

Proteomic analysis of the basic proteins in 5-fluorouracil resistance of human colon cancer cell line using the radical-free and highly reducing method of two-dimensional polyacrylamide gel electrophoresis

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Abstract. 5-Fluorouracil (5-FU) is widely used for the treatment of patients with advanced colon cancers and it is the mainstay of chemotherapy. However, the acquisition of resistance to 5-FU is one of the most prominent obstacles to successful chemotherapy. The purpose of this study was to identify the novel biological basis of 5-FU resistance in colon cancer cells. This study is the first comparative proteomic analysis of basic proteins between the DLD-1 human colon cancer cell line and DLD-1/5-FU its 5-FU resistant sub-line using the radical-free and highly reducing method of two-dimensional polyacrylamide gel electrophoresis, which has a superior ability in the separation of basic proteins and the quantification of post-translational modification. A densitometric analysis was performed to quantify the modulated proteins, and protein spots showing significant changes were identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry. Six basic proteins significantly modulated between DLD-1 and DLD-1/5-FU were identified. All of them showed up-regulated expression in DLD-1/5-FU in comparison to DLD-1. The six identified spots, corresponding to five different proteins included heterogeneous nuclear ribonucleoprotein G, mitochondrial trans-

cription factor A, histone H2B, histone H4 and ribosomal protein L3. Among the 5 basic proteins, several proteins are potentially related to 5-FU resistance by protecting the cells from DNA damage.

Introduction

5-Fluorouracil (5-FU) is widely used for the treatment of patients with advanced colon cancers and is still the mainstay of chemotherapy. However, this anticancer effect varies widely among individual patients (1,2). Some patients suffer adverse effects of 5-FU chemotherapy, while others do not. Human colon cancers show heterogeneous behavior at the molecular and cellular levels, and the tumor cells can develop resistance to chemotherapy (3,4). Resistance to 5-FU is one of the most prominent obstacles to successful chemotherapy. Therefore, the identification of the novel biological markers of 5-FU resistance in tumor cells is one of the most important steps for developing optimal treatment strategies. Furthermore, understanding the mechanisms how 5-FU causes tumor cell death and how tumor cells become resistant to 5-FU is an essential step towards predicting or overcoming this resistance.

To identify the predictive biomarkers for 5-FU resistance, several comprehensive studies have investigated 5-FU resistance by DNA microarray analysis technique (5-7). However, the genome is only a carrier of genetic information, whereas the protein is the executor of living activities. Moreover, changes at the mRNA level do not always correlate with alterations in the protein level, and genomics does not analyze the regulation of protein-protein interactions and post-translational modifications that contribute to the protein changes. In this respect, a proteome-based approach that can identify quantitative and qualitative protein changes associated with 5-FU resistance is an important and useful research approach. However, few studies have used

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2-D PAGE

proteomic-based approach, or specifically, comprehensive proteomics using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to screen proteins from human colon cancer cell lines with induced 5-FU resistance (8,9).

In comparative proteomics for identifying novel biomarkers of drug resistance, 2-D PAGE have been performed mainly using an immobilized pH gradient (IPG) method as the mainstream procedure. Actually, the IPG method can separate proteins within a limited range of isoelectric points, but it can not separate basic proteins with isoelectric points greater than pH 10. On the other hand, with the radical-free and highly reducing (RFHR) 2-D PAGE, the migration rate of protein is determined by the net charge of the protein at pH 8.6 in the first dimension electrophoresis, and the net charge at pH 3.6 and molecular sieving effect in the second dimension electrophoresis. Therefore, separation of proteins in the RFHR 2-D PAGE is not limited by the isoelectric points of protein. Basic proteins, even if the isoelectric points are very high, can be sufficiently separated by the RFHR 2-D PAGE as well as neutral and acidic proteins (10,11). Furthermore, changes of the net charge of proteins by post-translational modification are detected as shifts of protein spots by RFHR 2-D PAGE. This high potential for separation of basic proteins in RFHR 2-D PAGE has been utilized through much of the analysis of the ribosomal proteins (RPs) (11).

5-FU is transformed into two active metabolites, fluorouridine triphosphate (FUTP) and fluorodeoxyuridine monophosphate (FdUMP), and induces tumor cells to undergo apoptosis in response to DNA and RNA damage. FUTP is incorporated into RNA instead of uridine triphosphate, and interferes with the maturation and finally the function of RNA; FdUMP forms a covalent ternary complex with thymidylate synthase (TS) and 5, 10-methylene tetrahydrofolate, thereby inhibiting deoxythymidine monophosphate synthesis from deoxyuridine monophosphate by TS and finally, DNA synthesis (12). Because the basic proteins can bind to DNA/RNA with a positive protein charge, it may be thought that identification of basic proteins related to DNA and RNA damage by 5-FU, using the RFHR 2-D PAGE, could provide further evidence for elucidation of the 5-FU resistant mechanism in colon cancer cells.

This study is the first comparative proteomic analysis between DLD-1 human colon cancer cell line and its 5-FU resistant line, DLD-1/5-FU, using an improved RFHR 2-D PAGE. An analysis of the enzyme activity analysis associated with 5-FU metabolism indicated that orotate phosphoribosyl transferase (OPRT), a key enzyme of 5-FU anabolism, was lower in DLD-1/5-FU than DLD-1 (13). In the cells with lower OPRT, a decreased metabolism of FUTP from 5-FU induces the attenuation of RNA damage (12). Six basic proteins which were modulated between DLD-1 and DLD-1/5-FU were identified. Among these proteins, several proteins had altered expressions potentially related to resistance to 5-FU induced DNA damage. The identification of the novel biological markers related to 5-FU resistance may therefore greatly help to achieve not only an improvement in both chemotherapeutic regimens and the patient prognosis, but it may also help in the development of novel idea for pharmaceutical products.

Materials and methods

Tumor cell lines. The human colon cancer cell line, DLD-1 and its 5-FU resistant sub-cell line, DLD-1/5-FU, were used in this study. DLD-1/5FU was selected by stepwise increases in 5-FU concentrations from 0.2 to 100 μ M (13). Cells were cultured in RPMI-1640 supplemented with 10% FBS at 37°C with 5% CO₂ in a humidified atmosphere.

In vitro cytotoxicity assay. A colorimetric assay using the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), was employed to assess the suppression of cell proliferation by 5-FU (provided by Taiho Pharmaceutical) (14). In brief, the cells (5×10^2 cells/well) were seeded into 96-well microplates. After 24 h, the cells were cultured with 200 μ l ZnSO₄ supplemented in growth medium and treated with varying doses of 5-FU. At 4 days after exposure to drugs, 0.1 mg of MTT was added to each well and incubated at 37°C for an additional 2 h. The plates were centrifuged at 450 x g for 5 min at room temperature and medium removed. DMSO (0.15 ml) was added to each well to solubilize the crystals and the plates were read at 540 nm using a microplate reader (Bio-Rad Laboratories Inc.). All experiments were done six times and the mean and SD of the IC₅₀ (μ M) values were calculated. The differences of cytotoxic effects of 5-FU were evaluated by the non-parametric Mann-Whitney's U test using Microsoft Office Excel software. The statistical significance was set at P-value <0.05.

Protein preparation. For protein preparation, 6 sheets of 150 cm² flask (80% confluent) of DLD-1 and DLD-1/5-FU were used, respectively. The nuclear, mitochondrial, high salt washed ribosomal and post-ribosomal supernatant fractions were prepared from the cell extract, essentially according to the method of Madjar (15). These fractions were mixed again, and the total proteins were prepared using the acetic acid method (16). After dialysis against 2% acetic acid at least five times, the proteins were lyophilized and stored at -80°C until use.

2-D PAGE and image analysis. The extracted proteins (~2 mg/gel) were analyzed by the RFHR 2-D PAGE as previously described with some modifications (10,11). The apparatus for the RFHR procedure was obtained from Nihon Eido (Tokyo, Japan). Briefly, protein samples were dissolved in a lysis buffer containing 10 M urea and 0.2 M β -mercaptoethanol, and incubated at 37°C for 60 min. Pyronine-G (Merck Ltd. Germany) and recrystallized acridine orange (Wako Purified Reagent, Kyoto, Japan) were added to the sample solution as a migration marker for sample charging electrophoresis. After a prerun was performed at 100 V for 60 min, sample charging electrophoresis was carried out on a 5% polyacrylamide gel containing 9 M urea, 12 mM KOH and 0.074% acetic acid at 100 V for 30 min. Pyronine-G and acridine orange in the sample solution concentrated rapidly and migrated into the gels, and the sample proteins followed them. When Pyronine-G and acridine orange had run inside the gels for at least 10 mm, the run was finished. Sample gel pieces containing proteins were obtained by cutting the gels

with a razor at a position immediately below the band of Pyronine-G and within a length of 10 mm. Subsequently, the first dimensional (1-D) separation was performed on a 7% polyacrylamide gel containing 9 M urea, 400 mM Tris, 500 mM boric acid and 21.5 mM EDTA-2Na. After a prerun was performed at 100 V for 6 h, the sample gel pieces were inserted into the spaces that were cut out with a ground spatula to the same length as the sample gel pieces. 1-D electrophoresis was performed at 170 V for 20 h with air cooling. The second dimensional (2-D) separation was then carried out on a 14% polyacrylamide gel containing 9 M urea, 50 mM KOH and 5% acetic acid (16x16x0.2 cm). After a prerun was performed at 100 V for 15 h, the 1-D gels and the sample gels were put on the 2-D gels. The 2-D electrophoresis was performed at 100 V for 20 h with air cooling. Gels were stained with Coomassie Brilliant Blue (CBB) G-250 (Nacalai Tesque, Kyoto, Japan). The gels were scanned with a GS-800 Calibrated Densitometer for differential analysis, using PDQuest™ version 7.1. software (Bio-Rad Laboratories Inc.). In addition the gel images were analyzed by the Quantity One™ version 4.5.0. software program (Bio-Rad Laboratories Inc.). For each gel, spot detection, area calculation and background subtraction was done, and unmodulated spots on each gel (identified as Elongation factor 1- α 1, Gene name EF1A1 in Swiss-Prot) were used as internal controls. The detection of changes in protein maps between the two cell lines was confirmed by visual inspection. The spots were considered significantly up- (or down-) regulated if the corresponding volumes showed an increase (or decrease) by a factor of at least two. For each of the cell lines, 3 lysates (from 3 independent cultures) were analyzed in duplicate (i.e., six experiments for each cell line).

In-gel digestion. The protein spots of interest were excised for digestion with sequence-grade modified trypsin (Promega) or lysyl endopeptidase Lys-C (Wako Purified Reagent) using the following procedures. The gel pieces were destained by incubation in 25 mM ammonium bicarbonate, 30% methanol for 10 min. For reductive alkylation, gels dehydrated with 100% acetonitrile for 10 min at room temperature were incubated for 45 min at 55°C in 50 μ l of 10 mM dithiothreitol, 25 mM ammonium bicarbonate, followed by replacement of the dithiothreitol solution with 50 μ l of 50 mM iodoacetamide, 25 mM ammonium bicarbonate and incubation for 30 min at room temperature in the dark. Gel pieces were washed with 5% acetic acid, 50% methanol, dehydrated in 100% acetonitrile and completely dried in a SpeedVac device. Dried pieces were rehydrated for 30 min at 4°C in 8-20 μ l of a solution of 50 mM ammonium bicarbonate containing 50-100 ng of trypsin or Lys-C. After incubation for 16 h at 37°C, peptides were extracted with 0.1% trifluoroacetic acid in 30% acetonitrile for 10 min at room temperature with sonication. The second extraction of the peptides was performed with 0.1% trifluoroacetic acid in 60% acetonitrile. The extracts were concentrated and desalted using ZipTip C18 micropipette tips (Millipore Corp.).

Protein identification by mass spectrometry. For identification using the peptide mass fingerprinting (PMF) method, 0.7 μ l aliquots were deposited on a target plate (Bruker

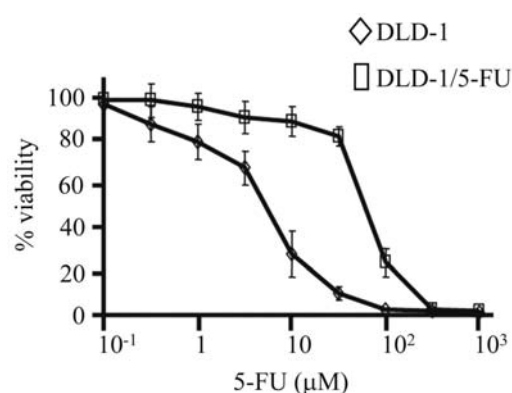


Figure 1. DLD-1 and DLD-1/5-FU drug sensitivities. DLD-1 and DLD-1/5-FU were exposed to varying concentrations of 5-FU for 4 days. Growth inhibition was determined by incubation with the tetrazolium dye MTT and by measurement of absorbance at 540 nm. The data represent the means and SD of six replicates at each concentration.

Daltonik GmbH, Bremen, Germany) followed by deposition of 0.7 μ l of matrix (10 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid). The identification of each protein was carried out by PMF analysis using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and by tandem mass spectrometry (MS/MS) analysis using Ultraflex (Bruker Daltonik GmbH). The proteins were identified by matching the PMF results with the Swiss-Prot protein database using the Mascot Search engine. Database searches were carried out using the following parameters: taxonomy, *homo sapiens*; enzyme, trypsin or LysC; and allowance of one missed cleavage. Carbamidomethylation was selected as a fixed modification and oxidation of methionine was allowed to be variable. The peptide and fragment mass tolerance were set at 100 ppm and 0.5 Da, respectively.

Results

Cytotoxic effects by 5-FU treatment in human colon cancer cell lines. The cytotoxic effects of 5-FU were determined in DLD-1 and DLD-1/5-FU. Following *in vitro* treatment of the cells with 5-FU with several different drug concentrations for 4 days, cell proliferation was evaluated using the MTT assay, and the dose-response curves were plotted (Fig. 1). The degree of resistance to 5-FU was estimated at the ratio of the IC₅₀, the concentration inhibiting growth by 50%, of the resistant line and to that of its parent line. The IC₅₀ values of 5-FU were 7.02 \pm 1.0 μ M and 74.1 \pm 4.7 μ M in DLD-1 cells and DLD-1/5-FU cells, respectively. The cytotoxic effects of 5-FU against the 5-FU resistant line was 10.6-fold lower than that against the parental cells ($P < 0.3 \times 10^{-6}$) (Table I). The degree of resistance to 5-FU of the resistant line seems to be quite stable because the IC₅₀ was consistent even after sequential passages without 5-FU (data not shown).

Separation of proteins by RFHR 2-D PAGE. To analyze the underlying mechanisms and identify downstream mediators that are unique to the effects of 5-FU resistance in colon cancer cells, a comparative proteome analysis of the two cell

Table I. Cytotoxic effect of 5-FU for human colon cancer cell line (DLD-1) and 5-FU resistant cell line (DLD-1/5-FU).

Cell lines	IC ₅₀ (μM) ^a for 5-FU	Relative resistance ^b
DLD-1	7.02±1.02	10.56
DLD-1/5-FU	74.14±4.71	

^aIC₅₀ is defined as the drug concentration that produced a 50% reduction in 540-nm absorbance compared with untreated controls in MTT assays.
^bFold changes in 5-FU IC₅₀ in resistant cells compared with parent cells.

lines was conducted. In each cell line, the basic proteins were separated by RFHR 2-D PAGE with high reproducibility. The analytical CBB staining was performed for the detection of differentially expressed basic proteins for each cell line. Typical separations of basic proteins isolated from DLD-1 and DLD-1/5-FU are shown in Fig. 2. In this figure, all of the proteins that have been significantly detected as modulated proteins by the densitometric analysis with confirmed by visual inspectional from each 3 lysates were marked with arrows. To identify the differentially expressed basic proteins, 6 proteins that showed significant changes were chosen, and

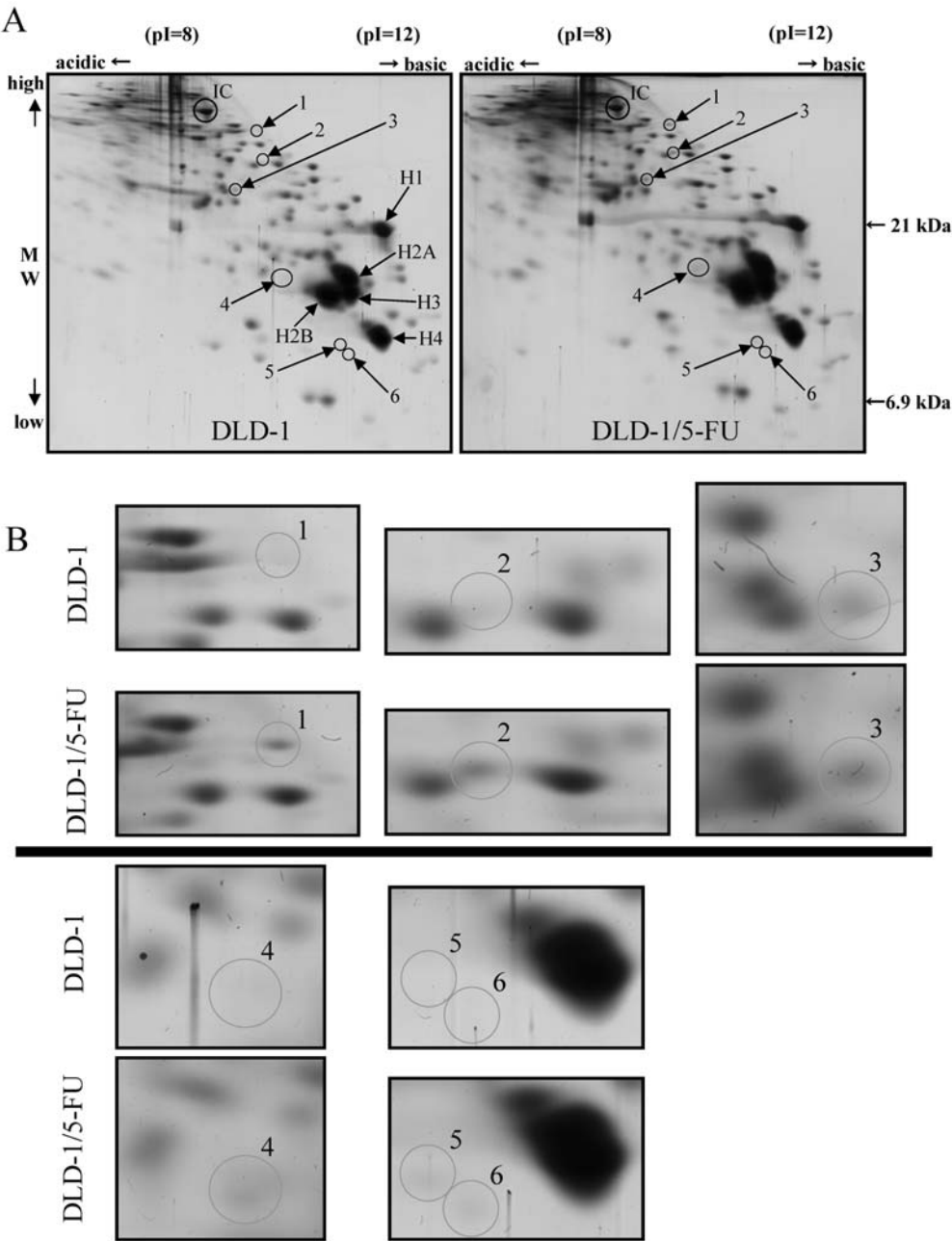


Figure 2. RFHR 2-D PAGE maps of DLD-1 and DLD-1/5-FU. Proteins were separated and visualized by CBB staining. Protein spots marked on the maps were considered differentially expressed and identified by Ultraflex MALDI TOF/TOF MS. Elongation factor 1- α 1 was used as an internal control (IC) for normalization purposes. Each cell line from 3 independent cultures was analyzed in duplicate. (A) The arrows and circles indicate the modulated expression of basic proteins between DLD-1 and DLD-1/5-FU. H1, H2A, H2B, H3 and H4 indicate histone H1, histone H2A, histone H2B, histone H3 and histone H4, respectively. All of significantly modulated spots showed up-regulated expression in DLD-1/5-FU in comparison to DLD-1. (B) Magnification of the spots containing the modulated expression. The corresponding identities are listed in Table II.

Table II. MALDI-TOF/TOF MS identification of the modulated expression of basic proteins in DLD-1/5-FU.

Spot no.	Identification	Gene name	Mass (Da)	pI	% Sequence coverage ^a	Mscot score ^b	Function	Change factor ^c
1	Ribosomal protein L3	RL3	46,109	10.20	39 983.517 (10-18)	144	60S ribosomal protein	2.54
2	Heterogeneous nuclear ribonucleoprotein G	HNRPG	42,306	10.06	47 1,513.660 (233-244)	242	Nucleic acid binding protein	3.15
3	Transcription factor A, mt precursor	TFAM	29,306	9.74	70 1,171.619 (160-169)	267	Maintenance and replication of mtDNA	2.37
4	Histone H2B C/D/H/K/M/N/F	H2B1	13,811	10.31	44 953.541 (101-109)	123	Necessary for the condensation of nucleosome	2.3
5	Histone H4	H4	11,360	11.36	37 1,211.650 (93-103)	75	Necessary for the condensation of nucleosome	2.42
6	Histone H4	H4	11,360	11.36	46 1,211.650 (93-103) 1,734.021 (46-60)	117	Necessary for the condensation of nucleosome	2.39

^aIdentified/confirmed by MS/MS analysis (TOF/TOF), thus indicated fragments are indicated with their scores. ^bThese values show the combined Mascot score, representing the total score of PMF and MS/MS (TOF/TOF) score. ^cThe means calculated from each 3 lysates of DLD-1 and DLD-1/5-FU.

all of them showed up-regulated expression in DLD-1/5-FU in comparison to DLD-1 as determined by a spot volume.

Identification of differentially expressed basic proteins between DLD-1 and DLD-1/5-FU. The peptides were extracted from the excised gel after in-gel digestion and then analyzed using Ultraflex MALDI-TOF/TOF MS. The acquired spectra were processed and searched against a Mascot Search engine based on the Swiss-Prot protein database with high confidence based on high scores and sequence coverage. An example [heterogeneous nuclear ribonucleoprotein G (hnRNP G)] of the PMF pattern of a spot and an excised MS/MS pattern obtained by MALDI-TOF/TOF are shown in Fig. 3. Table II presents these identifications with gene name, theoretical molecular weights, pI, sequence coverage rates, as well as the combined Mascot score that represents the total score of PMF and MS/MS (TOF/TOF) score. These proteins can be classified according to their functions, including RNA splicing proteins (heterogeneous nuclear ribonucleoproteins), mitochondrial DNA replication and maintenance proteins (mitochondrial transcription factor), histones and RPs.

Discussion

Six spots were identified by MALDI-TOF/TOF MS which were modulated between DLD-1 and DLD-1/5-FU. The five identified proteins included hnRNP G, mitochondrial transcription factor A (TFAM), histone H2B, histone H4 and RP L3. All of the basic proteins showed up-regulated expression in DLD-1/5-FU in comparison to DLD-1.

The RFHR 2-D PAGE in this study was developed as an improvement of the Kaltschmidt and Wittmann's method with the aim of separating and quantifying proteins that are small, basic and rich in disulphide bridges (17). Extremely small basic proteins are difficult to separate because of their exceedingly high mobility in conventional IPG method. In the RFHR method, the proteins are separated primarily by their net charges at pH 8.6 in 7% polyacrylamide gels in the first dimension, whereas in the second dimension separation is based mainly on molecular weight and net charge at pH 3.6 in 14% polyacrylamide gels. Preruns using radical scavengers were carried out to eliminate residual free radicals from the gels and thereby decreasing the loss of proteins caused by such radicals. The sample charging electrophoresis, before the first dimensional electrophoresis, was also designed to limit protein loss and, in addition, to concentrate a dilute sample solution as a sharp band. The second dimension electrophoresis was carried out at a more acidic pH 3.6 instead of pH 4.5 to obtain a better separation of extremely small basic proteins. In addition, even protein alterations such as post-translational modifications can be clearly separated and quantified (10,11), the RFHR 2-D PAGE protocol has been improved by decreasing the polyacrylamide and increasing the urea concentration of the gels in comparison with the original method, which held the sample loss to a minimum during the sample charging electrophoresis. On the other hand, the total proteins used in the RFHR method were prepared by mixing each fraction after cell fractionation as described in Materials and methods. Some proteins did not migrate in the gel during sample charging electrophoresis because of protein aggregation, and

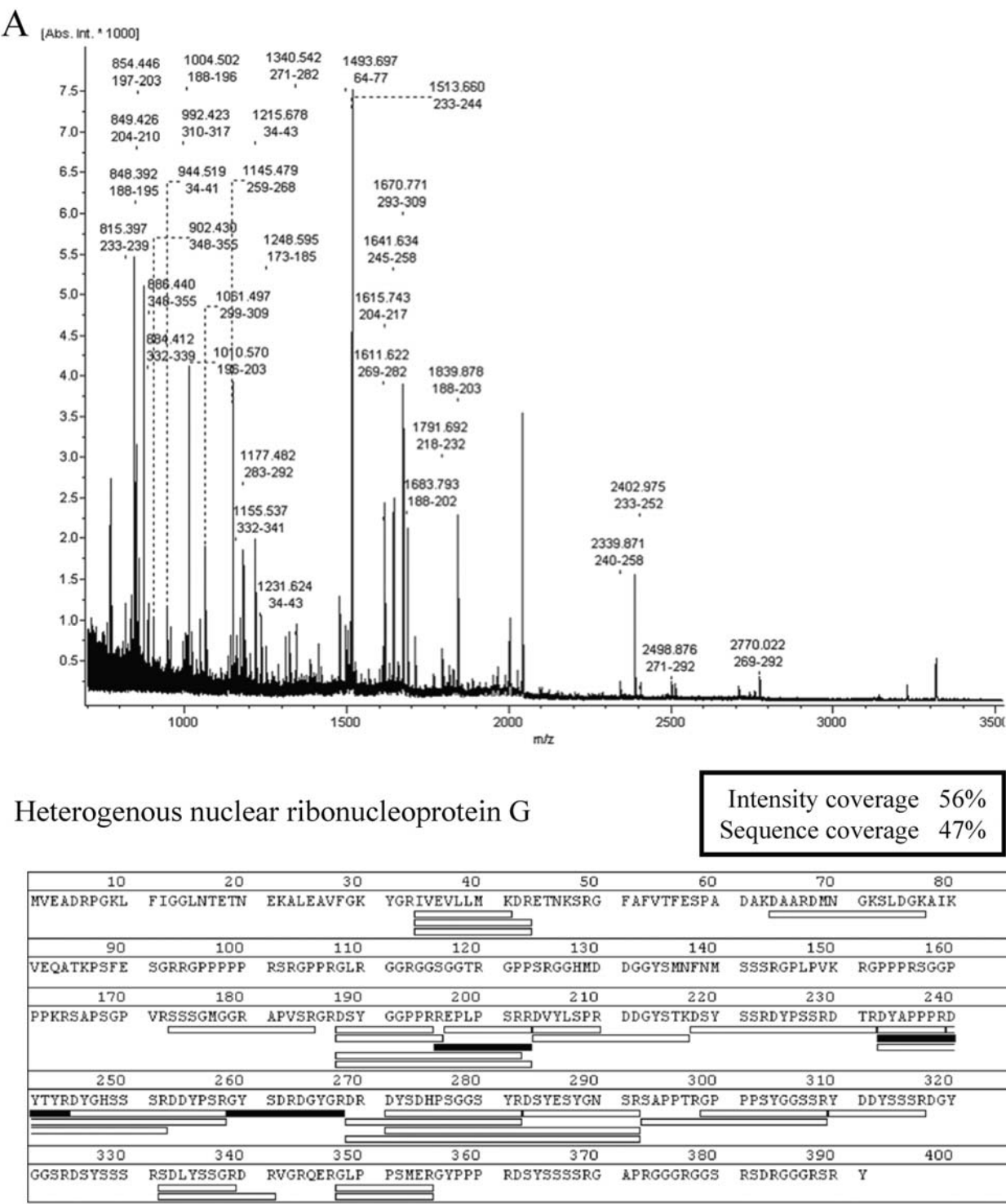


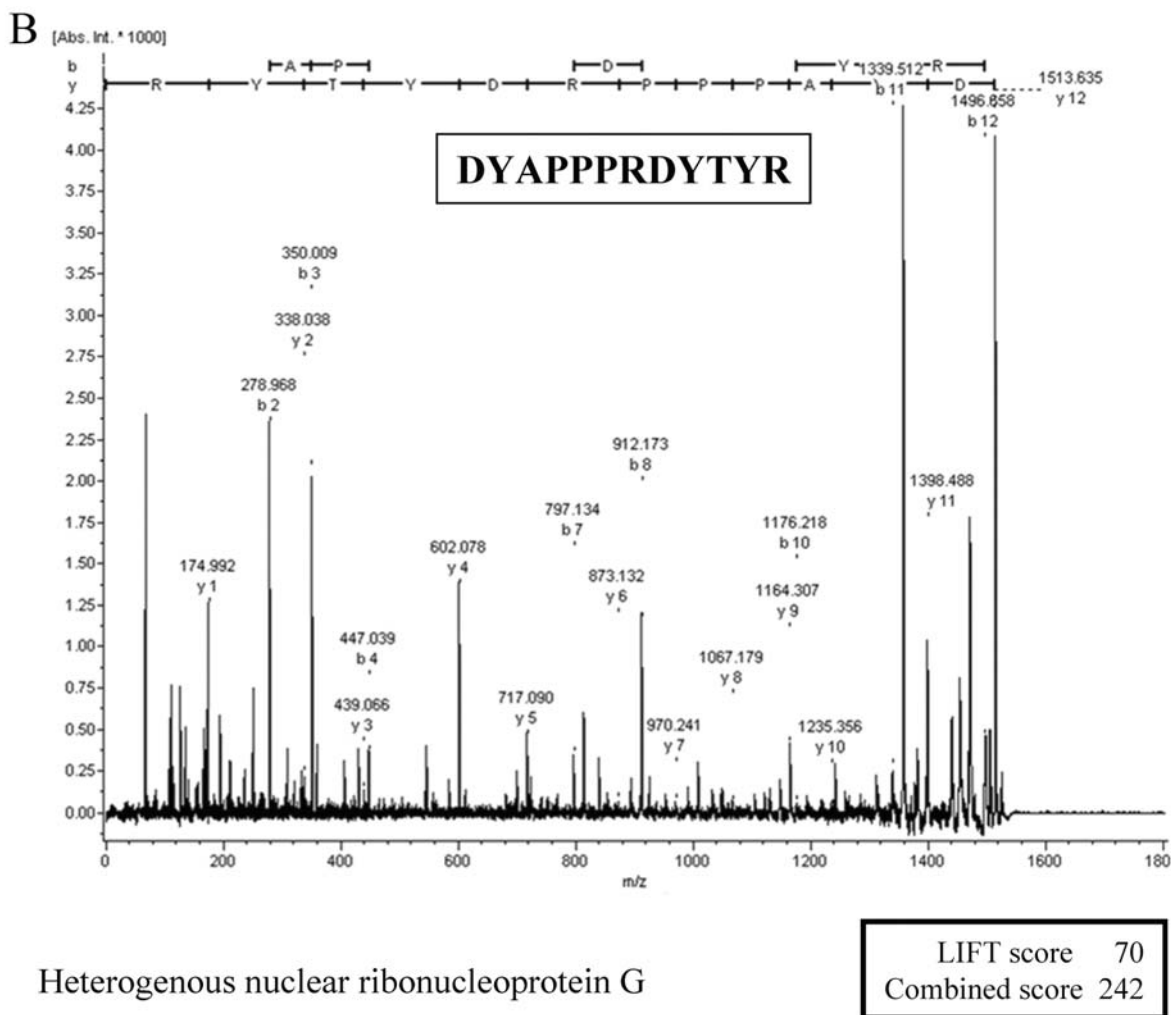
Figure 3. (A) An example of identification from the PMF patterns of a spot excised from a 2-D gel.

it was recognized that it depended on non-specific protein-protein interactions among each fraction. To avoid these interactions, the sample preparation method was improved, and this yielded reproducible results.

The results confirmed the lower level of OPRT in DLD-1/5-FU in comparison to DLD-1 that had previously been demonstrated by an enzyme activity analysis (13). In addition, TS expression was at the same level and OPRT expression was down-regulated in DLD-1/5-FU in comparison to DLD-1

in Western blot analysis (data not shown). This indicates that the down-regulated expression of OPRT induces 5-FU resistance by avoiding RNA damage, in addition the anti-tumor action of 5-FU on DLD-1/5-FU may occur mainly via DNA-directed affects. In this study, two basic proteins, hnRNP G and TFAM, associated with DNA damage by 5-FU showed up-regulated expression in DLD-1/5-FU.

hnRNP G belongs to the family of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). The



Heterogenous nuclear ribonucleoprotein G

1 MVEADRPGLK FIGGLNTETN EKALEAVFGK YGRIVEVLLM KDRETNKSRG
 51 FAFVTFESPA DAKDAARDMN GKSLDGKAIK VEQATKPSFE SGRRGPPPPP
 101 RSRGPPRGLR GGRGGSGGTR GPPSRGGHMD DGGYSMNFM SSSRGPLPVK
 151 RGPPPSGGP PPKRSAPSGP VRSSSGMGGR APVSRGRDSY GGPPRREPLP
 201 SRRDVYLSR DDGYSTKDSY SSRDYPSSRD TRDYAPPPRD YTYRDYGHSS
 251 SRDDYPSRGY SDRDGYGRDR DYSDHPSGGS YRDSYESYGN SRSAPPTRGP
 301 PPSYGGSSRY DDYSSSRDGY GGSRDYSSSS RSDLYSSGRD RVGRQERGLP
 351 PSMERGYPPP RDSYSSSSRG APRGGGRGGS RSDRGGGRSR Y

Figure 3. (B) An example of the MS/MS pattern obtained by MALDI TOF/TOF. The result of hnRNP G is shown. The mass peak in the PMF pattern as indicated in (A), 1,513.660, was analyzed by an MS/MS analysis.

hnRNPs constitute a large family of nucleic acid-binding proteins with more than 30 different members. They were first described as chromatin-associated RNA binding proteins with a major role in RNA processing (18). Recent studies showed that the biological functions of hnRNPs are extremely diverse and include RNA turnover, telomere biogenesis, oncogenesis, and spermatogenesis (19). Moreover, the involvement of p53 in the repair of DNA double-strand breaks via a DNA non-homologous end-joining (NHEJ) process, indicate that hnRNP G is associated with a protein complex with the DNA ends in p53 containing human RKO

colorectal cancer cells, but is not associated with the DNA ends with a p53 knockdown based on a DNA end-protection assay. Meanwhile, it was demonstrated that the ectopic expression of hnRNP G significantly enhances the fidelity of DNA NHEJ and the protection of DNA ends in human cancer cells lacking hnRNP G protein or containing mutant hnRNP G by binding to the DNA ends and protecting them from degradation by nuclease. In addition, the depletion of endogenous hnRNP G notably diminished the fidelity of NHEJ (20). The slower exonuclease activity in the hnRNP G overexpressing cells was attributable to an increased delay

in the initiation of DNA degradation possibly caused by hnRNP G binding and protection of the DNA ends against exonuclease activity (20).

TFAM is a member of a high-mobility group (HMG) of proteins named on the basis of their electrophoretic mobility in polyacrylamide gels, and was first cloned as a transcription factor of the mitochondrial DNA. This group is composed of non-histone chromatin proteins and transcription factors that can bind DNA either non-specifically or in a sequence-dependant manner (21). TFAM is encoded in the nucleus and is synthesized on cytoplasmic ribosomes as a precursor, which is converted, upon mitochondrial importation, to a mature form. The mature form contains two HMG boxes, HMG-like domain 1 (HMG1) and HMG-like domain 2 (HMG2), joined by a basic 36 amino acid linker and followed by a basic 27 amino acid tail (22). Most of the TFAM proteins have been shown to stimulate transcription of mitochondrial DNA by binding to the D-loop region, and it is thought that TFAM not only contributes to the mitochondrial DNA stability by packaging of the mitochondrial DNA, but also maintains the mitochondrial DNA (23). Recently, it was shown that TFAM preferentially recognizes cisplatin-damaged DNA and oxidized DNA. In addition, increased apoptosis has been observed in cells lacking mitochondrial DNA gene expression, thus suggesting that TFAM is involved in apoptosis because mitochondria act as a pivotal decision center in many types of apoptotic response (24,25). Yoshida *et al* reported that TFAM expression was up-regulated in mitochondria interacted by p53 after human epidermoid cancer cells (KB cells) were treated with cisplatin. Up-regulated expression of TFAM was recognized in mitochondria when HCT116 colon cancer cell was treated with 5-FU (26). Nuclear HMG-box protein, HMG1, has been shown to be enhanced binding of cisplatin-damaged DNA in association with p53 (27), and cisplatin-damaged DNA binding of TFAM is activated by p53. Therefore, since in TFAM transfected HeLa cell, the susceptibility of three chemotherapeutic drugs, etoposide, camptothecin and cisplatin, are decreased in comparison to the parental cell line, up-regulated expression of TFAM may contribute to the prevention of chemotherapeutic drug-induced apoptosis (28).

The up-regulated expression of hnRNP G and TFAM in DLD-1/5-FU, and the fact that hnRNP G is involved in the repair of DNA, and TFAM is engaged in the mitochondrial DNA, indicates that these proteins may possibly be associated with the protection of not only DNA but also mitochondrial DNA from the damage affected by 5-FU and maintain the chemoresistance in this 5-FU resistant cell line. Although hnRNP G and TFAM may possibly be novel candidates for 5-FU resistance in these colon cancer cell lines, the regulation of the translational expression and their association with mechanisms of 5-FU resistance must be fully characterized.

Over 70 RPs must assemble with the rRNAs in the nucleolus to form the small and large ribosomal subunits (40S and 60S, respectively). A large number of RPs do not appear to be essential for ribosome function, and their probable task is to enhance the function of the rRNA (29). Currently, scarce data are available on human RP L3, but in plants the silencing of this gene causes alterations in growth, development and ribosome biogenesis (30). Bergqvist *et al* have

shown that the telomerase activity levels were positively correlated with RP L3 gene expression in 10 esophageal carcinoma cell lines by DNA microarray analysis (31). Telomerase is a reverse transcriptase that synthesizes telomeric repeats on chromosomal ends to compensate for telomere shortening during DNA replication (32). Telomerase is repressed in the majority of somatic cells but activated in approximately 85% of human malignancies (33). Telomerase activity levels are up-regulated during cancer progression in several malignancies, and, in addition, inhibition of telomerase activity can increase chemosensitivity (34).

Histones, including core histones and linker histones, are highly basic proteins, and maintain chromatin structures in an electrically neutral state, since DNA is acidic. Two molecules of each of the 4 core histone types (H2A, H2B, H3 and H4) interact to form a histone octamer. DNA is coiled around this octamer, which forms a nucleosome, considered to be the minimum and basic structure of chromatin (35). In contrast, histone H1, a linker histone, normally binds to the linker DNA between nucleosomes, sealing off two turns of DNA around a histone octamer, and is involved in gene activation, since these areas easily accessible to transcription factors (35). Recently, it has become clear that these core histone N-terminal tails can be post-translationally modified by processes such as acetylation, methylation, phosphorylation, ubiquitination and sumoylation, that target specific amino acid residues (36,37), and gene regulation is controlled by epigenetic modifications that define the chromatin state, mainly via histone modification. It appears that each histone octamer is uniquely modified according to different combinations of post-translational modifications. ATP-dependent chromatin remodeling complexes are primarily responsible for the rearrangement of nucleosomal arrays, according to signals defined by histone modifications (38,39). The sliding of histone octamers, around which DNA is coiled, is facilitated by ATP-dependent chromatin remodeling complexes, thereby exposing new naked DNA regions. The best characterized of the histone modifications is histone acetylation and deacetylation. Co-regulator complexes with histone acetyltransferase activate the transcription of target genes through the acetylation of histones, while histone deacetyltransferase complexes deacetylate histones and serve as co-repressors (38,39). Histone methylation is also induced by co-regulator complexes. Furthermore, histone phosphorylation is conspicuously observed in cells that undergo DNA damage or apoptosis introduced by UV-irradiation and chemotherapeutic drugs (40). Three protein spots were identified as histone H2B and H4 among the up-regulated proteins expressed in DLD-1/5-FU by MALDI-TOF/TOF MS (Fig. 2). However, these up-regulated spots on the 2-D gel were located on the acidic side of the histone clusters. This may have been the result of post-translational modification of H2B and H4. In other words, through the parts of these histones underwent post-translational modification such as the acetylation of lysine/threonine residues or the phosphorylation of serine residues. Changes in the protein net charge by post-translational modifications are detected as shifts of protein spots by RFHR 2-D PAGE (11), and this is one of the greatest advantages of separation and quantification of post-translational forms in RFHR 2-D PAGE. Nevertheless, it is

thought that some genes that control the 5-FU resistant mechanism are constantly up-regulated in DLD-1/5-FU, and further research is therefore necessary to identify how the gene expression is affected as a result of these post-translational modifications of histone H2B and H4.

In conclusion, this study demonstrated the high reproducibility of basic protein maps of colon cancer cell lines by RFHR 2-D PAGE. The 5-FU resistant line and its parent cell lines clearly differed regarding in their protein expression profile. Five basic proteins which were modulated between DLD-1 and DLD-1/5-FU were identified. Among them, several proteins were associated with 5-FU pharmacologic DNA damage in tumor cells, and may potentially be associated with the biological basis of 5-FU resistance. These proteomic approaches may not only be a powerful tool that can lead to the identification of novel potential markers involved in 5-FU resistance of colon cancer cells, but also be useful in completing the understanding of these complex mechanisms. Moreover, these observations will facilitate the development of novel therapeutic strategies against colon cancer.

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