hRFI overexpressed in HCT116 cells modulates Bcl-2 family proteins when treated with 5-fluorouracil

SHIN SASAKI[1,2], TOSHIAKI WATANABE[1], TAKASHI KOBUNAI[2], TSUYOSHI KONISHI[1],
HIDEKI NAGASE[3], YOSHIKAZU SUGIMOTO[3], TOSHINORI OKA[3] and HIROKAZU NAGAWA[1]

Departments of [1]Surgical Oncology, [2]Systematic Clinical Oncology, the University of Tokyo,
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; [3]Optimal Medication Research Laboratory,
Taiho Pharmaceutical Co., Ltd., 224-2 Hiraishi-Ebisuno, Tokushima 771-9194, Japan

Abstract. Exogenous overexpression of hRFI, originally isolated in our laboratory, inhibits not only death receptor-mediated apoptosis but also the mitochondrial apoptosis induced by several chemotherapeutic agents including 5-fluorouracil (5-FU). Recently, it has become clear that hRFI targets and degradates caspase-8 and -10 in death receptor-mediated apoptosis by E3 ubiquitin activity in a ring finger domain homologous to that of X-chromosome-linked inhibitor of apoptosis protein (XIAP). However, the cellular mechanism of the inhibition of mitochondrial apoptosis by hRFI has not been fully elucidated. We prepared HCT116 overexpressing hRFI (HCT116/hRFI) cells and comprehensively analyzed the expression changes of 51 apoptosis-related genes with or without 5-FU treatment between HCT116/hRFI and mock cells using microfluidic low-density arrays. As a result, we identified four genes (Bcl-2, Bcl-XL, cIAP2, and CFLAR) whose expression was four or more times higher in HCT116/hRFI cells than in HCT116/LacZ cells, and found that Bcl-2 and the ratio of Bcl-2/Bax or Bcl-2/Bak were upregulated when HCT116/hRFI cells were treated with 5-FU. Furthermore, we also validated the up-regulation of Bcl-2 and Bcl-XL in HCT116/hRFI cells treated with 5-FU by Western blot analysis. Such evidence suggests that the modulation of Bcl-2 family proteins seen in 5-FU treatment plays an important role in the anti-apoptotic function of HCT116/hRFI cells.

Introduction

hRFI (accession no. AB084914), human ring finger homologous to IAP type, is a gene which we isolated by means of a two-hybrid yeast screening method using hTID-1, an apoptosis

---

Correspondence to: Dr Shin Sasaki, Department of Surgical Oncology, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
E-mail: sasaki-1su@h.u-tokyo.ac.jp

regulator protein, as bait (1-3) and subsequently determined the cellular and chromosomal localization. We also demonstrated that hRFI is preferentially expressed in several digestive tract cancers, such as of the esophagus (1), stomach (4), and colorectum (5). Especially in colorectal carcinogenesis, the expression of hRFI increases in the transition from the normal colorectal mucosa to adenoma, and from adenoma to carcinoma (5). From the structural point of view, hRFI has several functional domains, including a ring finger domain highly homologous to that of X-chromosome-linked inhibitor of apoptosis protein (XIAP) (1), which is the most potent inhibitor of apoptosis among the IAPs (6,7), and a phospholipid-binding FYVE domain (8).

Recently, it has been reported that the ring finger domain has E3 ubiquitin activity which targets caspase-8 and -10 in death receptor-mediated apoptosis (8), supporting our previous finding that exogenous overexpression of hRFI in colorectal cancer cells results in inhibition of the extrinsic apoptotic pathway (9). However, we found that exogenous overexpression of hRFI inhibited not only death receptor-mediated apoptosis but also the mitochondrial apoptosis induced by several chemotherapeutic agents, including 5-fluorouracil (5-FU) (10). The cellular mechanism of the inhibition of mitochondrial apoptosis by hRFI has not been established yet. Considering that resistance to the induction of apoptosis is one of the most important determinants of colorectal cancer cell chemoresistance to 5-FU (11,12), clarification of this mechanism is an important issue for strategic chemotherapy applications in colorectal cancer patients.

To identify the differentially expressed genes in HCT116 cells overexpressing hRFI and mock cells with or without 5-FU treatment, we screened 51 apoptosis-related genes by microfluidic low-density arrays.

Materials and methods

Establishment of a stable HCT116 cancer cell line consistently overexpressing the hRFI protein. HCT116 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in McCoy's 5A Medium Modified (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma, St. Louis,

MO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY) at 37°C in a humidified 5% CO_2 atmosphere.

A stable HCT116 cancer cell line consistently over-expressing the hRFI protein was established as described previously (9). In brief, the full-length cDNA of hRFI was subcloned into a pcDNA3.1/V5-His vector (Invitrogen), and HCT116 cells were transfected with 2 μg/ml plasmids using 5 μg/ml lipofectin (Invitrogen) in serum-free OPTIMEM medium (Invitrogen) according to the manufacturer's protocol. For stable transfection, cells were trypsinized and incubated in the presence of 1000 μg/ml G418 (BD Biosciences, Palo Alto, CA) for 2-3 weeks. Individual G418 resistant colonies were isolated and maintained in the presence of G418.

Preparation of mRNA and whole lysate of samples. HCT116 cells overexpressing hRFI (HCT116/hRFI) and mock (HCT116/LacZ) cells were plated in 10-cm culture dishes at a density of 2x10⁶/dish. Twenty-four hours after plating, two dishes of each cell were subjected to isolation of mRNA and whole lysate as the no 5-FU treatment. For others, 1 or 5 μg/ml of 5-FU was added. Cells harvested at 24 and 48 h after the treatment were also subjected to mRNA and cell lysate preparation. To isolate mRNA, we used Isogen (Nippongene) according to the manufacturer's protocol. To isolate whole lysate for Western blotting, cells were scraped and lysed for 1 h 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. Cell lysates were cleared by centrifugation. The concentration of proteins was determined using BCA protein assay reagent (Pierce Biomedical Company, Rockford, IL).

Western blotting. Aliquots (40 μg) of whole cell lysates were electrophoresed in 15% Ready Gel J (Bio-Rad, Hercules, CA). Separated proteins were electrophoretically transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) and incubated with each antibody. Proteins were detected and visualized using an ECL detection system (Amersham Pharmacia Biotech). A mouse anti-V5 monoclonal antibody was purchased from Invitrogen; a mouse anti-ß-actin mono-clonal antibody from Sigma; a mouse anti-Bak monoclonal antibody from BD Biosciences (San Diego, CA); a mouse anti-Bax, Bcl-XL monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA); and a mouse anti-Bcl-2 monoclonal antibody from Dako Cytomation Denmark A/S (Glostrup, Denmark).

Microfluidic low-density arrays. We selected 51 apotosis-related genes from the source of European Bioinformatics Institute (EBI) GeneOntology. The categories used for the selection are as follows; apoptosis activator activity (GO:0016506), apoptosis inhibitor activity (GO:0008189), and apotosis regulator activity (GO:0016329). For 51 selected genes as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we ordered microfluidic low-density arrays from Applied Biosystems. The detectors for each gene used in this study are shown in Table I. The observed Ct values of genes were normalized by the ΔΔCt methods, using the GAPDH gene as an endogenous control and mock cell lines as calibrator samples (13,14).

Results

Identification of differentially expressed apotosis-related genes according to hRFI expression. The analysis of the fold change of gene expression between HCT116/hRFI and HCT116/LacZ cells using microfluidic low-density arrays identified 4 genes (Bcl-2, Bcl-XL, cIAP2, and CFLAR) whose expression levels were four or more times higher in HCT116/hRFI cells than in HCT116/LacZ cells (Table I).

Analysis of the fold change of the ratio of gene expression at 48 h to that at 24 h treatment with 5-FU. When cells were treated with 1 μg/ml or 5 μg/ml of 5-FU, the fold change of the ratio of gene expression at 48 h to that at 24 h exhibited the highest level in Bcl-2 (14.118 and 7.393, respectively). Survivin and TNFRSF6 also exhibited remarkably high fold changes. Compared to Bcl-2, Bcl-XL exhibited small changes (1.187 and 1.386, respectively). The results are shown in Table I.

Evaluation of the ratio of expression of anti-apoptotic genes to that of pro-apoptotic genes. We analyzed the expression ratio of Bcl-2 to Bax, Bcl-2 to Bak, Bcl-XL to Bax, and Bcl-XL to Bak. The fold change of the ratio at 48 h of 5-FU treatment to that at 24 h exhibited a high level in Bcl-2 to Bax and Bcl-2 to Bak, as shown in Table II. Especially when treated with 1 μg/ml of 5-FU, the fold change was remarkably high in these ratios (8.134 and 8.944, respectively). In contrast, the fold change in Bcl-XL to Bax and Bcl-XL to Bak was low.

Western blot analysis. Western blot analysis revealed that the expression of Bcl-XL in HCT116/hRFI cells was much higher than that in HCT116/LacZ cells; however, there was no remarkable alteration in the expression of other proteins (Bcl-2, Bax, and Bak) between the two cell lines. When cells were treated with 5-FU at concentrations of 1, and 5 μg/ml for 24 h, Bcl-XL was remarkably induced only in HCT116/hRFI cells.

Bcl-2 was gradually induced in a time-dependent manner after treatment with 1 μg/ml of 5-FU; however, the induction of Bcl-2 reached the highest level after 24 h treatment with 5 μg/ml of 5-FU and its expression decreased after 48 h treatment. Exogenous hRFI detected with anti-V5 antibody, like Bcl-XL, was induced in HCT116/hRFI cells. In contrast, the expression of pro-apoptotic proteins, Bax and Bak, exhibited no remarkable alteration in either cell line (Fig. 1).

Discussion

Fluoropyrimidines, including 5-fluorouracil (FU), are the most common drugs used for the treatment of colorectal cancer, and prediction of the response to 5-FU at the molecular level is very important in effective clinical use. Previously, we reported that overexpression of hRFI significantly inhibited several chemotherapeutic agents, including 5-FU, with decreased activities of caspase-3, -8, and -9 (10). Furthermore, we found that high expression of hRFI correlated with resistance to

Table I. Fold change of 51 apotosis-related genes' expression with or without 5-FU treatment between HCT116/hRFI and HCT116/LacZ cells.

Ref. Seq.	Genbank	Gene symbol	Description	Detector	Fold change (HCT116/h RFI to HCT116/LacZ)		
					5-FU no treatment	5-FU 1 μg/ml 48/24 h	5-FU 5 μg/ml 48/24 h
NM_013229	AB007873	APAF1	Apoptotic protease activating factor	APAF1-Hs00559441_m1	1.073	3.756	3.408
NM_000038	BC034955	APC	Adenomatosis polyposis coli	APC-Hs00181051_m1	2.361	4.061	3.461
NM_000484	AF282245	APP	Amyloid ß (A4) precursor protein	APP-Hs00169098_m1	2.534	1.717	2.861
NM_004322	AF031523	BAD	BCL2-antagonist of cell death	BAD-Hs00188930_m1	1.142	1.709	3.235
NM_00428	BC014656	BAG3	BCL2-associated athanogene 3	BAG3-Hs00188713_m1	1.630	5.176	2.494
NM_001188	BC004431	BAK1	BCL2-antagonist/killer 1	BAK1-Hs00832876_g1	0.975	1.579	2.47
NM_138763	L22474	BAX	BCL2-associated X protein	BAX-Hs00180269_m1	0.724	1.736	1.290
NM_000633	BC027258	BCL2	B-cell CLL/ lymphoma 2 (Bcl-2)	BCL2-Hs00608023_m1	4.750	14.118	7.393
NM_001191	Z23116	BCL2L1	BCL2-like 1 (Bcl-XL)	BCL2L1-Hs00169141_m1	4.856	1.187	1.386
NM_197966	BC033634	BID	BH3 interacting domain death agonist	BID-Hs00609630_m1	1.154	5.123	6.150
NM_001197	U49730	BIK	BCL2-interacting killer	BIK-Hs00609635_m1	2.468	1.810	1.675
NM_004536	U19251	BIRC1	Baculoviral IAP repeat-containing 1 (NAIP)	BIRC1-Hs00244967_m1	N.D.	N.D.	N.D.
NM_001166	BC028578	BIRC2	Baculoviral IAP repeat-containing 2 (cIAP1)	BIRC2-Hs00357350_m1	2.243	4.750	5.695
NM_001165	BC037420	BIRC3	Baculoviral IAP repeat-containing 3 (cIAP2)	BIRC3-Hs00154109_m1	9.829	2.256	2.131
NM_001167	U32974	BIRC4	Baculoviral IAP repeat-containing 4 (XIAP)	BIRC4-Hs00745222_s1	1.860	1.557	2.554
NM_001168	AF077350	BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	BIRC5-Hs00153353_m1	1.579	9.838	10.737
NM_016252	AF265555	BIRC6	Baculoviral IAP repeat-containing 6 (apollon)	BIRC6-Hs00212288_m1	1.585	1.977	2.015
NM_13931	AF301009	BIRC7	Baculoviral IAP repeat-containing 7 (livin)	BIRC7-Hs00223384_m1	N.D.	N.D.	N.D.
NM_033341	BC039318	BIRC8	Baculoviral IAP repeat-containing 8	BIRC8-Hs00364262_s1	2.426	0.655	0.405
NM_003879	AF041462	CFLAR	CASP8 and FADD-like apoptosis regulator	CFLAR-Hs00153439_m1	4.071	2.769	2.469
NM_003805	U79115	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	CRADD-Hs00187009_m1	2.560	3.797	2.382
NM_001350	AF039136	DAXX	Death-associated protein 6	DAXX-Hs00154692_m1	1.501	3.363	4.218
NM_004401	AF087573	DFFA	DNA fragmentation factor, 45 kDa, α polypeptide	DFFA-Hs00189336_m1	1.585	N.D.	N.D.
NM_004402	AB013918	DFFB	DNA fragmentation factor, 40 kDa, ß polypeptide	DFFB-Hs00237077_m1	N.D.	N.D.	N.D.
NM_004435	BC016351	ENDOG	Endonuclease G	ENDOG-Hs00172770_m1	1.476	2.978	2.275
NM_003824	X84709	FADD	Fas (TNFRSF6)-associated via death domain	FADD-Hs00538709_m1	1.378	3.041	2.952
NM_131917	AK094843	FAF1	Fas (TNFRSF6) associated factor 1	FAF1-Hs00169544_m1	2.149	2.498	5.497

Table I. Continued.

Ref. Seq.	Genbank	Gene symbol	Description	Detector	Fold change (HCT116/h RFI to HCT116/LacZ)		
					5-FU no treatment	5-FU 1 μg/ml 48/24 h	5-FU 5 μg/ml 48/24 h
NM_001924	M60974	GADD45A	Growth arrest and DNA-damage-inducible, α	GADD45A-Hs00169255_m1	1.965	3.520	2.097
NM_002746	Z11696	MAPK3	Mitogen-activated protein kinase 3	MAPK3-Hs00385075_m1	1.556	5.531	2.999
NM_005938	X93996	MLLT7	Myeloid/lymphoid or mixed-lineage leukemia	MLLT7-Hs00172973_m1	1.675	2.998	4.842
NM_002467	BC058901	MYC	v-myc myelocytomatosis viral oncogene homolog	MYC-Hs00153408_m1	1.176	1.334	1.438
NM_002598	AK055180	PDCD2	Programmed cell death 2	PDCD2-Hs00751277_sH	0.701	1.859	3.595
NM_145813	AF131759	PDCD8	Programmed cell death 8	PDCD8-Hs00377585_m1	1.294	3.289	4.452
NM_000326	J04213	RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	RIPK1-Hs00169407_m1	1.799	1.646	2.121
NM_025126	AF306709	RIPK2	Receptor-interacting serine-threonine kinase 2	RIPK2-Hs00169419_m1	1.851	4.419	3.452
NM_003576	AF024636	STAT1	Signal transducer and activator of transcription 1	STAT1-Hs00234829_m1	1.904	8.507	3.527
NM_015975	AF220509	STK24	Serine/threonine kinase 24	STK24-Hs00269168_m1	1.664	1.847	0.978
NM_001065	BC010140	TNFRSF10A	Tumor necrosis factor receptor supf., member 10a	TNFRSF10A-Hs00269491_m1	1.876	2.199	1.440
NM_001066	BC052977	TNFRSF10B	Tumor necrosis factor receptor supf., member 10b	TNFRSF10B-Hs00187196_m1	1.404	0.781	0.830
NM_148972	AY358309	TNFRSF1A	Tumor necrosis factor receptor supf., member 1A	TNFRSF1A-Hs00533560_m1	1.587	0.834	1.326
NM_003810	BC020220	TNFRSF25	Tumor necrosis factor receptor supf., member 25	TNFRSF25-Hs00237054_m1	1.326	1.089	2.501
NM_153012	BC019047	TNFRSF6	Tumor necrosis factor receptor supf., member 6	TNFRSF6-Hs00531110_m1	3.674	8.282	9.999
NM_000639	D38122	TNFSF10	Tumor necrosis factor (ligand) supf., member 10	TNFSF10-Hs00234356_m1	0.120	0.028	0.002
NM_003286	U07806	TNFSF12	Tumor necrosis factor (ligand) supf., member 12	TNFSF12-Hs00611242_m1	0.743	0.598	0.526
NM_001067	J04088	TNFSF6	Tumor necrosis factor receptor supf., member 6	TNFSF6-Hs00181225_m1	N.D.	N.D.	N.D.
NM_003789	BT006934	TP53	Tumor protein p53	TP53-Hs00153340_m1	3.130	4.474	4.221
NM_145726	L38509	TRADD	TNFRSF1A-associated via death domain	TRADD-Hs00601065_g1	1.445	1.110	0.927
NM_001071	BC018858	TRAF2	TNF receptor-associated factor 2	TRAF2-Hs00377454_m1	0.939	0.372	2.233
NM_003342	BT007416	TRAF3	TNF receptor-associated factor 3	TRAF3-Hs00377462_m1	1.227	4.130	3.845
		GAPDH[a]	Glyceraldehyde-3-phosphate dehydrogenase	4342376-GAPDH			

Supf., superfamily; N.D., not determined, [a]used as an endogenous control.

fluoropyrimidines in human colon cancer cell lines and in human cancer xenografts (15). These lines of evidence clearly suggest that hRFI might be not only a novel predictive marker for 5-FU but also a therapeutic target for gene therapy in colorectal cancer. For clinical applications, the mechanism by which hRFI inhibits 5-FU-induced mitochondrial apoptosis

Table II. The fold change of the expression ratio of Bcl-2 to Bax, Bcl-2 to Bak, Bcl-XL to Bax, and Bcl-XL to Bak when treated with 5-FU.

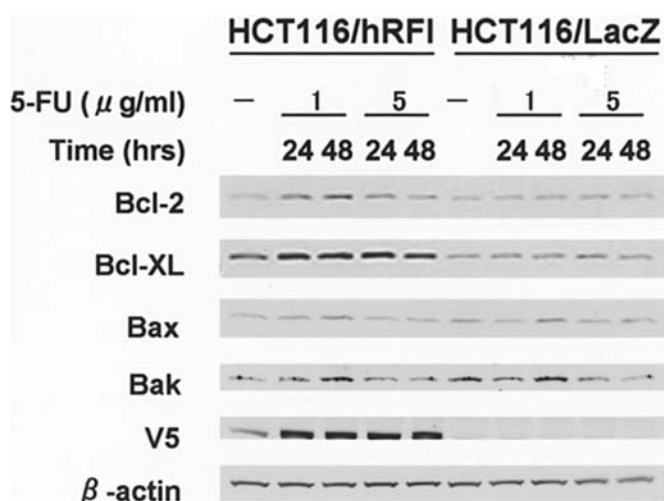| 5-FU	1 μg/ml			5 μg/ml		
	24 h	48 h	48/24 h	24 h	48 h	48/24 h
Bcl-2/Bax	0.185	1.508	8.134	0.345	0.985	2.854
Bcl-2/Bak	0.207	1.853	8.944	0.415	1.335	3.214
Bcl-XL/Bax	0.423	0.289	0.684	0.307	0.212	0.691
Bcl-XL/Bak	0.473	0.356	0.752	0.369	0.287	0.778

Figure 1. Expression of anti- (Bcl-2 and Bcl-XL) and pro- (Bax and Bak) apoptotic proteins belonging to the Bcl-2 family as well as expression of extrinsic hRFI in HCT116/hRFI and HCT116/LacZ cells without or with treatment of 5-FU by Western blot analysis. ß-actin was used as a control.

needs to be elucidated. Hence, we decided to comprehensively analyze 51 apoptosis-related genes which were selected from EBI GeneOntology and investigated by means of microfluidic low-density arrays. The microfluidic low-density array was developed by Applied Biosystems (Foster City, CA, USA), and mRNA expression of up to 384 target genes can be analyzed on a single card on the basis of real-time reverse transcription PCR (RT-PCR). The target genes can be randomly selected and researchers can utilize this method as a custom-made DNA microarray. Abruzzo LV *et al* have described the practical procedures of this method and reported reproducible analysis with a wide dynamic range compared to other high-throughput forms of analysis (14).

As a result of the study of the 51 apoptosis-related genes' expression between HCT116/hRFI and HCT116/LacZ cells, we identified 4 genes (Bcl-2, Bcl-XL, cIAP2, and CFLAR) whose expression is four or more times higher in HCT116/hRFI cells than in HCT116/LacZ cells. Bcl-2 and Bcl-XL are the anti-apoptotic members of the Bcl-2 family, sharing the conserved Bcl-2 homology (BH) regions BH1, BH2 and BH3 (7,16,17), and act as stabilizers of mitochondrial membrane integrity by blocking the release of cytochrome c (and smac/

DIABLO) and subsequent caspase-9 and -3 activation, thus regulating the mitochondrial apoptotic pathway (7,18,19). cIAP2 is also known as HIAP1, one of the inhibitors of apoptosis protein (IAP), and inhibits apoptosis by binding to the tumor necrosis factor receptor-associated factors, TRAF1 and TRAF2 (20,21). CFLAR has several nomenclature designations (FLIP, CASPER, FLAME1, CASH, CLARP, and MRIT), interacts with FADD, TRAF1, caspase-8, and -3 and regulates cell death pathways (22,23). It is of great interest that the expression of these genes, which mainly exert inhibition of apoptosis, was higher in HCT116/hRFI than in HCT116/LacZ cells. Furthermore, the analysis of the fold change of each gene's expression between the two cell lines, not only in periods of quiescence but also in the time course after treatment with 5-FU, helps shed light on the cellular mechanisms of the anti-apoptotic effects of hRFI. Our previous report revealed that HCT116/hRFI cells significantly inhibited 5-FU-induced apoptosis compared to HCT116/LacZ cells after treatment with 5-FU at 0.3, 1, and 3 μg/ml for 48 h (10). Hence, we analyzed the expression of genes for HCT116/hRFI and HCT116/LacZ cells with or without treatment of 5-FU at 1 and 5 μg/ml for 48 h. When treated with 5-FU, the fold change of the ratio of gene expression at 48 h to that at 24 h was the highest in Bcl-2. Survivin and TNFRSF6 also exhibited remarkably high changes. Unlike Bcl-2, Bcl-XL didn't exhibit remarkable alterations of fold change of the ratio (at 48 h:24 h) between the two cell lines. This evidence suggests that up-regulation of Bcl-2 may have an effect on the anti-apoptotic function of hRFI during treatment 5-FU.

Recently, there have been reports that a dynamic interplay among the Bcl-2 family of proteins has an effect on the response to some chemotherapeutic agents. In particular, the relative ratio of anti-apoptotic (Bcl-2 or Bcl-XL) to pro-apoptotic (Bax or Bak) genes is one of the determinants of the response to DNA-damaging agents and 5-FU (11,24-26). We also previously reported that the Bcl-XL to Bax ratio significantly correlates with resistance to 5-FU in colorectal cancer cell lines (26). Hence, we analyzed four kinds of ratio change (Bcl-2:Bax, Bcl-2:Bak, Bcl-XL:Bax, and Bcl-XL: Bak) during 5-FU treatment. As shown in Table II, we found that the fold change of the ratio at 48 h to that at 24 h treatment of 5-FU at a concentration of 1 μg/ml was high in Bcl-2 to Bax and Bcl-2 to Bak. Especially when treated with 1 μg/ml of 5-FU, the fold change was remarkably high among these ratios (8.134 and 8.944, respectively). The expression of Bcl-2 family genes in a steady-state varies extremely widely in colorectal cancer cell lines, as has been reported previously (26). Which genes are predominantly induced or suppressed by 5-FU treatment apparently also varies in different cells; however, it is suggested in this study that the expression of pro- or anti-apoptotic genes belonging to the Bcl-2 family generally have the greatest effect on 5-FU chemosensitivity.

Finally, we performed Western blot analysis to validate the change of expression at protein level. Western blotting revealed that, after treatment with 5-FU at concentrations of 1 and 5 μg/ml for 24 h, Bcl-XL was remarkably induced in HCT116/hRFI cells, and that Bcl-2 was gradually induced in a time-dependent manner after treatment with 1 μg/ml of 5-FU. These results imply that Bcl-XL is initially upregulated and,

later, Bcl-2 is gradually upregulated when apoptosis is induced by 5-FU treatment in hRFI overexpressing cells and that such upregulation of anti-apoptotic genes might play an important role in the anti-apoptotic function of hRFI for 5-FU induced apoptosis.

Recently, McDonald ER *et al* reported two genes which bind to and negatively regulate death effector domain (DED) caspases (caspase-8 and -10) by ubiquitination (8). Interestingly, one of these, CARP1, is hRFI itself, and they clarified that hRFI (CARP1 in their terminology) has E3 activity via a ring finger domain at the C terminus, leading to the proteasomal degradation of DED caspases when hRFI is overexpressed. This finding supports our previous data that overexpression of hRFI inhibits apoptosis induced by tumor necrosis factor-α (TNF-α) or TNF-related apotosis-inducing ligand (TRAIL) in colorectal cancer cells (9), and might account for the cellular mechanism of inhibition of death receptor-mediated apoptosis by hRFI. Considering the mechanism in mitochondrial apoptotic pathway, there is the possibility that hRFI regulates certain apoptosis activating proteins through proteasomal degradation by its E3 activity. This might also clarify hRFI overexpression and the manner in which hRFI upregulates Bcl-2 and Bcl-XL and elevates the relative ratio of Bcl-2 to Bax or to Bak during 5-FU treatment.

Acknowledgements

References

1. Sasaki S, Nakamura T, Arakawa H, *et al*: Isolation and characterization of a novel gene, hRFI, preferentially expressed in esophageal cancer. Oncogene 21: 5024-5030, 2002.
2. Syken J, Macian F, Agarwal S, Rao A and Munger K: TID1, a mammalian homologue of the *Drosophila* tumor suppressor lethal(2) tumorous imaginal discs, regulates activation-induced cell death in Th2 cells. Oncogene 22: 4636-4641, 2003.
3. 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.
4. Sasaki S, Kitayama J, Watanabe T, Konishi T and Nagawa H: Diffuse expressions of hRFI is correlated with blood vessel invasion in gastric carcinoma. Jpn J Clin Oncol 34: 584-587, 2004.
5. 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.
6. Holcik M, Gibson H and Korneluk RG: XIAP: apoptotic brake and promising therapeutic target. Apoptosis 6: 253-261, 2001.
7. Yang L, Cao Z, Yan H and Wood WC: Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res 63: 6815-6824, 2003.
8. McDonald ER III and El-Deiry WS: Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth. Proc Natl Acad Sci USA 101: 6170-6175, 2004.
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. Konishi T, Sasaki S, Watanabe T, Kitayama J and Nagawa H: Exogenous expression of hRFI induces multidrug resistance through escape from apoptosis in colorectal cancer cells. Anticancer Res 25: 2737-2741, 2005.
11. Violette S, Poulain L, Dussaulx E, *et al*: Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. Int J Cancer 98: 498-504, 2002.
12. Pritchard DM, Potten CS, Korsmeyer SJ, Roberts S and Hickman JA: Damage-induced apoptosis in intestinal epithelia from bcl-2-null and bax-null mice: investigations of the mechanistic determinants of epithelial apoptosis *in vivo*. Oncogene 18: 7287-7293, 1999.
13. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] method. Methods 25: 402-408, 2001.
14. Abruzzo LV, Lee KY, Fuller A, *et al*: Validation of oligonucleotide microarray data using microfluidic low-density arrays: a new statistical method to normalize real-time RT-PCR data. Biotechniques 38: 785-792, 2005.
15. Sasaki S, Watanabe T, Kobunai T, Konishi T, Kitayama J and Nagawa H: High hRFI expression correlates with resistance to fluoropyrimidines in human colon cancer cell lines and in xenografts. J Exp Clin Cancer Res 24: 397-403, 2005.
16. Cory S and Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2: 647-656, 2002.
17. Chang BS, Kelekar A, Harris MH, Harlan JE, Fesik SW and Thompson CB: The BH3 domain of Bcl-x(S) is required for inhibition of the antiapoptotic function of Bcl-x(L). Mol Cell Biol 19: 6673-6681, 1999.
18. Eskes R, Desagher S, Antonsson B and Martinou JC: Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol 20: 929-935, 2000.
19. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD: The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132-1136, 1997.
20. Uren AG, Pakusch M, Hawkins CJ, Puls KL and Vaux DL: Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci USA 93: 4974-4978, 1996.
21. Liston P, Roy N, Tamai K, *et al*: Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 379: 349-353, 1996.
22. Shu HB, Halpin DR and Goeddel DV: Casper is a FADD- and caspase-related inducer of apoptosis. Immunity 6: 751-763, 1997.
23. Han DK, Chaudhary PM, Wright ME, *et al*: MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. Proc Natl Acad Sci USA 94: 11333-11338, 1997.
24. Oltvai ZN and Korsmeyer SJ: Checkpoints of dueling dimers foil death wishes. Cell 79: 189-192, 1994.
25. Shi X, Liu S, Kleeff J, Friess H and Buchler MW: Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. Oncology 62: 354-362, 2002.
26. Nita ME, Nagawa H, Tominaga O, *et al*: 5-fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. Br J Cancer 78: 986-992, 1998.