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-fluouracil — 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 deoxyxuridine 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 studies6 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 line7 was supplied by Health Science Research Resources Bank (Tokyo); DLD-1, a colon carcinoma cell line8 was purchased from Dainippon Pharmaceutical Co. (Osaka);
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 or MTT assay. 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-cm² 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 was performed, with slight modification. Briefly, mRNA was isolated from the cell samples with commercial kits: an RNaseasy 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 rRNasrin ribonuclease inhibitor (Promega), 10 μg of BSA, and 500 U of reverse transcriptase (SuperScript II RNase H⁺; GIBCO BRL, Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 0.5 mM dNTPs. Initially, the mRNA solution mixed with degenerate 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 (CGCAAGCTTGGAGCTATGGGCAAGCGAGGT) linked to the 5'-end of the primers used in the original method. 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 primers, and 2.5 U of Taq polymerase (Ex Taq, TaKaRa) in 5 μl of 10× Ex Taq buffer (TaKaRa), and 0.2 mM 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-% 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 prep (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 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

| Name     | Sequence         | Length (nt) |
|----------|------------------|-------------|
| HD-52    | CGCAAGCTTGGAGCTATGGGCAAGCGAGGT | 20          |
| ER-54    | GCCGAATTTCGAAACCCGCAAC | 20          |
| ER-55    | GCCGAATTTCGATGGAAAGCGT | 20          |
| ER-57    | GCCGAATTTCGGAAGCAGCT | 20          |
| ER-58    | GCCGAATTTCGAGTGGACGTT | 20          |
| ER-61    | GCCGAATTTCGGTGCACGGAGCGA | 20          |
| ER-62    | GCCGAATTTCGGCTCCATGCAACG | 20          |
| ER-70    | GCCGAATTTCGGCTCAGCAGCA | 20          |
| ER-73    | GCCGAATTTCGGCTCAGCT | 20          |
| ER-76    | GCCGAATTTCGGCTGTCACGC | 20          |
| ER-78    | GCCGAATTTCGGCTGTCACGC | 20          |
| ER-71    | GCCGAATTTCGGCTGTCACGC | 20          |

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

### RESULTS

#### Degree of resistance to 5FU

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 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. Each arrow indicates the gene-specific fragment upregulated 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.

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. The sequences revealed that three fragments upregulated in NUGC-3/5FU/L cells 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 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
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 kinase protein; cell adhesion molecule 1 (CD166); caveolin-1; and NIX, a nuclear gene encoding mitochondrial protein.

**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              | CDC/42-binding protein kinase β | AF128625 | 89 |
| 26              | SGLT1       | K03195 | 92 |
| 28              | general transcription factor III | 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 |
| 45              | dihydrolipoamide dehydrogenase-related protein-2 | AF042166 | 80, 85 |
| 48              | thyroid hormone receptor-associated protein | AF117756 | 85 |
| 50              | general β-spectrin | S65762 | 97 |
| 56, 63          | selenium binding protein 1, subfamily I | U29091 | 95, 90 |
| 60              | membrane glycoprotein 4F2 antigen heavy chain | J03569 | 83 |
| 66              | golgi-specific brefeldin A-resistance factor 1 | AF068755 | 80 |
| 68              | Ski-W for helicase | Z48796.1 | 84 |
| 71              | dystroglycan 1 | L19711 | 95 |
| 74              | elongation factor 1, α2 (EF-1, α2) | L10340 | 95 |
| 76              | methyl-CpG binding endonuclease (MED1) | AF114784.1 | 96 |
| 80              | secretory protein 24 | AJ31245.1 | 83 |
| 85              | NTHY        | U40490 | 89 |
| 93              | receptor-type protein tyrosine phosphatase γ | L09247 | 98 |
| 94, 99          | semaphorin IV | AC000063 | 86, 91 |
| 95              | Hsp 27      | U90096 | 87 |
| 97              | ENT1        | U81375 | 88 |
| 105             | putative transcription factor | AF04923.1 | 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 family 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 |
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...
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NUGC-3/5FU/L was lower than in the parent line. Many studies have investigated the mechanism of resistance to 5FU, but almost all of them have been limited to pyrimidine metabolism. 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. 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. 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. The cytotoxicity and clinical efficacy of gemcitabine (2′,2′-difluorodeoxycytidine) appear to be influenced by cellular expression of ENT1, and growth-inhibitory concentrations of 5FU have been reported to increase the expression of ENT1 in cells. 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. Though it has been reported that a series of adriamycin-resistant human cancer cell lines develop cross-resistance to 5FU, 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, but no report has described sensitivity to 5FU as being modulated by Hsp27.

![Fig. 2. Analysis by northern hybridization.](image-url)
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 deoxyxycytidine 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. 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.

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