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A Unique Gene Expression Signature of 5-fluorouracil

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Abstract

To understand the response of cancer cells to anticancer drugs at the gene expression level, we examined the gene expression changes in response to five anticancer drugs, 5-fluorouracil, cytarabine, cisplatin, paclitaxel, and cytochalasin D in NCI-H460 human lung cancer cells. Of the five drugs, 5-fluorouracil had the most distinctive gene expression signature. By clustering genes whose expression changed significantly, we identified three clusters with unique gene expression patterns. The first cluster reflected the up-regulation of gene expression by cisplatin, and included genes involved in cell death and DNA repair. The second cluster pointed to a general reduction of gene expression by most of the anticancer drugs tested. A number of genes in this cluster are involved in signal transduction that is important for communication between cells and reception of extracellular signals. The last cluster represented reduced gene expression in response to 5-fluorouracil, the genes involved being implicated in DNA metabolism, the cell cycle, and RNA processing. Since the gene expression signature of 5-fluorouracil was unique, we investigated it in more detail. Significance analysis of microarray data (SAM) identified 808 genes whose expression was significantly altered by 5-fluorouracil. Among the up-regulated genes, those affecting apoptosis were the most noteworthy. The down-regulated genes were mainly associated with transcription- and translation-related processes which are known targets of 5-fluorouracil. These results suggest that the gene expression signature of an anticancer drug is closely related to its physiological action and the response of caner cells.

Keywords: Anticancer drugs, Gene expression profile, Microarray, 5-fluorouracil, Transcription- and translation-related processes

Anticancer drugs inhibit the proliferation of cancer cells and induce apoptotic cell death by blocking essential cellular processes. The targets of anticancer drugs are macromolecules such as DNA, RNA, and enzymes. 5-fluorouracil is metabolized in cells and is then either incorporated into RNA or DNA, or inhibits thymidylate synthase[1]. Cytarabine is also incorporated into DNA[2], while cisplatin is a DNA cross-linker[3], and paclitaxel and cytochalasin D target the cytoskeleton. Paclitaxel inhibits the depolymerization of microtubules[4] and cytochalasin D inhibits actin polymerization[5]. Cells tend to respond to challenge with anticancer drugs by altering gene expression to maintain homeostasis and promote survival. Therefore, the gene expression profiles of cells treated with anticancer drug may reflect their physiological effects. Since the targets of drugs differ, their effects on gene expression should also differ. Although the biochemical pathways leading to damage to macromolecules by anticancer drugs are relatively well known, the effects of the drugs on genome-wide gene expression is largely unknown.

In the present study, we examined changes in gene expression in NCI-H460 human lung cancer cells in response to five anticancer drugs (5-fluorouracil, cytarabine, cisplatin, paclitaxel, and cytochalasin D). 5-fluorouracil, cisplatin, and paclitaxel are currently used for treatment of lung cancer. cytarabine is used for the treatment of leukemia and lymphoma. Therefore, the information of the effects of these drugs at gene expression may be helpful to understand the biology of tumors undergoing chemotherapy. Of five drugs examined, 5-fluorouracil had a unique gene expression signature, suggesting that its physiological action differs from those of the other drugs tested. We investigated its gene expression profile in more detail in replicate microarray experiments and by Significance Analysis of Microarray data (SAM). Many of the genes up-regulated were associated with apoptosis, whereas those down-regulated were mainly involved in RNA-related processes, which are known targets of 5-fluorouracil. These results suggest that the effect of an anticancer drug on gene expression is closely related to its physiological action and the response of cancer cells.

Discussion

Gene Expression Profiles in Cells Treated with Anticancer Drugs

In an effort to understand the response of cancer cells to anticancer drugs at the gene expression level, we examined the gene expression profile changes in response to five anticancer drugs, 5-fluorouracil, cytarabine, cisplatin, paclitaxel, and cytochalasin D in NCI-H460 human lung cancer cells. Gene expression profiles were obtained after 24 h of anticancer drug treatment to examine the later response that is related to the physiological change of the cells. Hierarchical clustering analysis for all genes showed that the gene expression patterns were relatively similar for the cells exposed to paclitaxel, cytarabine, and cytochalasin D. However, the expression pattern of the 5-fluorouracil treated cells was quite different (Fig. 1A). To eliminate noise from the genes with uniform expression patterns across drugs, we selected genes with significant variation at $p = 0.001$ and obtained 993 genes. Hierarchical clustering analysis showed that the clustering pattern of drugs was quite similar to that of the unfiltered genes (Fig. 1B). To examine the similarities between the drug effects, we also calculated the correlation between the gene expression profiles of the cells treated with each drug (Fig. 1C). The correlation coefficients between the gene expression profile of the 5-fluorouracil-treated cells and the profiles of cells treated with the other drugs were around 0.1. This was much lower than the correlations between the profiles obtained for the other drugs. Evidently the gene expression profile of the 5-fluorouracil treated cells is quite different from the others.

Induction of Genes Related to Apoptosis and DNA Repair by Cisplatin

From the clustering pattern in Fig. 1B, we identified five major clusters of expression patterns. Three of the clusters can be related to the physiological effects of the drugs. Genes belonging to cluster 1 (C1) are highly expressed in cisplatin-treated cells but not in others. GO information is available for 84 of the 112 C1 genes. Of these, ten are involved in cell death, six in DNA repair, and six in negative regulation of cell proliferation. Up-regulation of genes related to DNA repair is expected since cisplatin cross-links cellular DNA that needs to be repaired for cell survival. Cell death-related genes in C1 include CDKN1A (encoding $p21^{cip1}$), TNFSF7 (tumor necrosis factor superfamily, member 7), TNFRSF12A and TNFRSF6 (tumor necrosis factor receptor superfamily members 12A and 6, respectively) that are known targets of transcriptional activation by p53. The induction of these genes by cisplatin in NCI-H460 cells, which contain wild type $p53$[12], is consistent with the involvement of apoptosis mediated by p53 and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) in the anticancer activity of cisplatin[13-15].

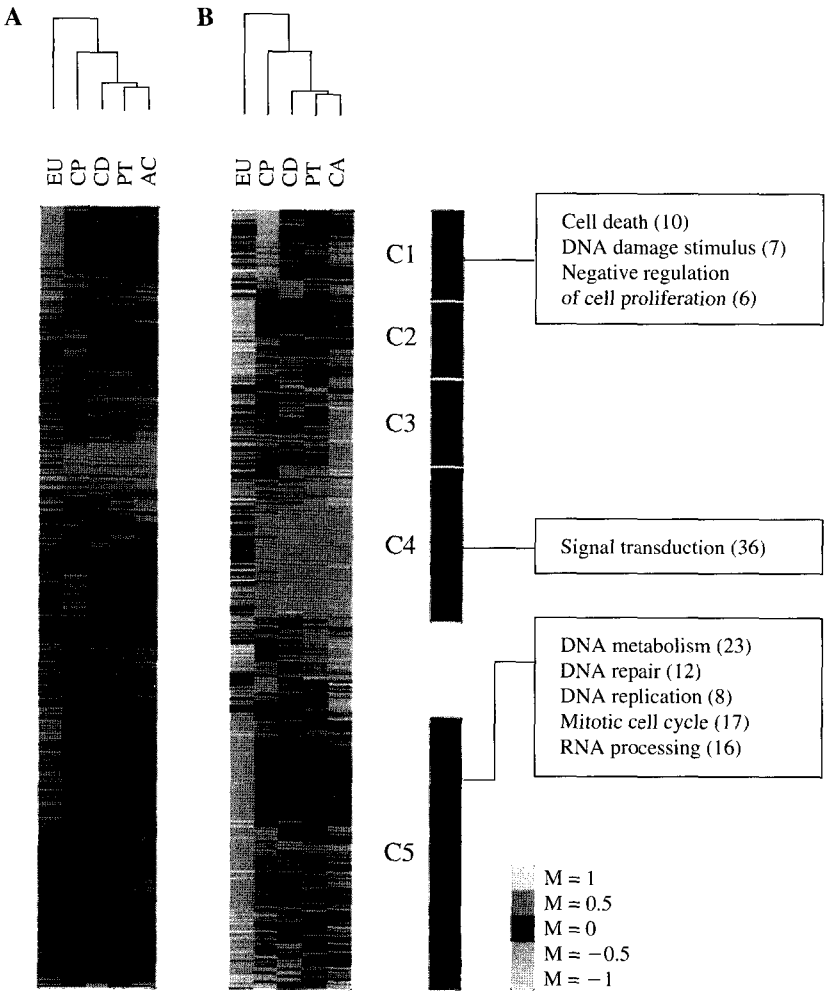
Reduced Expression of Genes Related to Signal Transduction

Genes in cluster 4 (C4) were down-regulated in cells treated with cisplatin, cytochalasin D, paclitaxel, and cytarabine but not with 5-fluorouracil. Genes involved in signal transduction are prevalent in this cluster. Of the 207 genes, GO annotation was available for 119, and, of these, 36 are involved in signal transduction. Inhibition of expression of genes related to signal transduction should reduce the interaction of cells with their environment. Although the expression of genes in this cluster was not reduced in 5-fluorouracil treated cells, 46 signal transduction-related genes are included in cluster 5 whose expression was suppressed specifically in 5-fluorouracil-treated cells. Signal transduction related genes in C4 include CXCL10 (chemokine (C-X-C motif) ligand 10), TGFBR2 (transforming growth factor, beta receptor II (70/80 kDa)), CCR3 (chemokine (C-C motif) receptor 3), GFRA1 (GDNF family receptor alpha 1), and PDGFRA (platelet-derived growth factor receptor, alpha polypeptide. Signal transduction related genes in cluster 5 include PRKCA (protein kinase C, alpha), TGFBR1 (transforming growth factor, beta receptor 1), IL6ST (interleukin 6 signal transducer), and BMPR2 (bone morphogenetic protein receptor, type II). These results suggest that suppression of genes involved in signal transduction is a general response to anticancer drugs but that the signals affected vary with the drug used.

Reduced Expression of Genes Involved in DNA Metabolism, Cell Cycle, and RNA Processing in Response to 5-fluorouracil

Genes belonging to cluster 5 (C5) were down-regulated specifically in 5-fluorouracil-treated cells. GO annotation information was available for 251 of them. Of these, 23 are involved in DNA metabolism (twelve in DNA repair and eight in DNA replication), seventeen in the cell cycle, and sixteen in RNA processing. The reduced expression of genes related to RNA processing may reflect a response to damaged cellular RNA, while the reduced expression of cell cycle and DNA replication genes seems to be related to the inhibition of cell proliferation. While the expression of DNA repair-related genes were reduced in

cisplatin-treated cells (cluster 1), the expression of this cluster was reduced in 5-fluorouracil-treated cells. Because 5-fluorouracil may also damages DNA, the reduced expression of DNA repair-related genes was unexpected. However, it is not clear that this actually reflects inhibition of DNA repair since many such genes are likely to be involved in DNA replication and other processes needed for cell proliferation. Of the twelve genes involved in DNA repair, several are involved in other processes coupled to DNA repair: ADPRT (ADP-ribosyltransferase polymerase) is also involved in transcription, while FRAP1 (FK506 binding protein 12-rapamycin associated protein 1 or mTOR) plays an important role in regulation of the cell cycle. RFC5 (replication factor C (activator 1) 5) is involved in DNA replication, and XAB2 (XPA binding protein 2) is also involved in RNA processing. The other explanation is that DNA damage is not main cytotoxic effect of 5-fluorouracil treatment in this cell line as reported in some cell



C

	FU	CA	CP	PT	CD
FU	1	0.10	0.14	0.12	0.13
CA		1	0.40	0.78	0.71
CP			1	0.46	0.41
PT				1	0.71
CD					1

Fig. 1. Clustering analysis of gene expression changes induced by five anticancer drugs. Designations of drugs are: FU, 5-fluorouracil; AC, cytarabine; CP, cisplatin; PT, paclitaxel; and CD, cytochalasin D. Increase in gene expression is indicated in red and decrease in green. A, Clustering of drugs with unfiltered genes. B, Clustering of drugs with genes showing significant changes in expression across drugs ($p<0.001$, Materials and Methods). Three clusters of genes are evident, and the functional categories that may be related to the clusters are indicated, with the number of genes in each category in parenthesis. C, Correlation coefficients between the gene expression profiles for each drug pair.

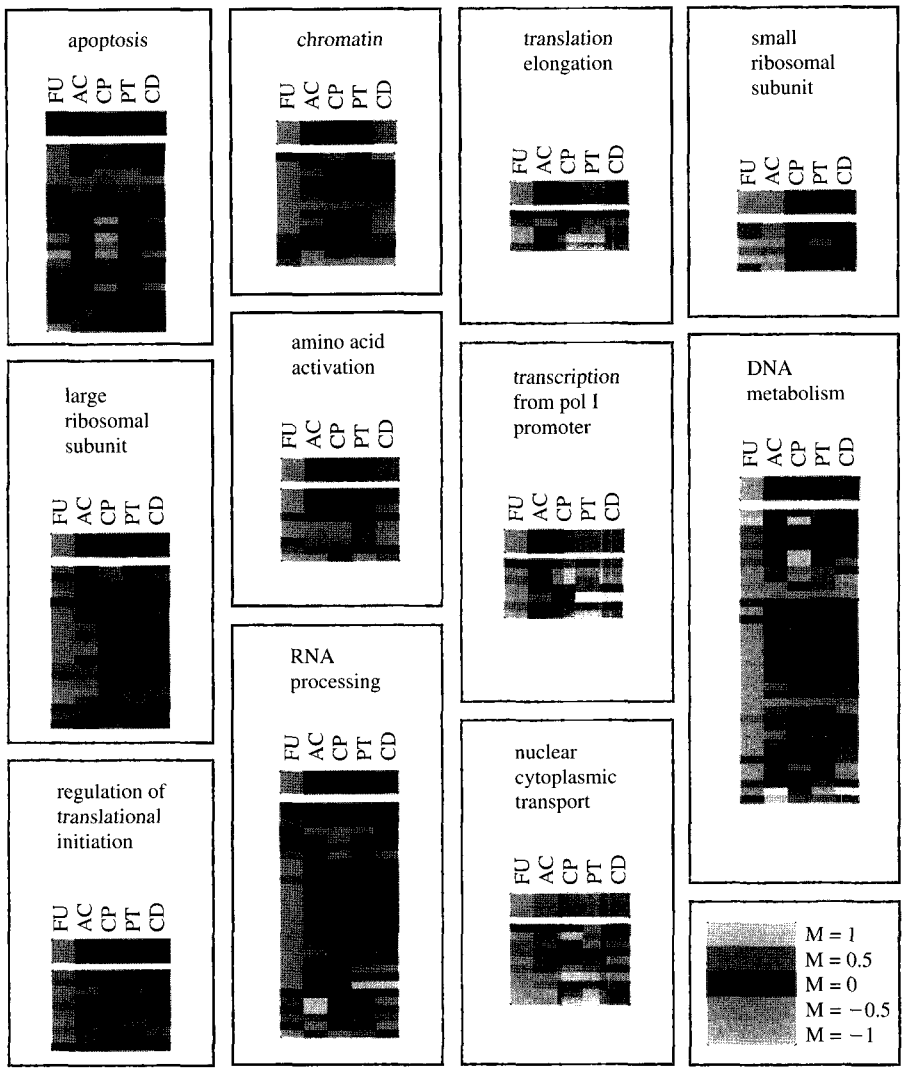


Fig. 2. Comparison of gene expression changes by treatment of five anticancer drugs in GO categories with altered gene expressions by 5-fluorouracil. The gene expression changes resulting from treatment with the five anticancer drugs are represented by heat maps. Genes belonging to GO categories that are significantly associated with 5-fluorouracil-induced gene expression changes are shown. The averages of the gene expression changes in a given category are shown as color bars above the heat maps. Designations of drugs and color codes are as in Fig. 1.

culture systems[16,17].

Selection of 5-fluorouracil Responsive Genes

Although clustering analysis is useful for comparing gene expression patterns induced by anticancer drugs, it is insufficient for a systematic analysis of the effect of the anticancer drugs on gene expression. Since clustering analysis showed that 5-fluorouracil was unique in its effect on gene expression, we set out to characterize these effects more systematically. We therefore examined the gene expression changes in 5-fluorouracil-treated cells twice by means of dye-swapping experiments, thus generating data in quadruplicate. The 5-fluorouracil-responsive genes were identified by SAM with a cut-off of 5% q value, and this yielded 223 up-regulated genes and 585 down-regulated genes.

To gain an overall picture on the biological functions of the gene expression changes, we again related the gene expression data to the annotation information of the Gene Ontology (GO) Consortium. For this purpose we calculated the probability (P) that a given number of genes in a functional group (GO category) is larger than expected by chance. Thus, a smaller P value indicates a greater statistical significance of the association of a group of genes with a biological function. GO categories with $P < 0.01$ containing at least five genes were considered significantly associated with 5-fluorouracil-induced gene expression changes. A simplified list is presented in Table 1.

Induction of Apoptosis-related Genes in 5-fluorouracil Treated Cells

Of the up-regulated genes, those involved in apoptosis (GO : 0006915) showed the strongest association

Table 1. Gene Ontology categories associated with 5-fluorouracil-induced gene expression changes

GO number	Ontology name	Ratio*	*P* value
Up-regulation			
GO:0006915	apoptosis	12/106	0.004715
Down-regulation			
GO : 0015934	large ribosomal subunit	18/21	0
GO : 0006446	regulation of translational initiation	10/15	0.000006
GO : 0000785	chromatin	15/33	0.000019
GO : 0006418	amino acid activation	9/14	0.000026
GO : 0006396	RNA processing	26/90	0.000253
GO : 0006414	translational elongation	5/7	0.00102
GO : 0006360	transcription from Pol I promoter	7/16	0.004468
GO : 0006913	nucleocytoplasmic transport	10/29	0.005544
GO : 0015935	small ribosomal subunit	6/13	0.006563
GO : 0006259	DNA metabolism	31/141	0.008998

*Ratio of the number of genes in a given category whose expression changes divided by the number of genes in that category on the microarray.

(12 of 106 genes changed) (Table 1). CASP3 (caspase 3), ARHGEF6 (Rac/Cdc42 guanine nucleotide exchange factor 6), ACINUS (apoptotic chromatin condensation inducer in the nucleus), EI24 (etoposide induced 2.4 mRNA), BAK1 (BCL2-antagonist/killer 1), and TNFRSF6 (tumor necrosis factor receptor superfamily, member 6, FAS antigen) are positive effectors of apoptosis, while SIRT1 (sirtuin 1, S. cerevisiae sir2 homolog), TNFAIP3 (tumor necrosis factor, alpha-induced protein 3), BAG1 (BCL2-associated athanogene), and IER3 (immediate early response 3) are negative effectors. The increase of BAK1 in response to 5-fluorouracil treatment was previously described[18]. Although the increase in gene expression does not necessarily indicate that the relevant gene products are involved in apoptosis, these results are suggestive that the pathway involving FAS, BAK1, and caspase 3 participates in 5-fluorouracil induced apoptosis. Since apoptosis is controlled by interactions between pro- and anti-apoptotic machineries, the up-regulation of negative effectors of apoptosis may also contribute to regulating 5-fluorouracil-induced apoptosis.

Reduced Expression of Genes Involved in Processes of Transcription and Translation in 5-fluorouracil Treated Cells

The genes whose expression was most markedly reduced in 5-fluorouracil treated cells are those associated with translation (Table 1). Among them, the reduced expression of ribosome subunit genes was most apparent. Of 21 genes encoding large ribosomal subunits (GO : 0015934), 18 genes had reduced expression, and of 13 genes encoding small ribosomal subunits (GO:0015935), six showed reduced expression. Genes for translation initiation factors were also significantly down-regulated. Of 15 such genes (GO : 0006446), 10 were reduced in expression. The expression of genes involved in translation elongation (GO : 0006414) was also remarkably reduced (five of seven genes reduced). Genes involved in amino acid activation (GO : 0006418) were also down-regulated: of fourteen genes involved in this process (mostly encoding aminoacyl-tRNA synthetases), nine were down-regulated. These results show that, in 5-fluorouracil treated cells, the expression of genes involved in translation and related process is reduced to the greatest extent.

Transcription is another process affected by 5-fluorouracil. A number of genes involved in transcription from pol I (GO : 0006360) and pol II promoters (GO : 0006366) were down-regulated, whereas genes involved in transcription from pol III promoters (GO : 0006383) were less affected. Seven of sixteen genes involved in transcription from pol I promoters and 24 of 142 genes involved in transcription from pol II promoters were down-regulated, whereas only one of nine genes involved in transcription from pol III promoters was down-regulated.

RNA processing-related genes were also significantly reduced in expression. The genes involved in mRNA processing (14 of 52 genes), RNA modification (1 of 2 genes), RNA splicing (13 of 43 genes), and rRNA processing (6 of 13 genes) were down-regulated.

The expression of genes involved in nucleocytoplasmic transport (GO : 0006913) necessary for import of ribosomal subunits into the nucleus and export of the assembled ribosomes to the cytoplasm, were also reduced. Of the 29 such genes in the microarray, 10 showed reduced expression.

Fifteen out of 33 genes encoding chromatin (GO :

0000785) components (histone and other chromatin components) were also reduced in expression, as were genes related to DNA metabolism (GO : 0006259). The latter genes are involved in DNA recombination, repair, replication, and packaging. The reduced gene expression in these categories suggests that processes involving maintenance and synthesis of DNA are affected by 5-fluorouracil treatment.

Most of the Reductions in Gene Expression are Unique to 5-fluorouracil

The most interesting feature of the 5-fluorouracil-induced gene expression changes is the reduction of expression of genes involved in target-related processes. In the case of cisplatin, which crosslinks DNA, the expression of genes related to DNA metabolism and repair increased. This difference in the pattern of gene expression changes can be explained by the mode of action of the drugs. Since 5-fluorouracil is incorporated into RNA and produces abnormal RNA, continued transcription, RNA processing, and translation will result in the accumulation of abnormal proteins. Hence suppression of transcription and translation, and related processes might promote the survival of 5-fluorouracil treated cells.

To examine the effect of the other drugs on the genes affected by 5-fluorouracil, we compared the gene expression changes in each category induced by all the tested drugs (Fig. 2). The general tendency of the gene expression changes in 5-fluorouracil treated cells was down-regulation. With the other drugs, some genes also showed reduced expression but this tendency was much weaker. These results show that the gene expression signature of 5-fluorouracil is unique and related to the cellular targets of 5-fluorouracil.

Gene Expression Profiles and the Response of Cancer Cells to Anticancer Drugs

In this study we examined the gene expression changes induced by five anticancer drugs with different cellular targets. Clustering analysis showed that the gene expression profile changes are related to their activities of treated drugs and the cellular responses. From the GO analysis, we identified several functional categories that are significantly associated with 5-fluorouracil-induced gene expression changes. Most of these categories can be linked to RNA, the main target of 5-fluorouracil. This result underlines the relationship between gene expression signature and drug activity.

We have also identified a group of genes whose expression was jointly inhibited by four anticancer drugs with different cellular targets. This suggests that some biological processes may be affected regardless of the drug used. Since anticancer drugs block processes essential for cell proliferation and maintenance, the cells may try to adapt to the toxic environment, and this may be reflected in their gene expression profiles. The shared suppression of expression of signal transduction-related genes by several anticancer drugs with different targets may be an example of this response.

Since we used established cell line rather than primary tumor cells or tumor tissue in animal model, the gene expression profile obtained in this study may not represent real biological response in vivo situation. However, the gene expression profile observed in 5-fluoruracil treated cells is consistent with that in tumor specimens from 5-fluorouracil treated colorectal cancer patients[19]. Therefore, the gene expression profile obtained in this study may be useful information for the understanding of the physiology of the cells treated with anticancer drugs.

Methods

Sample Preparation for cDNA Microarray

NCI-H460 human lung cancer cells were maintained in RPMI-1640 medium (InVitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. To prepare samples for microarrays, exponentially growing cells were treated with 1 μM 5-fluorouracil, 0.2 μM cytarabine, 5 μM cisplatin, 5 nM paclitaxel, or 0.2 μM cytochalasin D for 24 h. The concentrations of the applied drugs were GI20 at 48 h of treatment. Controls were treated like the experimentals, apart from the drug exposure. Total RNA was prepared with TriZol (InVitrogen, Carlsbad, CA) reagent and further purified with an RNeasy column (Qiagen, Valencia, CA). The integrity of the RNA was checked by capillary electrophoresis with a Bioanalyzer 2001 (Agilent, Palo Alto, CA).

cDNA Microarray Hybridization

The cDNA microarrays (GenePlorer™TwinChip™ Human 8 K, Digital Genomics, Seoul, Korea) used in this study contain 8,256 cDNA elements representing 7,194 genes. The PCR-amplified cDNA was spotted on both upper and lower halves of individual slides so that each slide had two identical arrays. For hybridization, 20 μg of total RNA was reverse-transcribed with aminoallyl-modified dUTP and chemically coupled with Cy3 or Cy5 fluorescent dyes. Pairs of labeled samples were combined and hybridized on the duplicate arrays. Tiff image files were quantified with GenePix 3.0 (Axon Instruments, Union City,

CA).

The microarray data can be found in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo with accession number GSE2182.

Experimental Design and Data Analysis

Except for the 5-fluorouracil treatment, control RNA was labeled with Cy3 and RNA from drug-treated cells, with Cy5. The experiments with 5-fluorouracil were performed by dye-swapping. After quantification of the tiff image data, each microarray spot was transformed to M and A values according to the equation:

$$M = \log_2\left(\frac{R}{G}\right) \quad A = \log_2 \sqrt{R \times G}$$

where R is the background-subtracted Cy5 signal and G, the background-subtracted Cy3 signal. The data were normalized in an intensity- and location-dependent manner using 'lowess' implemented in the software package S-plus (InSightful, Seattle, WA). Within-print tip group normalization was performed, followed by scale normalization between the print pin groups. The normalizations were performed as described by Yang *et al.*[6], and the user-defined parameter *f* for the 'lowess' function was set at 0.2.

Hierarchical clustering analysis was performed with Cluster, and the results were visualized with TreeView[7]. Uncentered correlation was used for similarity metric, together with average linkage clustering. To identify genes whose expression varied across experiments, we tested the statistical significance at p = 0.001 of the difference between the variance of each gene and the median of the gene-specific variance. The identification was performed with BRB ArrayTools v3.0 (NCI). Specifically, the quantity $(n-1)Var_i/Var_{med}$ is computed for each gene I, where Var_i is the variance of the log intensity for gene i across the entire set of n arrays, and Var_{med} is the median of these gene-specific variances. This quantity is compared to a percentile of the chi-square distribution with n-1 degrees of freedom. The correlation between microarray data was calculated with Microsoft Excel 2000 (Microsoft, Redmond, WA).

To identify genes whose expression changes in response to 5-fluorouracil treatment, four sets of microarray data (from two arrays per slide together with dye swapping) were analyzed with SAM[8].

GO Analysis

The biological meaning of the gene expression patterns was analyzed using the annotation information from the Gene Ontology (GO) consortium[9]. To identify the functional categories significantly associated with a given list, we calculated the probability (*P*) that a given, or greater, number of genes belonging to a given GO category would be observed by chance in a selected gene list. Genes not annotated in the GO database were not considered in this analysis. To calculate the *P* value, we employed the hypergeometric distribution[10,11] in which the probability (*P*) of observing by chance at least *k* genes from a gene list of size *n* in a category containing *C* genes out of a total number of genes, *G*, is given by: *C* genes out of a total number of genes, *G*, is given by:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{C}{i}\binom{G-C}{n-i}}{\binom{G}{n}}$$

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