Jpn. J. Cancer Res. **92**, 696–703, June 2001

Screening of Differentially Expressed Genes in 5-Fluorouracil-resistant Human Gastrointestinal Tumor Cells

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To identify genes differentially expressed in association with resistance to 5-fluorouracil (5FU), an mRNA differential display (DD) analysis was used to compare transcripts from the NUGC-3 human gastric tumor cell line and the NUGC-3/5FU/L line, which had acquired 208-fold resistance as a consequence of repeated exposure to escalating concentrations of 5FU. The 110 cDNA fragments differentially expressed in the DD analysis of either the NUGC-3 or NUGC-3/5FU/L cells were sequenced and subjected to a homology search, and 29 overexpressed and 22 underexpressed genes were identified in NUGC-3/5FU/L as a result. To confirm whether the changes in the gene expression levels in the NUGC-3/5FU/L cells were shared by other 5FU-resistant cells, 35 genes were analyzed by northern hybridization in 3 pairs of parent/5FU-resistant human gastrointestinal tumor cell lines. The analysis revealed 20 overexpressed and 10 underexpressed genes in at least one of the three 5FU-resistant cells as compared with those in the parent cells. Among them, P-glycoprotein, equilibrative nucleoside transporter 1, and methylenetetrahydrofolate dehydrogenase were highly expressed in two of the three 5FU-resistant cells and cytidine deaminase and integrin α3 were underexpressed. The acquisition of resistance to 5FU by tumor cells may result from multiple changes in cellular functions.

Key words: Resistance to 5-fluorouracil — Profiling of gene expression — Human gastrointestinal tumor cells — mRNA differential display — Northern hybridization

5-Fluorouracil (5FU) is widely used in the treatment of solid tumors, but the inherent or acquired resistance of certain tumors to 5FU therapy is a major clinical problem. Various mechanisms of resistance to 5FU have been proposed: overexpression of the target enzyme thymidylate synthase (TS), depletion of folate cofactors, an increase in the level of the competing substrate deoxyuridine monophosphate, altered drug transport/metabolism, and alterations in DNA repair/cell-cycle control pathways.[1–5]

Following repeated exposure to escalating concentrations of 5FU, a 5FU-resistant human gastric tumor cell line we established, NUGC-3/5FU/L, developed a 208-fold increase in resistance to 5FU compared with the parent line. Our previous studies[6] showed a significant decrease in the inhibitory effect of 5FU on intracellular TS activity and the incorporation of 5FU into the RNA of NUGC-3/5FU/L cells compared to parent cells. Assays for several enzymes involved in 5FU metabolism or anabolism revealed that the activities of uridine phosphorylase (UP), uridine kinase (UK), and orotate phosphoribosyltransferase (OPRT) were about 70% lower in NUGC-3/5FU/L cells than in the parent line. It is possible that other as yet unknown molecular mechanisms are also involved in 5FU resistance, since there appear to be considerable differences between the degree of resistance and the rates of inhibition of these 5FU-activating enzymes.

In this study, an mRNA differential display (DD) were performed to screen for genes expressed in association with 5FU resistance. The results revealed overexpression of 29 genes and underexpression of 22 genes in NUGC-3/5FU/L cells. To confirm whether the changes in gene expression in the NUGC-3/5FU/L cells were shared by other 5FU-resistant cells, northern blot analysis was performed with 3 pairs of parent/5FU-resistant human gastrointestinal tumor cell lines.

MATERIALS AND METHODS

Tumor cell lines NUGC-3, a human gastric cancer cell line,[7] was supplied by Health Science Research Resources Bank (Tokyo); DLD-1, a colon carcinoma cell line,[8] was purchased from Dainippon Pharmaceutical Co. (Osaka);

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Abbreviations: LIG3, DNA ligase III; PARP, poly(ADP-ribose) polymerase; SGLT1, glucose transporter; NTHY, NAD(P) transhydrogenase; DPD, dihydropyrimidine dehydrogenase; TK, thymidine kinase; TP, thymidine phosphorylase; RRM1, ribonucleoside-diphosphate reductase M1 subunit; RRM2, ribonucleoside-diphosphate reductase M2 subunit; TOP1, topoisomerase 1; TOP2A, topoisomerase 2α; TOP2B, topoisomerase 2β; ERCC1, DNA excision repair protein ERCC1; UNG, uracil-DNA glycosylase; MRP1, multidrug resistance-associated protein 1; ENT2, equilibrative nucleoside transporter 2; VEGFB, vascular endothelial growth factor β.

and HT-29, a colon carcinoma cell line, was from the American Type Culture Collection (Rockville, MD). 5FU-resistant sublines of each line (NUGC-3/5FU/L, DLD-1/5FU, and HT-29/5FU) were established by repeated continuous (3- to 5-day) exposure of the cultures to escalating concentrations of 5FU. Cells were maintained in RPMI 1640 (ICN Biomedicals Inc., Aurora, OH) containing 10% fetal bovine serum.

Cytotoxicity assay Cells were exposed to various concentrations of 5FU for 4 days, and the growth inhibition rate was assessed by SRB assay[9] or MTT assay.[10] The drug concentrations that caused 50% growth inhibition as compared with the control (without drug), i.e., the IC_{50}, was calculated from the regression lines. The degree of resistance to 5FU was estimated as the ratio of the IC_{50} for each resistant line to that for the parent line.

Cell samples Tumor cells grown in 175-cm^2 flasks without 5FU were harvested by trypsinization before reaching confluence (about 70% confluence). The cells were then washed twice in phosphate-buffered saline, immersed in liquid nitrogen, and stored at −80°C until analyzed.

mRNA differential display To compare the transcripts from NUGC-3 and NUGC-3/5FU/L cells, a rapid and simple PCR-based DD method using agarose gel electrophoresis[11] was performed, with slight modification. Briefly, mRNA was isolated from the cell samples with commercial kits: an RNeasy midi kit (QIAGEN Inc., Chatsworth, CA) for total RNA isolation followed by a PolyATtract mRNA Isolation System (Promega Co., Madison, WI) for poly A^+ selection.

Reverse transcription with 1 μg of mRNA was carried out in a total volume of 100 μl containing 250 pmol of fully degenerate 6-mer oligonucleotide, 80 U of rRNasin ribonuclease inhibitor (Promega), 10 μg of BSA, and 500 U of reverse transcriptase (SuperScript II RNase H^-; GIBCO BRL, Gaithersburg, MD) in 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, and 0.5 m*M* dNTPs. Initially, the mRNA solution mixed with degenerate 6-mer oligonucleotide was heated at 70°C for 10 min and immediately chilled on ice, then the other reagents were added. First-strand cDNAs were obtained after 15 min at 20°C, 60 min at 42°C, 6 min at 99°C, and 5 min at 95°C. The sample was then digested with 120 U of ribonuclease H (TaKaRa, Shiga) at 37°C for 20 min and heated at 95°C for 5 min.

The PCR primers, summarized in Table I, were designed as fixed 10-mer sequences (CGCAAGCTTG or GCCGAATTCG) linked to the 5′-end of the primers used in the original method.[11] First-round PCR was carried out in a final volume of 50 μl containing 1 μl of cDNA solution (10 ng as mRNA), 50 pmol of each of one or two primer(s), and 2.5 U of *Taq* polymerase (Ex Taq, TaKaRa) in 5 μl of 10× Ex Taq buffer (TaKaRa), and 0.2 m*M* dNTPs. The PCR profile consisted of a 3-min initial denaturation at 94°C, then 2 cycles at 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min, followed by 43 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and finally, a 10-min extension at 72°C. The PCR products were separated by electrophoresis in 1.5–4% gel (depending on their size) prepared with low-melting-point agarose and stained with ethidium bromide. DNA bands were visualized on a 302-nm wavelength UV transilluminator and cut from the gel. The DNA was isolated from the gel pieces with Wizard PCR preps (Promega) and eluted in 50 μl of distilled water. Second-round PCR was carried out in the same reaction mixture as first-round PCR, with 1 μl of the first-round PCR product solution being used as the template. The PCR profile consisted of a 3-min initial denaturation at 94°C, 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and finally, a 10-min extension at 72°C. The second-round PCR products were separated by agarose gel electrophoresis and isolated in the same manner as described above.

The isolated DNA fragments were directly sequenced on a DNA sequencer (ABI PRISM 377; PE Biosystems, Foster City, CA) by using a Big Dye Termination Cycle Sequencing Ready Reaction (PE Biosystems), and the sequences were subjected to a homology search (Basic BLAST, http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

Northern blot analysis Total RNA (5 μg) extracted from each parent and 5FU-resistant cell line was separated on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary action under 20× SSC. Gene-specific cDNA fragments containing the

Table I. Primers Used for the PCR Amplification and Sequencing of Second-round PCR Products

Name	Sequence	Length (nt)
HD-52	CGCAAGCTTGCAAGCGAGGT	20
ER-54	GCCGAATTCGAACGCGCAAC	20
ER-55	GCCGAATTCGGTGGAAGCGT	20
ER-57	GCCGAATTCGGGAAGCAGCT	20
ER-58	GCCGAATTCGCAGTGAGCGT	20
ER-61	GCCGAATTCGG(C)TCACGGACG[a]	20
ER-62	GCCGAATTCGG(C)CCATGCACG[a]	20
ER-70	GCCGAATTCGGAGCTATGGC	20
ER-73	GCCGAATTCGAGCCTGTGTC	20
ER-76	GCCGAATTCGCTGGTCACAC	20
ER-78	GCCGAATTCGCACAGTGAGC	20
ER-71	GCCGAATTCGGAGCTATGGCATG	23

Primers were designed as fixed 10-mer sequences linked to the 5′-end of the arbitrary but fixed primers (underlined sequences) used in the original method.[11]

a) Degenerate primers with both a G and a C in the G(C) position.

Table II. Sensitivity of Parent and 5FU-resistant Human Tumor Cells to 5FU

Cell line	IC_{50} of 5FU (μM)		Degree of resistance to 5FU
	Parent cells	5FU-resistant cells	
NUGC-3 vs. NUGC-3/5FU/L	2.5	520	208
DLD-1 vs. DLD-1/5FU	8.1	353	44
HT-29 vs. HT-29/5FU	6.2	141	23

IC_{50} refers to the 5FU concentration causing 50% growth inhibition as compared to the control (no treatment). The degree of resistance to 5FU is expressed as the ratio of the IC_{50} of the resistant cells to that of the parent cells.

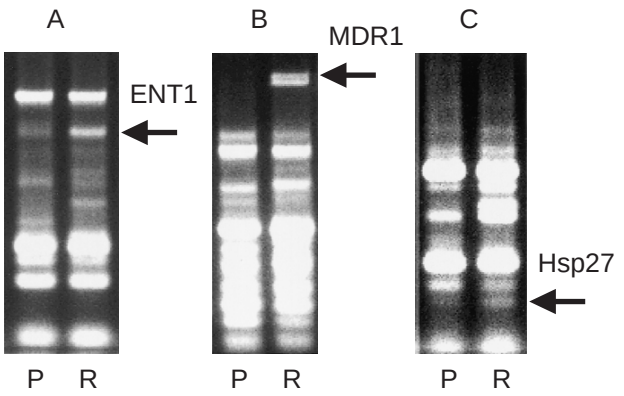

Fig. 1. Analysis by mRNA differential display. PCR products obtained from reverse-transcribed mRNA isolated from NUGC-3 cells (P) and NUGC-3/5FU/L cells (R) were fractionated by agarose gel electrophoresis. The primers used for PCR amplification were as follows: A, ER-61 and ER-73; B, ER-70 alone; and C, ER-61 and ER-71, respectively. Each arrow indicates the gene-specific fragment overexpressed in NUGC-3/5FU/L cells as follows: ENT1 of about 1100 bp, MDR1 of about 2100 bp, and Hsp27 of about 250 bp, respectively.

entire coding regions (approximately 300–900 bp) for use as hybridization probes were obtained by PCR amplification. cDNA probes labeled xwith [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech) were synthesized with a Rediprime II DNA labelling system (Amersham Pharmacia Biotech) and subjected to column chromatography to remove unincorporated nucleotides. Membranes were prehybridized at 65°C in hybridization buffer (RapidHyb Buffer, Amersham Pharmacia Biotech) for at least 30 min prior to probe addition. Hybridization was performed with the probe at 65°C for about 2 h in a rolling bottle.

RESULTS

Degree of resistance to 5FU The degree of resistance to 5FU was estimated as the ratio of the IC_{50} of each resistant line to that of its parent line when cells were exposed to various concentrations of 5FU for 4 days. As shown in Table II, although their degree of resistance to 5FU varied, each of the resistant lines had acquired relatively high resistance to 5FU. The degree of resistance to 5FU of each resistant line seems to be quite stable because the IC_{50} of each was consistent even after sequential passages without 5FU.

Analysis by mRNA differential display The purpose of this study was to identify known, rather than unknown, genes that were differentially expressed in association with resistance to 5FU. Analysis of unknown genes by the usual mRNA DD method requires sub-cloning which is a relatively laborious process. We tried to directly sequence the second-round PCR products without sub-cloning and to identify the gene sequences by a database homology search. Since the primers used in the original method[11] were too short, i.e., their melting temperature (T_m) was too low (about 32°C calculated from 2AT+4GC), to be diverted for sequencing, we designed 12 primers that had fixed 10-mer sequences attached to the 5′-end of the original primers (Table I). Our primers were not only arbitrary enough to amplify various cDNAs but also long enough (T_m of about 65°C) to be subjected to sequencing.

A total of 110 cDNA fragments were judged to be differentially expressed by visual inspection in either NUGC-3 or NUGC-3/5FU/L cells in the displays of first-round PCR products, and they were isolated after the second-round PCR and directly subjected to sequencing. Fig. 1 shows three representative photographs of agarose gel electrophoresis of the first-round PCR products, in which we were able to identify several differentially expressed cDNA fragments. A homology search (Basic Blast) of the sequences revealed that three fragments overexpressed in NUGC-3/5FU/L cells (indicated by arrows in Fig. 1) were identical to equilibrative nucleoside transporter 1 (ENT1), P-glycoprotein (MDR1), and heat-shock protein 27 (Hsp27). As a result, we were able to determine more than 84% of the entire sequence of 83 fragments out of the 110 (75%), but we failed to sequence the other 27 fragments (25%). The 83 sequences were applied to Basic Blast with the following results: 6 (5%), no hit to published sequences; 17 (15%), genomic DNA; and 60 (55%, including 7 duplicates), human mRNA. Two mRNAs out of the 7 duplicates were judged to be false-positive because the pattern of expression of the genes was inconsistent in either NUGC-3 or NUGC-3/5FU/L cells, and

Table III. Overexpressed Genes in NUGC-3/5FU/L Cells by Analysis of mRNA Differential Display. Homology of cDNA Fragments to Known Human mRNA Sequences

cDNA fragment #	Homology to	Database accession	Homology (%)
8, 89	autoantigen calreticulin	M84739	84, 91
10	MDR1	M14758	89
25	CDC42-binding protein kinase β	AF128625	99
26	SGLT1	K03195	92
28	general transcription factor IIIC	U02619	98
33	α-tubulin isoform 1	AF081484	86
36	breast tumor autoantigen	U24576.1	81
39	agrin precursor	AF016903	88
41	lipoprotein receptor related protein 5	AF077820	94
42	cytochrome P450, subfamily I	U56438	88
45	dihydropyrimidinase related protein-2	D78013	93
48	thyroid hormone receptor-associated protein	AF117756	85
50	general β-spectrin	S65762	97
56, 63	selenium binding protein 1	U29091	95, 90
60	membrane glycoprotein 4F2 antigen heavy chain	J03569	83
66	golgi-specific brefeldin A-resistance factor 1	AF068755	80
69	Ski-W for helicase	Z48796.1	84
72	dystroglycan 1	L19711	95
74	elongation factor 1, α-2 (EF-1, α2)	L10340	95
76	methyl-CpG binding endonuclease (MED1)	AF114784.1	96
80	secretory protein 24	AJ131245.1	83
85	NTHY	U40490	89
93	receptor-type protein tyrosine phosphatase γ	L09247	98
94, 99	semaphorin IV	AC000063	86, 91
95	Hsp 27	U90906	87
97	ENT1	U81375	88
105	putative transcription factor	AF104923.1	98
107	NIX, nuclear gene encoding mitochondrial protein	AF067396.1	87
108	KIAA0068	D38549	80

Table IV. Underexpressed Genes in NUGC-3/5FU/L Cells by Analysis of mRNA Differential Display. Homology of cDNA Fragments to Known Human mRNA Sequences

cDNA fragment #	Homology to	Database accession	Homology (%)
1, 96	β-filamin	AF042166	80, 85
3	MT-MMP (MMP-14)	U41078	95
9	zinc finger protein 76	M91592	87
15	uPA	M15476	84
19	KIAA0710 protein	AB014610	87
21	DNA replication licensing factor	D83986	90
23	α-catenin	D13866	99
27	PPARG	U63415	81
29	HSPC025	AF083243.1	94
31	caveolin	Z18951	99
37	cleavage stimulation factor, 50 kDa subunit	L02547	99
40	LIG3	U40671	94
43	sec61 homolog	AF084458.1	89
46	β adaptin	L13939	95
49	Alu-J subfamily consensus sequence	U14567	90
54	splicing factor Prp8	AF092565	95
70	KIAA0934 protein	AB023151	98
81, 82	ITGA3	M59911	81, 94
90	breast carcinoma fatty acid synthase	U29344	97
100	PARP	J03473	81
102	palmitoyl-protein thioesterase	L42809	93
103	AXL	M76125	92

we ultimately identified 29 overexpressed (Table III) and 22 underexpressed (Table IV) genes in NUGC-3/5FU/L cells.

Analysis by northern hybridization Northern hybridization was performed using total RNAs isolated from 3 pairs of parent/5FU-resistant human gastrointestinal tumor cell lines to confirm whether the changes in the gene expression levels in the NUGC-3/5FU/L cells were also shared by other 5FU-resistant cells. The relative expression of 35 genes was analyzed, including 2 housekeeping genes, i.e., glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, as internal standards, 13 genes that were differentially expressed in DD analysis, and 20 genes in which we are interested that were not detectable in DD analysis. An at least 1.6-fold difference in expression between parent and resistant cells was considered essential, since the average relative TS-expression in the three 5FU-resistant cells as compared with the parent cells was 1.6. This analysis revealed 20 overexpressed and 10 underexpressed genes in at least one of the three 5FU-resistant cells as compared with the parent cells (Table V). Three of the genes, MDR1, ENT1, and methylenetetrahydrofolate dehydrogenase (MTHFD) were highly expressed in two of the three 5FU-resistant cells and expression of the genes encoding integrin α3 (ITGA3) and cytidine deaminase (CDA) was low (Table V and Fig. 2).

The expression patterns of the following 5 genes in 3 pairs of parent/5FU-resistant cell lines were inconsistent: urokinase-type plasminogen activator (uPA); AXL tyrosine

Table V. Relative Gene Expression in Various 5-FU-resistant Cell Lines Compared with the Parent Cell Lines by Northern Hybridization Analysis

	Relative expression level			Over-[a] expressed	Under-[a] expressed	DD analysis
	NUGC-3/5FU/L	DLD-1/FU	HT-29/FU			
Genes differentially expressed in DD analysis						
MDR1	10.4	2.4	1.3	2	0	O[b]
ENT1	3.9	1.7	1.1	2	0	O
Hsp27	1.5	1.5	4.4	1	0	O
NTHY	6.2	1.2	1.2	1	0	O
SGLT1	1.8	0.8	1.0	1	0	O
ITGA3	0.4	0.5	1.4	0	2	U[c]
MT-MMP	0.0	0.7	1.1	0	1	U
α-catenin	0.1	0.7	1.3	0	1	U
uPA	0.1	1.2	22.5	1	1	U
AXL	0.0	0.9	3.1	1	1	U
PPARG	0.3	1.2	1.8	1	1	U
PARP	1.5	1.2	1.3	0	0	U
LIG3	1.5	0.8	1.4	0	0	U
Other genes (not detected by DD analysis)						
TS	2.3	1.0	1.4	1	0	
DPD	P[d]	not detected	not detected	0	1	
OPRT	1.7	0.7	0.5	1	1	
TK	1.0	0.9	1.0	0	0	
UK	1.1	1.1	0.8	0	0	
TP	0.7	1.3	2.1	1	0	
UP	0.7	1.1	1.6	0	0	
CDA	0.0	0.8	0.0	0	2	
MTHFD	1.7	1.8	1.3	2	0	
RRM1	1.7	1.2	1.1	1	0	
RRM2	2.2	1.3	1.0	1	0	
TOP1	2.5	1.1	1.2	1	0	
TOP2A	0.9	1.2	1.4	0	0	
TOP2B	1.2	1.4	1.1	0	0	
ERCC1	0.9	1.1	1.6	1	0	
UNG	1.1	1.0	0.9	0	0	
p53	0.3	1.4	1.7	1	1	
MRP1	1.3	0.7	3.2	1	0	
ENT2	1.1	1.6	1.3	1	0	
VEGFB	0.7	1.6	1.3	1	0	
GAPDH	1.4	1.1	1.1	0	0	
β-actin	0.9	0.9	1.1	0	0	

a) Numbers of cell lines in which the gene was overexpressed or underexpressed in 5FU-resistant cells with an at least 1.6-fold difference in expression by northern hybridization.
b) Gene overexpressed in 5FU-resistant cells in DD analysis.
c) Gene underexpressed in 5FU-resistant cells in DD analysis.
d) Gene expression was detected in only NUGC-3 cells.

kinase receptor (AXL); peroxisome proliferator activated receptor γ (PPARG); OPRT; and p53 (Table V). The expression pattern of 10 genes (*MDR1*, *ENT1*, *NTHY*, *SGLT1*, *ITGA3*, *MT-MMP*, *α-catenin*, *uPA*, *AXL*, and *PPARG*) was consistent in either DD analysis or northern hybridization for NUGC-3 and NUGC-3/5FU/L cells (Table V).

DISCUSSION

The purpose of this study was to screen widely for genes differentially expressed in association with resistance to 5FU. Our previous studies, in which we focused on the process of 5FU-metabolism, demonstrated that the activity of several enzymes involved in 5FU-activation in

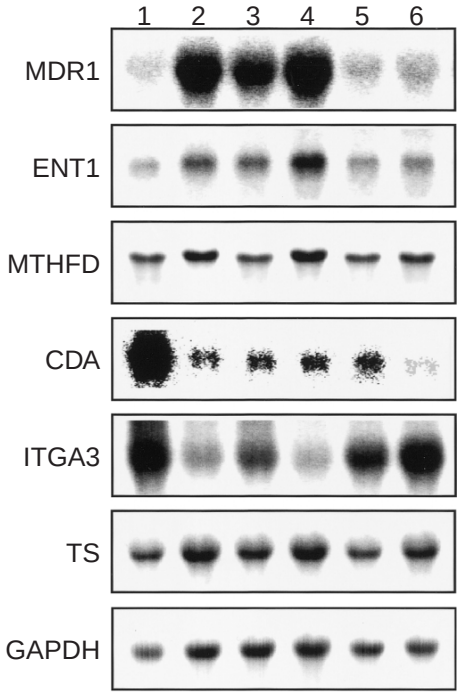

Fig. 2. Analysis by northern hybridization. Total RNAs isolated from 3 pairs of parent/5FU-resistant human gastrointestinal tumor cell lines were used to determine gene-expression patterns. Total RNA extracted from NUGC-3 cells (lane 1), NUGC-3/5FU/L cells (lane 2), DLD-1 cells (lane 3), DLD-1/5FU cells (lane 4), HT-29 cells (lane 5), and HT-29/5FU cells (lane 6) was separated on a 1.2% denaturing agarose gel and transferred onto a nylon membrane. The ^{32}P-labeled probes synthesized from each gene-specific cDNA fragment were hybridized to the RNA-blotted membrane. GAPDH was used as an internal standard.

NUGC-3/5FU/L was lower than in the parent line.[6] Many studies have investigated the mechanism of resistance to 5FU, but almost all of them have been limited to pyrimidine metabolism.[1–5] However, other as yet unknown molecular mechanisms may also be involved in the 5FU-resistance of NUGC-3/5FU/L cells, since there appear to be considerable differences between the degree of resistance (208-fold, see in Table I) and the rates of inhibition of these 5FU-activating enzymes.

In this study, DD analysis was performed to screen for genes differentially expressed in NUGC-3 and NUGC-3/5FU/L cells. The results revealed overexpression of 29 genes in NUGC-3/5FU/L cells and underexpression of 22 genes. To confirm whether the changes in gene expression in the NUGC-3/5FU/L cells were shared by other 5FU-resistant cells, northern blot analysis of 35 genes was performed in 3 pairs of parent/5FU-resistant human gastrointestinal tumor cell lines. Contrary to expectation, the gene expression patterns of these 5FU-resistant cells differed considerably. Only 5 genes, *MDR1*, *ENT1*, *MTHFD*, *CDA*, and *ITGA3*, were consistently expressed in two of the three 5FU-resistant cells, and 5 genes, *uPA*, *AXL*, *PPARG*, *OPRT*, and *p53* were inconsistent (Table V). We do not have any data to explain this variation, and it may be due to inherent characteristics of each parent cell line. In general, an established cancer cell line consists of a very heterogeneous cell population. Therefore it would be rather common that a drug-resistant subline has many different phenotypes and/or genotypes from the parent cell line, of which only a few may be involved in drug-resistance. The present profiling of gene expression, comparing a 5FU-resistant line with the parent line by DD analysis, is likely to screen not a few genes irrelevant to 5FU resistance. In any event, the results indicated that the expression profiles of cells with acquired 5FU resistance, rather than being simple, were complicated and varied.

Genes overexpressed in 5FU-resistant cells, such as MTHFD, ENT1 and MDR1 seem to be associated with tumor cell acquisition of resistance to 5FU. MTHFD (EC 1.5.1.5) is a trifunctional enzyme in human and regulates folates pool in cells.[12] TS loses its enzymatic activity by forming a covalent complex with 5-fluoro-2′-deoxyuridylate, an active metabolite of 5FU, and 5,10-methylenetetrahydrofolate, a reduced folate. The depletion of folate cofactors is thought to be one of the mechanisms of resistance to 5FU.[1] Since 5,10-methylenetetrahydrofolate is also catalyzed bidirectionally by MTHFD, overexpressed MTHFD may deplete reduced folate in cells and make them acquire resistance to 5FU. ENT1 is an equilibrative membrane transporter that is the route of cellular uptake for many natural nucleosides and cytotoxic nucleosides used in cancer chemotherapy.[13] The cytotoxicity and clinical efficacy of gemcitabine (2′,2′-difluorodeoxycytidine) appear to be influenced by cellular expression of ENT1,[14, 15] and growth-inhibitory concentrations of 5FU have been reported to increase the expression of ENT1 in cells.[16] It is possible that the elevation of ENT1 expression in cells may cause more cellular uptake of thymidine to rescue cells from thymineless death by TS inhibition. The *MDR1* gene encodes a membrane transporter P-glycoprotein that is an energy-dependent efflux pump of various molecules, and overexpression of P-glycoprotein confers resistance to multiple antitumor drugs such as vinblastine, adriamycin, etoposide, and taxol.[17, 18] Though it has been reported that a series of adriamycin-resistant human cancer cell lines develop cross-resistance to 5FU,[19] the evidence has not generally suggested yet that 5FU is involved in the multidrug-resistance system through P-glycoprotein. In addition, Hsp27, which was found to be overexpressed in HT-29/5FU cells by northern hybridization (Table III), also appeared to be associated with resistance to anthracyclins,[20, 21] but no report has described sensitivity to 5FU as being modulated by Hsp27.

Although the significance of underexpressed genes, such as those encoding CDA and ITGA3, in 5FU-resistant cells is unclear, these molecules may nevertheless play a considerable role. It is supposed that the cytidine pool is maintained at a relatively high level when expression of CDA is reduced. A high level of cytidine in cells may suppress the cytotoxicity of 5FU. When cytidine treatment replenished the deoxycytidine triphosphate pool, there was partial reversal of cytotoxicity induced by combined exposure to 5FU/interferon α/N-(phosphonacetyl)-L-aspartate in two human colon cancer cell lines.[22] As regards ITGA3, it was interesting that a preliminary cDNA array analysis using Atlas Human Cancer 1.2 Array (Clontech Laboratories, Inc., Palo Alto, CA) revealed underexpression of integrin β4 in NUGC-3/5FU/L cells (data not shown), since integrin β4, as well as ITGA3, recognizes the adhesion molecule laminin-5 as a ligand. Thus, quite a few molecules are probably expressed differentially in association with tumor cell acquisition of resistance to 5FU, even if the molecules are not directly involved in the 5FU resistance, and it would be worth investigating correlations between levels of expression of these genes and sensitivity to 5FU in clinical samples.

(Received February 21, 2001/Revised April 3, 2001/Accepted April 6, 2001)

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