Genomic Medicine, Biomarkers, and Health Sciences (2012) 4, 21–27

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SHORT COMMUNICATION

Microarray analysis and establishment of drug screening platform using 5-fluorouracil resistance HCT116 colon cancer cells

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Received 12 March 2012; accepted 30 March 2012
Available online 5 May 2012

KEYWORDS
chemoresistance;
colorectal cancer;
gene expression microarray

Abstract A systemic approach was used to identify the possible mechanisms underlying the development of 5-fluorouracil (5FU)-induced resistance on HCT116 colon cancer cells. From microarray analysis, HCT116 high-dose 5FU-resistant subclones showed differential gene expression compared to HCT116-sensitive clones. According to gene ontology, and Kyoto Encyclopedia of Genes and Genomes pathways, the up-regulated genes were related to cell death and lupus erythematosus, respectively. On the other hand, the down-regulated genes were related to cell division or DNA replication. Connectivity map (cMAP) analysis revealed that the molecular drugs, such as antiasthmatic or antiallergy agents that have negative correlations with cMAP score, may have beneficial effect for the resistant subclones. Our findings suggested that the feasibility of cMAP combining microarray gene expression profile may help identify a potential drug that possibly will reverse the effect of 5FU-induced resistance.

Introduction

Colon cancer is the third most frequently diagnosed cancer[1] and also the third leading cause of cancer deaths in Taiwan.[2] Commonly, 5-fluorouracil (5FU) is the first choice in the treatment of colon cancer[3]; however, long-term exposure of 5FU to cancer cells may result in chemoresistant phenotype.[4] The reasons why cancer treatment becomes ineffective over periods of time is unknown; however, it has been found that cancer cells become resistant through the mechanisms of alteration of drug's specific target, drug inactivation, influx and efflux of drugs in the cells, drug-induced damage, and evasion of apoptosis.[5]

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2211-4254/$36
doi:10.1016/j.gmbhs.2012.04.001

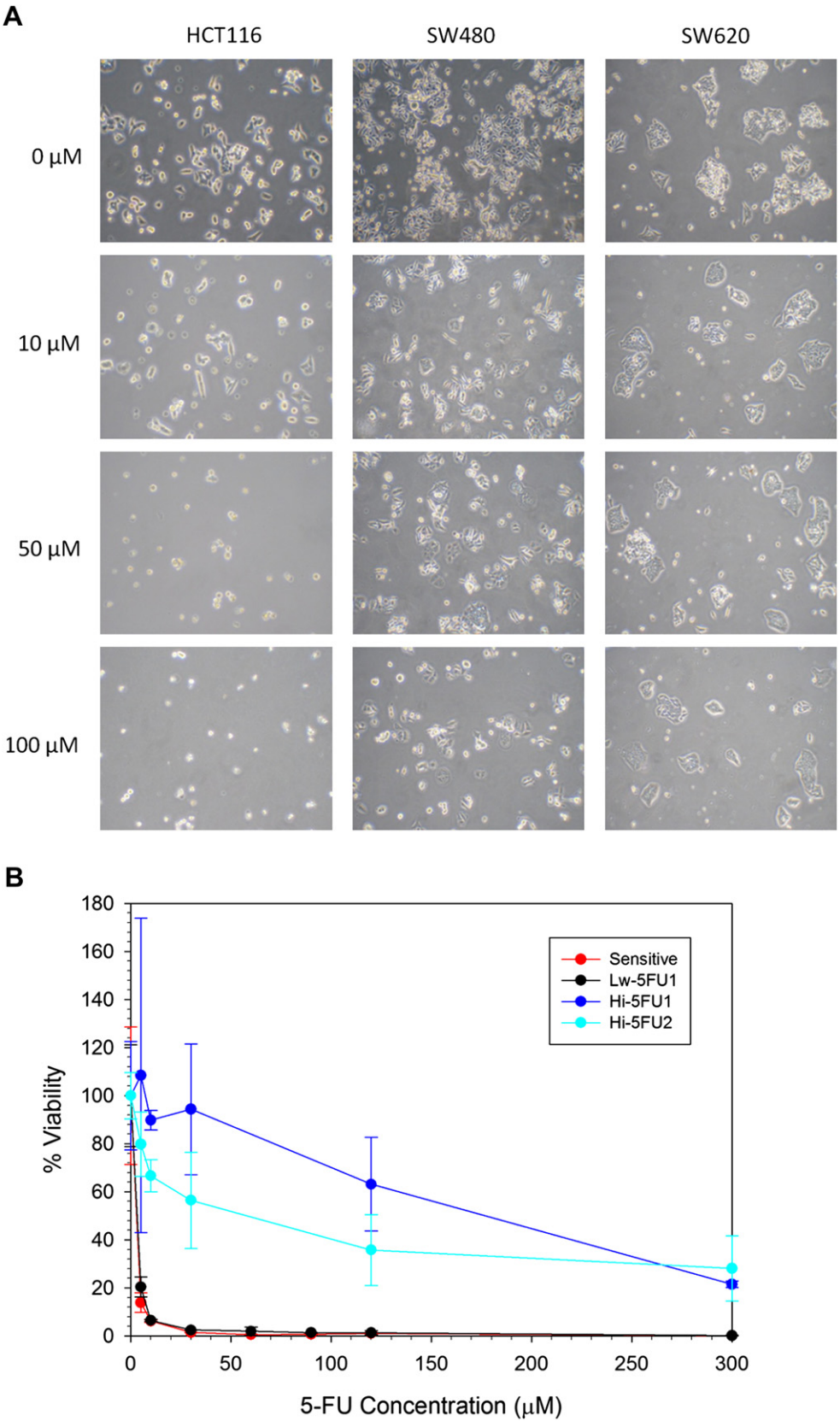

Figure 1 Effect of 5FU on the viability of HCT116-sensitive and HCT116-resistant cells. (A) Morphology of HCT116-, SW480-, and SW620-sensitive cells under microscope after 5 days. HCT116 cells were naturally sensitive to 5FU, and thus were chosen and trained to become 5FU resistant. SW-sensitive cells were naturally resistant to 5FU and thus were not chosen for this study. (B) Proliferative activity of the cells was treated with 0, 2, 5, 30, 60, 90, and 120 μM of 5FU assessed by MTT assay. The inhibitory effect of 5FU was dose -dependent. 5FU = 5-fluorouracil.

Table 1 IC_{50} of HCT116-sensitive and HCT116-resistant clones.

Clones	IC_{50} (μM)
Sensitive	3
Resistant	
Lw-5FU	3
Hi-5FU1	60
Hi-5FU2	180

IC_{50} = half maximal inhibitory concentration; 5FU = 5-fluorouracil.

Microarray technology and the associated databases provide a high throughput method for identifying differentially expressed gene profiles. In this study, two kinds of databases, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), were used for biological processes and pathways analysis, respectively. Connectivity map (cMAP) was used as a useful tool for the identification of any drugs that may have beneficial effects on the resistant subclones.

In the present report, we have successfully cultured HCT116 resistant subclones and, when performing cell viability assay (MTT), we proved that Hi-5FU1 and Hi-5FU2 were resistant toward high concentrations of 5FU. Using microarray technology, the differential gene expression reveals possible mechanisms that are related to chemoresistance, and, hopefully, the potential drugs identified from cMAP can reverse the resistance of HCT116 subclones and become the candidate drug for combined therapy in cancer retreatment.

Materials and methods

Cell lines and culture

The colon cancer cell lines HCT116 were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2:95% air. Three subclones of HCT116 (Lw-5FU1, Lw-5FU2, and Lw-5FU3), which are resistant to 2 μM of 5FU, and two subclones of HCT116 (Hi-5FU1, Hi-5FU2, and Hi-5FU3), which are resistant to 50 μM of 5FU, were used in this study. The resistant subclones were generated by adding 2 and 50 μM of 5FU continuously over a period of time.

MTT assay

The protocol was described by Kang et al.[6] In brief, cells were plated at 3×10^3/200 μL in 96-well plates in McCoy's 5A with 10% FBS and allowed to attach overnight. Then the medium was replaced with varying concentrations (0, 2, 10, 30, 60, 90, 120, and 300 μM) of 5FU. After 5 days of exposure, the number of viable cells in each well was estimated

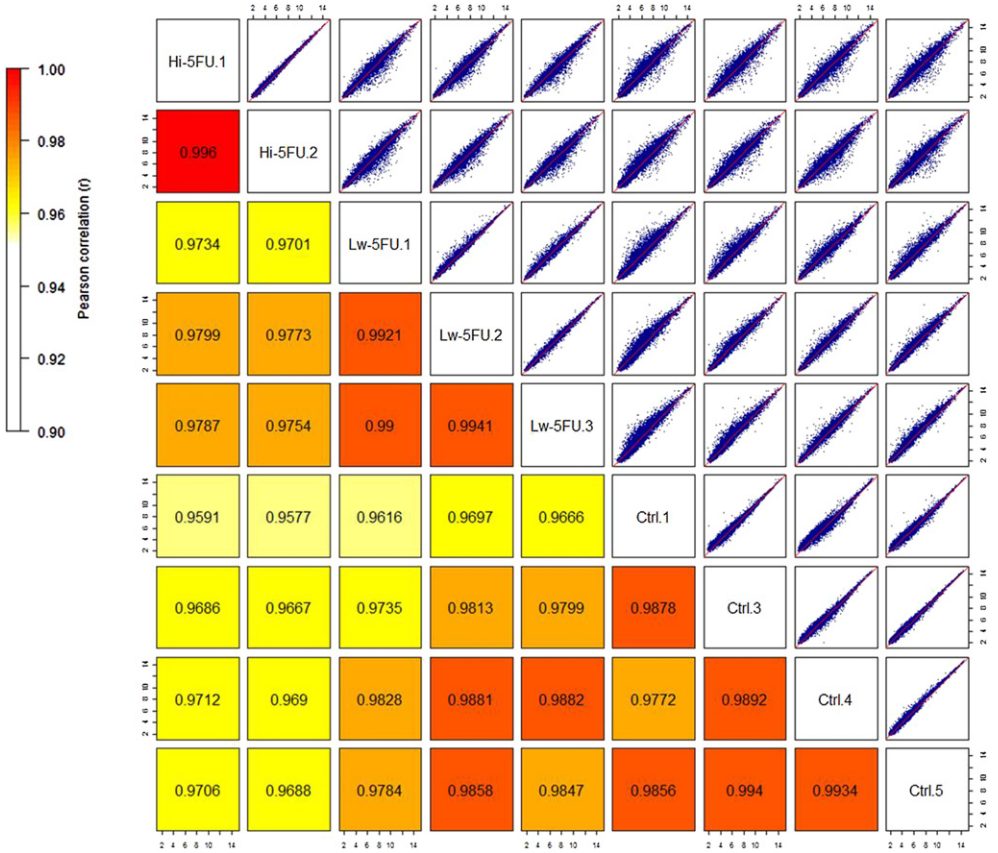

Figure 2 Correlation coefficient analysis between gene expression profiles. Nine samples from the three treatment groups showed good reproducibility within each treatment group.

by adding 10 μL of MTT solution and incubated for 5 hours at 37°C. All assays were performed in triplicates.

Processing of microarray chips

Total RNA was extracted using Illustra TriplePrep kit (GE Healthcare, Buckinghamshire, UK) from cells treated with or without 5FU, according to the manufacturer's protocol. Concentration and purity of RNA were determined using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). The gene expression data were generated using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). The cRNA synthesis and labeling were carried out according to Affymetrix GeneChip protocol (Affymetrix), and arrays were scanned with Affymetrix GeneChip 3000 7G (Affymetrix).

Quality assessment of the microarray data and identification of differentially expressed genes

The $\log_2$-transformed expression intensities with robust multichip average (RMA) normalization from nine microarrays were used to calculate the correlation coefficient in each cluster set, and a heatmap was constructed. The genes differentially expressed in this study were selected using R language bioconductor. The *t* test *p* value and fold change comparing the treatment and control group were calculated. A *p* value of greater than 0.01 and a fold change of greater than 3 were removed, and the remaining was considered as differentially expressed.

Identification of biological processes and pathways enriched with differentially expressed genes

The list of differentially expressed genes was imported to GO (http://www.geneontology.org/) and KEGG (http://www.genome.jp/kegg/) databases. The biological processes and pathways enriched with the differentially expressed genes were according to *p* values.

cMAP analysis

Hi-5FU2 was analyzed using cMAP in an attempt to link genes associated with therapeutic agents. The imported query was compared with predefined signatures of therapeutic compounds and ranked according to the connectivity score (+1 to −1).[7]

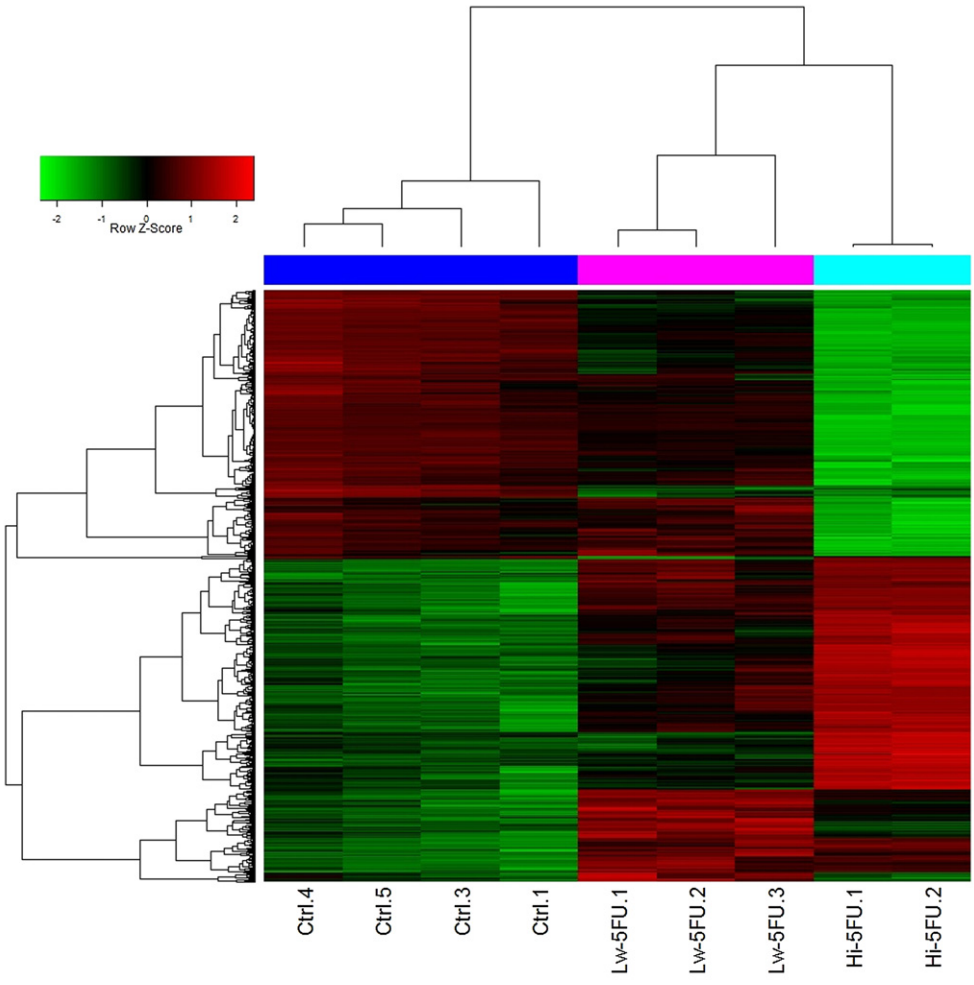

Figure 3 Two-dimensional hierarchical clustering analysis of genes that were treated with 2 μM of 5FU (Lw-5FU) and 50 μM of 5FU (Hi-5FU), compared to control. This heatmap is divided into three clusters: down-regulated, up-regulated, and intermediate down-regulated, based on the expression level in the 50 μM 5FU cluster. 5FU = 5-fluorouracil.

Results

Effect of 5FU on proliferative activity of HCT116-sensitive and HCT116-resistant cells

HCT116 cells were selected for resistance training because it is more sensitive to 5FU than other colon cancer types such as SW480 and SW620 (Fig. 1A). To prove that HCT116-resistant subclones were obtained successfully, treatments of HCT116-sensitive and HCT116-resistant cells were tested using MTT assay (Fig. 1B). At 2 μM of 5FU, the cell viability of HCT116-sensitive and Lw-5FU had decreased to 20% and 15%, respectively. With the increase in concentrations of 5FU, the inhibition of cell growth reached 100%.

Table 2 Top GO and KEGG pathways with respect to differentially expressed genes.

Cluster	GO	*p*	KEGG pathways	*p*
Up-regulated				
	Nucleosome assembly	4.92E−06	Systemic lupus erythematosus	5.62E−06
	DNA packaging	0.00012	P53 signaling pathway	2.92E−05
	DNA damage response	0.00017	Bladder cancer	0.00306
	Death	0.00024	Vitamin B6 metabolism	0.00332
	Chromatin assembly or disassembly	0.00031	Chronic myeloid leukemia	0.00724
	Regulation of programmed cell death	0.00056	Pathways in cancer	0.01007
	Induction of apoptosis by intracellular signals	0.00060		
	Induction of programmed cell death	0.00256		
	Negative regulation of cell death	0.00258		
	Regulation of cell cycle arrest	0.00292		
Down-regulated				
	Organelle fission	8.63E−61	Cell cycle	1.26E−23
	Cell division	6.90E−51	DNA replication	2.93E−18
	M phase	1.13E−50	Oocyte meiosis	8.27E−09
	Mitotic cell cycle	2.44E−38	Mismatch repair	2.11E−07
	Mitosis	8.39E−38	Progesterone-mediated oocyte maturation	3.82E−07
	Chromosome segregation	5.43E−26	Base excision repair	6.63E−05
	DNA replication	1.01E−17	One carbon pool by folate	0.00015
	Cellular component organization	1.06E−16	Pyrimidine metabolism	0.00021
	Cell cycle	2.55E−12	Nucleotide excision repair	0.00044
	Chromosome organization	6.66E−12	Colorectal cancer	0.00075
			P53 signaling pathway	0.00139
			Homologous recombination	0.00148
			Citrate cycle	0.00206
			Glycine, serine, and threonine metabolism	0.00280
			Cysteine and methionine metabolism	0.00372
			Bacterial invasion of epithelial cells	0.00635
			Pathogenic *Escherichia coli* infection	0.00730
			Cyano amino acid metabolism	0.01945
			Metabolic pathways	0.02041
			Pathways in cancer	0.03033
			Synthesis and degradation of ketone bodies	0.03196
			Peroxisome	0.03474
			Folate biosynthesis	0.04682
Intermediate down-regulated				
	Epidermis development	1.91E−05	P53 signaling pathway	3.43E−06
	Organ development	5.69E−05	Renal cell carcinoma	0.00441
	Regulation of hippo signaling cascade	0.00011	Hypertrophic cardiomyopathy	0.00813
	Positive regulation of endothelial cell migration	0.00012	Glycerolipid metabolism	0.01122
	Release of cytochrome c from mitochondria	0.00014	Focal adhesion	0.01152
	Localization of cell	0.00016	Adipocytokine signaling pathway	0.02627
	Regulation of cell adhesion	0.00018	Complement and coagulation cascades	0.02732
	Response to biotic stimulus	0.00020	Chronic myeloid leukemia	0.03288
	Blood vessel development	0.00031	B-cell receptor signaling pathway	0.03648
	Negative regulation of plasminogen activation	0.00032	Pathways in cancer	0.03677
			TGF-beta signaling pathway	0.04563
			Small cell lung cancer	0.04844

GO = gene ontology; KEGG = Kyoto Encyclopedia of Genes and Genomes; TGF = transforming growth factor.

The inhibitory effect of 5FU on Hi-5FU subclones was dose dependent. At a dose of 300 μM 5FU, Hi-5FU1 and Hi-5FU2 still maintained its resistance and reached an inhibition level of 60—70%. Both Hi-5FU1 and Hi-5FU2 subclones possess high resistance with a half maximal inhibitory concentration (IC_{50}) of 60 and 180 μM (Table 1), respectively.

Quality assessment of microarray data

Hierarchical clustering analysis was used to assess the quality of the microarray data. There is a high correlation between the gene expression profiles in each group (Fig. 2), based on Pearson correlation. This satisfactory result warrants further analysis.

The heatmap was divided into three groups: up-regulated, down-regulated, and intermediate down-regulated, according to color indication in high-dose 5FU cluster (Fig. 3). The three groups clustered tightly together and showed dramatically differential expression compared to control groups.

Biological process analysis in GO

To analyze the biological relevance of each cluster, the differentially expressed genes were grouped into GO (Table 2). In the up-regulated cluster, most of the genes are related to cell death at $p < 0.05$. Ten enriched biological processes were found in down-regulated cluster. Most of the processes are related to cell division or mitosis cell cycle.

Pathway analysis in KEGG database

There were 12 differentially expressed genes in the up-regulated cluster, which were involved with systemic lupus erythematosus and P53 signaling pathway. The pathways related to cell cycle or DNA replication were down-regulated in high-dose 5FU subclones. Comparing all the KEGG pathways in all three of the clusters listed in Table 2, most of the pathways were related to P53 signaling pathway, cell deaths, and cell cycle.

Gene expression profile and cMAP analysis

cMAP is an approach that compares the lists of gene expression profiles to the library of experiments assessing the effect of small molecules and genetic events on gene expression.[8] According to the query results, 20 negatively correlated drugs were identified (Table 3). We suspected that the drugs may have the potential to reverse the resistant subclones into its naive state. However, whether these agents may have beneficial effects on resistant subclones is still under investigation.

Discussion

Chemoresistance has posed several problems for patients who rely on chemotherapeutic drugs for cancer treatment. Based on microarray technologies, we integrated the differential gene expression data with computational approaches to identify pathways and rational drugs that may reverse the effect of chemoresistance. All the

Table 3 Results of cMAP query of the top 20 negatively correlated drugs based on cMAP score.

Rank	cMAP name	Dose	Cell	Score	Therapeutic uses
1	Zaprinast	15 μM	HL60	−1	Antiasthmatic, antiallergic
2	Acetazolamide	18 μM	PC3	−0.932	Diuretic, anticonvulsant
3	Riluzole	15 μM	PC3	−0.921	Anti-ischemic, anticonvulsant, antianxiety, hypnotic, anesthetic, psychotropic agent
4	Atractyloside	5 μM	HL60	−0.906	
5	Lansoprazole	11 μM	HL60	−0.904	Gastrointestinal agent
6	Vinblastine	100 nM	MCF7	−0.902	Antineoplastic
7	Harmol	16 μM	HL60	−0.898	
8	Diethylstilbestrol	15 μM	HL60	−0.896	Antineoplastic, vasodilator, hormonal agent
9	Podophyllotoxin	10 μM	MCF7	−0.889	Antineoplastic
10	Mafenide	18 μM	HL60	−0.882	Antibacterial
11	Fludrocortisone	9 μM	HL60	−0.882	
12	Diphenhydramine	14 μM	HL60	−0.876	Antihistamine
13	Proguanil	14 μM	HL60	−0.869	
14	Ioxaglic acid	3 μM	MCF7	−0.865	
15	Midodrine	14 μM	HL60	−0.864	Vasoconstrictor
16	Aciclovir	18 μM	HL60	−0.863	Antiviral
17	BCB000040	10 μM	MCF7	−0.858	
18	Fulvestrant	10 nM	HL60	−0.858	
19	Monorden	100 nM	HL60	−0.858	
20	Mimosine	20 μM	MCF7	−0.849	Antiproliferative

cMAP = connectivity map.

subclones were first tested for their resistance. The Hi-5FU1 and Hi-5FU2 clearly showed that they have high resistance toward 5FU.

Nine samples of array data were analyzed for quality assessment. The correlation within each data set has high correlations, indicating that there is a high reproducibility in the microarray experiment. The clusters showed different expression profiles when comparing high- and low-resistant subclones with control. In GO, most of the down-regulated genes in high-resistant clones were related to organelle fission and cell division. This finding supports our observation of slow growth of Hi-5FU1 and Hi-5FU2 in the laboratory.

Most of the up-regulated genes in our high-resistant sub-clones were related to death, and we suspect that it is related to autophagy since a study has demonstrated that the resistance of cells is associated with the switch from apoptosis to autophagy cell death.[9] The pathways related to down-regulated genes were mainly cell cycle or DNA replication, which correlates with the finding on GO. Whether the pathways in the up-regulated gene cluster were involved with lupus erythematosus is now under investigation in our laboratory. The molecular effect of potential drugs from cMAP query is important for cancer therapies. However, whether these drugs can potentiate the cancer cell resistance still awaits further studies.

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