis. Indeed, it has been demonstrated that transient transfection of hRFI into Hela cells results in a slight increase in cell survival after treatment with tumor necrosis factor alpha (TNF-α).

Previous reports have also revealed that hRFI is overexpressed in esophageal, gastric and colorectal cancers (1, 4, 5). Particularly in colorectal carcinogenesis, hRFI expression progressively increases in the transition from normal colorectal mucosas to adenomas, and from adenomas to carcinomas (4). This sequential increase of hRFI expression suggests that hRFI might play an essential role in colorectal tumor progression, probably related to its effects on anti-apoptotic transformation. However, the actual functional role of hRFI in colorectal cancer cells has not been elucidated.

The aim of this study was to investigate whether the hRFI molecule elicits resistance to the apoptosis induced by 5-FU, CDDP and CPT-11 in colorectal cancer cells.

Materials and Methods

Cells and culture conditions. HCT116 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and was maintained in McCoy's 5A Medium Modified (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS (Sigma, St. Louis, MO, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY, USA), at 37˚C in a humidified 5% CO_2 atmosphere.

Correspondence to: S. Sasaki, Department of Surgical Oncology, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel/Fax: 81-35800-8653/ 81-33811-6822, e-mail: sasaki-1su@h.u-tokyo.ac.jp

Key Words: hRFI, apoptosis, chemotherapy, cisplatin, irinotecan, 5-fluorouracil, colorectal cancer.

Establishment of a stable HCT116 cell line consistently expressing the hRFI protein. The full-length cDNA of hRFI was subcloned into a pcDNA3.1/V5-His vector (Invitrogen), as described previously (1). Cells were seeded at a density of $1x10^5$ /ml in 35-mm dishes 24 h before transfection. Cultures were then transfected with 2 μg/ml plasmids using 5 μg/ml lipofectin (Invitrogen) in a serum-free OPTIMEM medium (Invitrogen), according to the manufacturer's protocol. Transfectants were incubated for 6 h at 37˚C and, after replacement of the complete medium, maintained for an additional 48 h. For stable transfection, cells were trypsinized and incubated in the presence of 1,000 μg/ml G418 (BD Biosciences, Palo Alto, CA, USA) for 2-3 weeks. Individual G418-resistant colonies were isolated and maintained in the presence of G418.

Western blotting. Cells were scraped and lysed for 45 min at 4˚C in a lysis buffer containing phosphate-buffered saline (PBS) with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01 mg/ml aprotinin and 0.01 mg/ml leupeptin. The cell lysates were cleared by centrifugation. The concentration of protein was determined by the BCA protein assay reagent (Pierce Biomedical Company, Rockford, IL, USA). Twenty μg aliquots of whole cell lysates were electrophoresed in 15% Ready Gel J (Bio-Rad, Hercules, CA, USA). Separated proteins were electrophoretically transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and incubated with a rabbit anti-hRFI polyclonal antibody generated previously, an anti-V5 antibody (Invitrogen) and an anti-β-actin antibody (Sigma). Proteins were detected and visualized with the ECL detection system (Amersham Pharmacia Biotech).

Proliferation assay. Cells were seeded in 96-well plates at a density of $2x10^3$ /well, and the proliferative activity at 72, 96 and 120 h after seeding was determined by MTS assay, according to the manufacturer's instructions (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA), to monitor the number of viable cells. All experiments were performed in triplicate.

Flow cytometry analysis of apoptosis. Cells were plated at a density of $1x10^5$ /well in 6-well plates 24 h before the induction of apoptosis. After treatment with CDDP, CPT-11 or 5-FU for 48 h, the cells were harvested by trypsinization and double-stained with AnnexinV-FITC and propidium iodide (PI) using an AnnexinV-FITC Apoptosis Detection Kit (BioVision Research Products, Mountain View, CA, USA), according to the manufacturer's protocol. Samples were immediately analyzed using Becton Dickinson FACScan with CELLQuest software (Franklin Lakes, NJ, USA). The apoptosis rate was defined as the percentage of the AnnexinV-FITC-positive rate in apoptosis-induced cells. Assays were performed in triplicate.

Flow cytometry analysis of caspase-3 activity. Cells were plated at a density of $1x10^5$ /well in 6-well plates 24 h before the induction of apoptosis. After treatment with the chemotherapeutic agents for 48 h, the cells were harvested by trypsinization and caspase-3 activity was determined using a Caspase-3 Detection Kit (CALBIOCHEM, Darmstadt, Germany), according to the manufacturer's protocol. Samples were immediately analyzed using a Becton Dickinson FACScan with CELLQuest software. Assays were performed in triplicate.

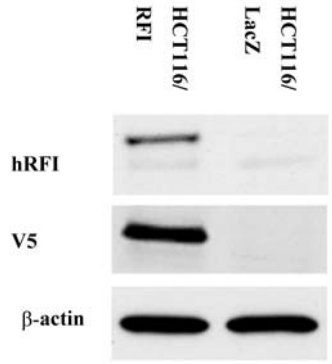

Figure 1. *Western blot analysis of hRFI expression in HCT116 cells stably transfected with hRFI or LacZ. HCT116 cells were transfected with an expression vector containing the full length cDNA of hRFI or LacZ using lipofectin. After 2-3 weeks selection in G418, resistant cells were isolated and analyzed for the hRFI protein by Western blot. An anti-hRFI polyclonal antibody and anti-V5-HRP antibody were used. Anti-β-actin was used as a loading control. The exogenously transfected hRFI with the V5-His tag was detected at a larger molecular weight than the native hRFI.*

Results

Selection of HCT116 cells stably expressing hRFI. HCT116 cell lines stably overexpressing hRFI were initially established, in order to evaluate the functional role of hRFI in human colon cancer cells. HCT116 cells were transfected with an expression vector containing full length hRFI cDNA (HCT116/hRFI cells) or a control vector containing LacZ (HCT116/LacZ). After selection in G418, resistant cells were isolated and the relative level of hRFI was determined by Western blot. The Western blot analysis revealed that HCT116/hRFI cells exhibited a marked increase of hRFI protein expression as compared to HCT116/ LacZ cells (Figure 1). The exogenously transfected hRFI with a V5-His tag was detected at a larger molecular weight than the native hRFI.

Effect of hRFI on cell proliferation in HCT116 cells. In order to determine whether hRFI expression affected cell proliferation, the *in vitro* cell proliferation of HCT116/hRFI and HCT116/LacZ cells was assessed by MTS assay. No significant differences in proliferation appeared between these transfectants at 72, 96 or 120 h after plating (data not shown).

Effect of hRFI on apoptosis induced by CDDP, CPT-11 and 5-FU in HCT116 cells. In order to investigate whether hRFI expression affected the apoptosis induced by the chemotherapeutic agents in HCT116 cells, HCT116/hRFI and HCT116/LacZ cells were treated with CDDP, CPT-11, or 5-FU at three different concentrations for 48 h, as shown

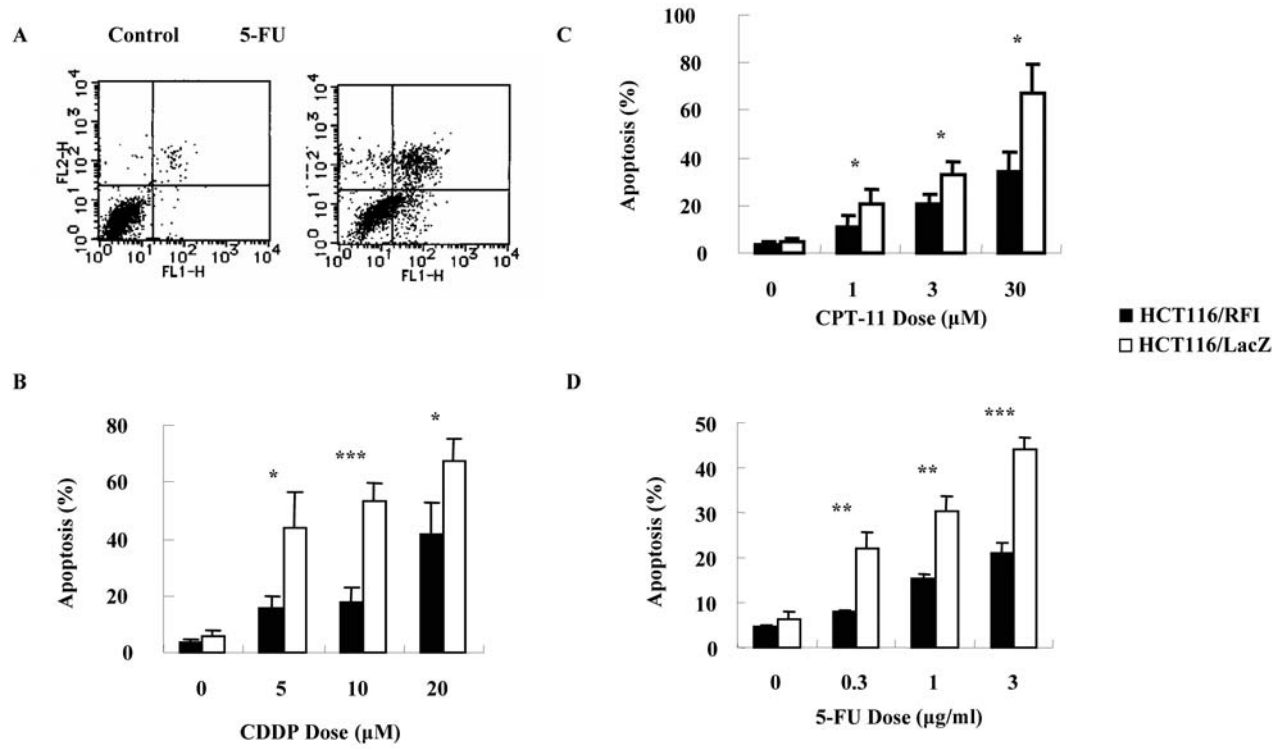

Figure 2. *Effect of hRFI expression on apoptosis after exposure to CDDP, CPT-11 and 5-FU in HCT116 cells. A) Representative result of flow cytometry analysis for the apoptosis. Cells without apoptosis did not exhibit any staining for AnnexinV-FITC or PI and cells that underwent apoptosis stained positive for AnnexinV after 48-h treatment with 3 μg/ml 5-FU. B-D) HCT116/hRFI and HCT116/LacZ cells were exposed for 48 h to the indicated concentrations of B) CDDP, C) CPT-11 and D) 5-FU and were analyzed with flow cytometry for the apoptosis rate after double-staining with AnnexinV-FITC and PI. Columns and bars indicate the mean and SD of values in triplicate. *, ** and *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared to the control, as determined by Student's t-test.*

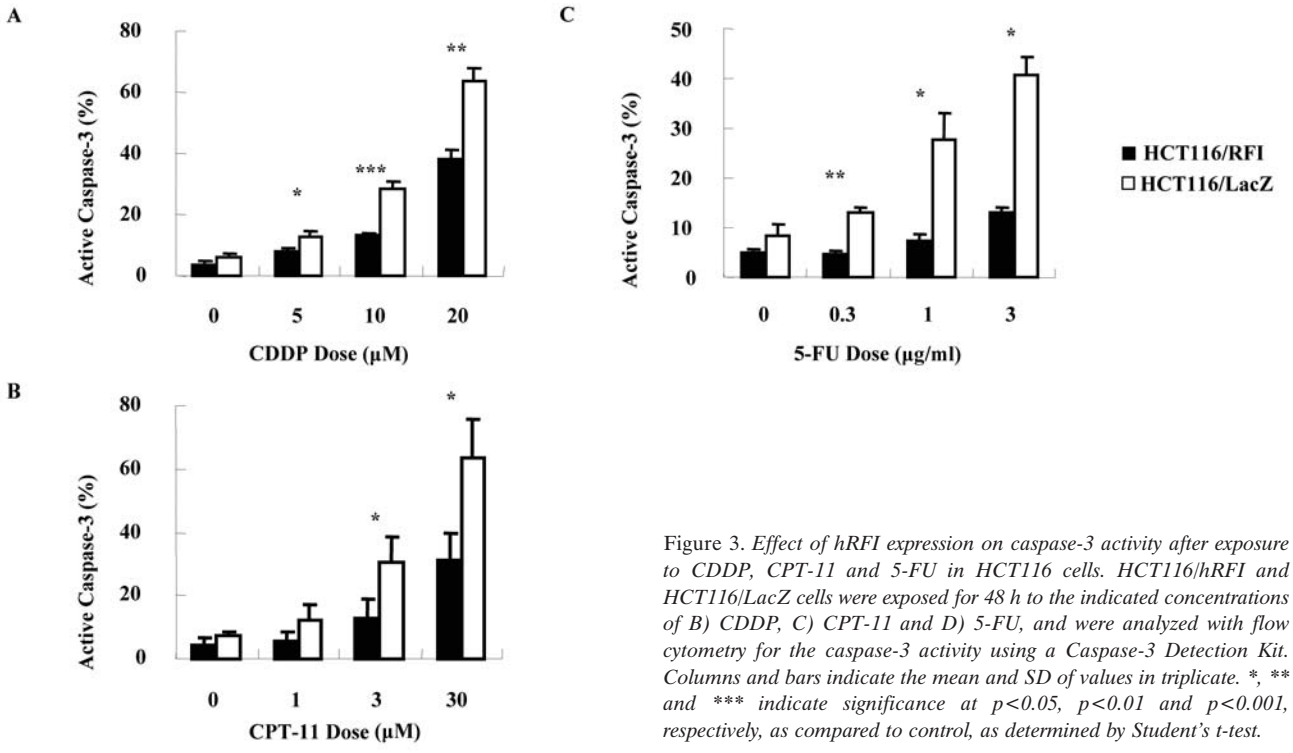

Figure 3. *Effect of hRFI expression on caspase-3 activity after exposure to CDDP, CPT-11 and 5-FU in HCT116 cells. HCT116/hRFI and HCT116/LacZ cells were exposed for 48 h to the indicated concentrations of B) CDDP, C) CPT-11 and D) 5-FU, and were analyzed with flow cytometry for the caspase-3 activity using a Caspase-3 Detection Kit. Columns and bars indicate the mean and SD of values in triplicate. *, ** and *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared to control, as determined by Student's t-test.*

in Figure 2 B-D, and were analyzed for the apoptosis rate after double-staining with AnnexinV-FITC and PI using flow cytometry. As shown in Figure 2A, cells without treatment exhibited basically no staining for either AnnexinV-FITC or PI, while cells that underwent apoptosis stained positive for AnnexinV. As shown in Figure 2 B-D, apoptosis was observed in a dose-dependent manner in both transfectants for the three chemotherapeutic agents. However, apoptosis rates in the HCT116/hRFI cells were significantly decreased compared to those in the HCT116/LacZ cells, at any of the administered doses of these chemotherapeutic agents. These results indicate that the overexpression of hRFI inhibited the apoptosis induced by CDDP, CPT-11 and 5-FU in HCT116 cells.

Effect of hRFI on caspase-3 activation induced by CDDP, CPT-11 and 5-FU in HCT116 cells. In order to determine whether the anti-apoptotic effect of hRFI was associated with the caspase-3 inactivation, caspase-3 activity was measured after each treatment with CDDP, CPT-11 or 5-FU. As shown in Figure 3, caspase-3 activity in the HCT116/hRFI cells was significantly decreased compared to that in the HCT116/LacZ cells. These results indicate that the inhibition of the apoptosis induced by the chemotherapeutic agents in the hRFI transfectant was associated with caspase-3 inactivation.

Discussion

The present study demonstrated that hRFI overexpression inhibits the apoptosis induced by each of the three agents CDDP, CPT-11 and 5-FU in colorectal cancer cells. However, the mechanisms which induce tumor cell death are quite different among these three agents. CDDP inhibits DNA replication and chain elongation by forming adducts to the DNA, which is believed to be the main cause of its antineoplastic activity, and its action is not dependent on the cell cycle (6). In contrast, CPT-11 is a topoisomerase-I inhibitor that interferes with DNA replication only in the S-phase (7). 5-FU inhibits the synthesis of both RNA and DNA, and its action is time-dependent (8). In spite of these different mechanisms among the three agents, apoptosis was similarly inhibited by hRFI overexpression. Indeed, hRFI has also been suggested to inhibit TNF-α-induced-apoptosis. The overexpression of hRFI in Hela cells is reported to exhibit a trend toward increasing survival of cells after treatment with TNF-α (1). Furthermore, in HCT116 colorectal cancer cells, our preliminary study has revealed that the overexpression of hRFI significantly inhibited TNF-α-induced-apoptosis (9). However, the known molecular mechanisms which induce apoptosis are different between TNF-α and the chemotherapeutic agents. TNF-α stimulates the death receptors and induces the activation of the extrinsic apoptotic pathway, which is triggered by the formation of death-inducing signaling complexes and the activation of caspase-8, finally resulting in the sequential cleavage of downstream effector caspases such as caspase-3 (10). In contast, the chemotherapeutic agents mainly work by means of the mitochondrial apoptotic pathway, which is triggered by the release of cytochrome c from mitochondria and the activation of the caspase-9-containing apoptosome complex, finally resulting in the common sequential cleavage cascades (11). The present study demonstrated that hRFI inhibited the apoptosis induced by multiple chemotherapeutic agents as well as TNF-α, namely, the apoptosis triggered by both pathways. Therefore, it is suggested that hRFI functions as an anti-apoptotic regulator in both the extrinsic and the mitochondrial pathways, or acts at a common downstream element in both.

From a structural point of view, hRFI posseses a Ring Finger domain highly homologous to that of XIAP, which is the most potent inhibitor of apoptosis among the known IAPs (3). Recent studies have demonstrated that the Ring Finger is associated with ubiquitin ligase activity. The Ring Finger of XIAP also exerts the ubiquitin ligase activity, which is responsible for the proteosomal degradation of caspase-3, smac and XIAP itself, thus regulating the anti-apoptotic effect of XIAP (12, 13). Considering that the Ring Finger domain of hRFI possesses a strong homology to that of XIAP, the results of this study suggest a possible relationship between hRFI and the ubiquitin ligase activity targeted to caspases, smac and XIAP. Another characteristic structure of hRFI is the DEDD sequence at the residues 230-233, where the hRFI protein was cleaved by caspase-3 protein *in vitro* (1). Although direct inhibition of the caspase-3 activity by the hRFI protein has yet to be examined, direct interactaction with caspase-3 is another possible mechanism of the anti-apoptotic property of hRFI.

Finally, the apoptosis regulators are known to be closely related to both chemosensitivity and prognosis in colorectal cancer. For example, the Bax/ Bcl-XL ratio positively correlates with chemosensitivity to 5-FU in colorectal cancer cells (14). Survivin expression correlates with a poorer prognosis in colorectal cancer, and gene therapy targeting survivin has been investigated (15, 16). In light of the finding that hRFI enhanced the anti-apoptotic property of colorectal cancer cells against chemotherapeutic agents, hRFI is a novel candidate for a predictive marker for chemosensitivity, and a therapeutic target for gene therapy in colorectal carcinomas.

In conclusion, hRFI plays an important role in the resistance of cancer cells to chemotherapeutic agent-induced apoptosis. The expression levels of hRFI may be a powerful predictor for chemosensitivity in colorectal cancer, and further investigation is needed to clarify its value for both this application and as a therapeutic target.

Acknowledgements

We thank all the members of the Tumor Biology Study Group, Dr Takeshi Kobunai (Dept. of Systematic Clinical Oncology) and Ms Uchikawa for their helpful discussions and exellent technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, Culture, and Technology of Japan and from the Ministry of Health, Labour and Welfare, and in part by the Public Trust of Surgery Research Fund.

References

1 Sasaki S, Nakamura T, Arakawa H, Mori M, Watanabe T, Nagawa H and Croce CM: Isolation and characterization of a novel gene, *hRFI*, preferentially expressed in esophageal cancer. Oncogene *21*: 5024-5030, 2002.

2 Syken J, De-Medina T and Munger K: TID1, a human homolog of the *Drosophila* tumor suppressor l(2)tid, encodes two mitochondrial modulators of apoptosis with opposing functions. Proc Natl Acad Sci USA *96*: 8499-8504, 1999.

3 Holcik M, Gibson H and Korneluk RG: XIAP: apoptotic brake and promising therapeutic target. Apoptosis *6*: 253-261, 2001.

4 Sasaki S, Watanabe T, Konishi T, Kitayama J and Nagawa H: Effects of expression of hRFI on adenoma formation and tumor progression in colorectal adenoma-carcinoma sequence. J Exp Clin Cancer Res *23*: 507-512, 2004.

5 Sasaki S, Kitayama J, Watanabe T, Konishi T and Nagawa H: Diffuse expression of hRFI is correlated with blood vessel invasion in gastric carcinoma. Jpn J Clin Oncol *34*: 584-587, 2004.

6 Crul M, van Waardenburg RC, Beijnen JH and Schellens JH: DNA-based drug interactions of cisplatin. Cancer Treat Rev *28*: 291-303, 2002.

7 Li LH, Fraser TJ, Olin EJ and Bhuyan BK: Action of camptothecin on mammalian cells in culture. Cancer Res *32*: 2643-2650, 1972.

8 Longley DB, Harkin DP and Johnston PG: 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer *3*: 330-338, 2003.

9 Konishi T, Sasaki S, Watanabe T, Kitayama J and Nagawa H: Overexpression of hRFI (human ring finger homologous to inhibitor of apoptosis protein type) inhibits death receptor-mediated apoptosis in colorectal cancer cells. Mol Cancer Ther *4*: 743-750, 2005.

10 Krammer PH: CD95's deadly mission in the immune system. Nature *407*: 789-795, 2000.

11 Roy S and Nicholson DW: Cross-talk in cell death signaling. J Exp Med *192*: 21-26, 2000.

12 Suzuki Y, Nakabayashi Y and Takahashi R: Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci USA *98*: 8662-8667, 2001.

13 MacFarlane M, Merrison W, Bratton SB and Cohen GM: Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination *in vitro*. J Biol Chem *277*: 36611-36616, 2002.

14 Nita ME, Nagawa H, Tominaga O, Tsuno N, Fujii S, Sasaki S, Fu CG, Takenoue T, Tsuruo T and Muto T: 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. Br J Cancer *78*: 986-992, 1998.

15 Kawasaki H, Toyoda M, Shinohara H, Okuda J, Watanabe I, Yamamoto T, Tanaka K, Tenjo T and Tanigawa N: Expression of survivin correlates with apoptosis, proliferation and angiogenesis during human colorectal tumorigenesis. Cancer *91*: 2026-2032, 2001.

16 Mesri M, Wall NR, Li J, Kim RW and Altieri DC: Cancer gene therapy using a survivin mutant adenovirus. J Clin Invest *108*: 981-990, 2001.

Received January 25, 2005
Accepted May 2, 2005