Gene expression analysis using human cancer xenografts to identify novel predictive marker genes for the efficacy of 5-fluorouracil-based drugs

Akio Ooyama,[1,3] Teiji Takechi,[2] Etsuko Toda,[1] Hideki Nagase,[1] Yoshihiro Okayama,[1] Kenji Kitazato,[2] Yoshikazu Sugimoto,[1] Toshinori Oka[1] and Masakazu Fukushima[1]

[1]Optimal Medication Research Laboratory, and [2]Cancer Research Laboratory, Taiho Pharmaceutical Company, 224-2 Ebisuno, Hiraishi, Kawauchi-cho Tokushima, 771-0194, Japan

(Received October 25, 2005/Revised February 13, 2006/Accepted February 14, 2006/Online publication May 11, 2006)

The development of a diagnostic method for predicting the therapeutic efficacy or toxicity of anticancer drugs is a critical issue. We carried out a gene expression analysis to identify genes whose expression profiles were correlated with the sensitivity of 30 human tumor xenografts to 5-fluorouracil (5-FU)-based drugs (tegafur + uracil [UFT], tegafur + gimeracil + oteracil [S-1], 5′-deoxy-5-fluorouridine [5′-DFUR], and N^4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine [capecitabine]), as well as three other drugs (cisplatin [CDDP], irinotecan hydrochloride [CPT-11], and paclitaxel) that have different modes of action. In the present study, we focused especially on the fluoropyrimidines. The efficacy of all anticancer drugs was assayed using human tumor xenografts in nude mice. The mRNA expression profile of each of these xenografts was analyzed using a Human Focus array. Correlation analysis between the gene expression profiles and the chemosensitivities of seven drugs identified 39 genes whose expression levels were correlated significantly with multidrug sensitivity, and we suggest that the angiogenic pathway plays a pivotal role in resistance to fluoropyrimidines. Furthermore, many genes showing specific correlations with each drug were also identified. Among the candidate genes associated with 5-FU resistance, the dihydropyrimidine dehydrogenase mRNA expression profiles of the tumors showed a significant negative correlation with chemosensitivity to all of the 5-FU based drugs except for S-1. Therefore, the administration of S-1 might be an effective strategy for the treatment of high dihydropyrimidine dehydrogenase-expressing tumors. The results of the present study may enhance the prediction of tumor response to anticancer drugs and contribute to the development of tailor-made chemotherapy. (*Cancer Sci* 2006; 97: 510–522)

Many chemotherapeutic agents have been used to treat cancer patients; however, the emergence of drug resistance has prevented successful treatment in many cases. A large population of cancer patients suffers from the adverse effects of chemotherapy without achieving any benefit in terms of a good response. Differences in the efficacy of anticancer drugs among patients have been associated with variations in polymorphisms and gene expression profiles in cancer cells,[1–3] and predicting tumor response based on valid markers is important because patients who are unlikely to respond to a treatment can avoid the adverse effects of unsuccessful treatments and be placed on alternative regimens. Furthermore, a maximal response during the course of the first regimen is important to avoid the acquisition of drug resistance. Hence, the development of tailor-made chemotherapy regimens, which would select a suitable regimen for each patient based on biological features (including genomic factors and gene expression profiles), is a very critical issue.

Combination therapy is now a standard treatment for cancer patients. The rationale for combination chemotherapy is to use suitable anticancer drugs that are active on different cell populations of cancer tissue, thereby increasing the possibility that more cancer cells will be killed. Although various drug combinations have been evaluated recently in clinical trials,[4–6] some combinations may not only decrease the prognosis, but may induce the adverse effects of the treatment. Hence, identifying genes that contribute to single or multiple drug resistance is important for selecting the optimal drug combination for each patient.

5-fluorouracil is one of the most commonly used anticancer drugs in chemotherapy against various solid tumors.[7] 5-FU has two main modes of action that are realized through its active metabolites: FdUMP (5-fluoro-2′-deoxyuridine-5′-monophosphate) and FUTP (fluorouridine-5′-triphosphate). FdUMP inhibits TS (Thymidylate synthase) by forming a covalent ternary complex with 5,10-methylentetrahydrofolate that subsequently suppresses DNA synthesis, whereas FUTP is incorporated into RNA, resulting in the distortion of gene expression.[8,9] DPD (dihydropyrimidine dehydrogenase), which is both an initial and a rate-limiting catabolic enzyme of 5-FU, has been reported to play an important role in the pharmacokinetics of 5-FU,[10] and 80% of 5-FU is catabolized rapidly into inactive metabolites by DPD in the liver. Furthermore, DPD not only inactivates 5-FU, but also produces fluoroacetate and fluorohy-droxypropionic acid, which have been reported to induce cardiotoxicities and neurotoxicities.[10] To resolve this problem, oral fluoropyrimidine derivatives were developed in the form of 5-FU prodrugs (e.g. tegafur, 5′-DFUR and capecitabine)[11] and both prodrugs and DPD inhibitors (e.g. S-1, UFT).[12,13] S-1 and UFT are classified as DIF (DPD inhibitory fluoropyrimidines) drugs. UFT is a combination drug consisting of 1 M tegafur and 4 M uracil that selectively inhibits the

[3]To whom correspondence should be addressed.
E-mail: akio-ooyama@taiho.co.jp

doi: 10.1111/j.1349-7006.2006.00204.x

degradation of 5-FU by DPD. Recently, a clinical study on the use of UFT confirmed that adjuvant chemotherapy with UFT effectively prolonged the survival periods of patients with resected adenocarcinoma of the lung.[14] S-1 is a newly developed DIF that consists of 1 M tegafur, 0.4 M gimeracil (a potent DPD inhibitor) and 1 M oteracil (an oratate phosphoribosyltransferase inhibitor) to protect against gastrointestinal toxicity. It showed a high clinical efficacy when used in patients with unresectable advanced gastric,[15] colorectal,[16] breast[17] and non-small-cell lung cancers.[18] However, some factors other than DPD might play important roles in the efficacy of 5-FU, but few studies have examined markers to predict the antitumor effects of various 5-FU-based drugs using genome-wide expression analysis.

Microarray technology has been used widely for global gene expression analysis, and several studies have examined the comprehensive gene expression profiles for predicting the response of cancer cells to anticancer drugs.[19–21] This technology has enabled us to identify new target genes that play a key role in drug efficacy, and has provided fundamental information for overcoming drug resistance.

In the present study, we carried out gene expression analysis to identify genes whose expression profiles were correlated with the sensitivity of 30 human tumor xenografts to 5-FU based drugs (DIF: UFT, S-1; non-DIF: 5′-DFUR, capecitabine). Furthermore we also examined some drugs (CDDP, CPT-11, and paclitaxel) that have different mechanisms of action, because these drugs have already been used in combination therapies with 5-FU based drugs, or may be used in the future. We have identified gene sets that showed a significant correlation with tumor sensitivity to each drug as well as candidate genes involved in multidrug resistance, and applied an ontological approach to extract genes that may be predictive markers of drug efficacy.

Materials and Methods

Nude mice and human tumor xenografts

Six gastric carcinoma xenografts (AZ-521, SC-2, ST-40, 4-1ST, SC-4 and OCUM-2MD3), six colon carcinoma xenografts (KM12C, HCT-15, KM20C, COL-1, KM12C/FU and CO-3), six breast carcinoma xenografts (MC-5, H-31, MC-2, MX-1, MDA-MB-435SHM and MDA-MD-231), seven lung carcinoma xenografts (GT3TKB, LC-11, Lu-99, LX-1, LC-6, Lu-134 and Lu-130) and five pancreatic carcinoma xenografts (PAN-3, PAN-4, PAN-12, H-48 and BxPC-3) were used in this study. KM12C and KM20C were kindly provided by Dr Kiyoshi Morikawa of the National Cancer Institute (Tokyo, Japan). KM12C/FU was established as described previously.[22] MDA-MB-435SHM was established from an *in vivo* xenograft.[23] LX-1 and MX-1 were kindly provided by Dr K. Inoue of the Cancer Chemotherapy Center (Tokyo, Japan). H-31 and H-48 were kindly provided by Dr Tetsuo Taguchi of the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan). AZ-521 and MDA-MB-231 were purchased from the Human Science Research Resource Bank (Osaka, Japan) and the American Tissue Culture Collection (Manassas, VA, USA), respectively. HCT-15 and BxPc-3 were purchased from Dainippon Pharmaceutical Company (Tokyo, Japan). The other lines were provided by the Central Institute for Experimental Animals (Kawasaki, Japan). Male BALB/c-*nu*/*nμ* nude mice (5 weeks old; 18–20 g) were purchased from CLEA Japan, (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions, and were provided with sterile food and water *ad libitum*. Each human tumor xenograft (2-mm cubic fragment) or cultured cell line was implanted subcutaneously into nude mice.

Chemicals

UFT, S-1 and capecitabine were synthesized in our laboratory. 5′-DFUR, CDDP, CPT-11 and paclitaxel were purchased from Nippon Roche (Tokyo, Japan), BristolMyers Squibb (Tokyo, Japan), Yakult Honsha KK (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. [6-^{14}C]-5-FU (1.85 GBq/mmol) and [6-^{3}H]-FdUMP (625 GBq/mmol) were obtained from Moravek Biochemicals (Brea, CA, USA). All other reagents were commercially available and of the highest quality.

Examination of antitumor activity

When the estimated tumor volume ($0.5 \times \text{length} \times \text{width}^2$) reached 100–300 mm^3, the tumor-bearing mice were allocated randomly to a test group (day 0, $n = 5$). UFT, S-1, 5′-DFUR and capecitabine were administered orally once a day from day 1 to day 14 (q.d.), as per the reported maximal tolerated dose.[24] The maximal tolerated doses of the other drugs used in each schedule were determined in a pre-experiment (data not shown). The RTV (relative tumor volume) was calculated on day 15 as follows: tumor volume on day 15/tumor volume on day 0. The antitumor effect (inhibition rate [%]) was calculated as follows: inhibition rate [%] = (1 − mean RTV of drug-treated group/mean RTV of untreated group) × 100. The tumor growth inhibition rate value on day 15 was regarded as representing the antitumor effect. All animal experiments were carried out according to the Guidelines for the Welfare of Animals in Experimental Neoplasia.[25]

Extraction of total RNA and genechip hybridizations

Total RNA was extracted from each xenograft using the RNeasy mini kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. The total RNA yields and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. cDNA and biotinylated cRNA were synthesized according to the standard protocols provided by Affymetrix (Santa Clara, CA, USA). Briefly, 5–10 μg of total RNA was reverse transcribed with a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) in the presence of an oligo dT-T7 primer. After phenol–chloroform extraction and ethanol precipitation, the cDNA pellet was air dried and resuspended in 12 μL of RNase-free water. Ten microliters were used for the *in vitro* transcription–amplification reaction in the presence of biotinylated nucleotides (Enzo Diagnostics, Farmingdale, NY, USA). Fifteen micrograms of biotinylated cRNA were then fragmented in a solution of 40 mM Tris–acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate at 94°C for 35 min and hybridized to HG Focus GeneChip arrays (Affymetrix) containing probe sets that represent approximately 8500 transcripts. Chip hybridization, washing and staining were carried out according to Affymetrix-recommended protocols.

Clustering analysis of drug sensitivity

We carried out a clustering analysis based on drug sensitivity. We then calculated the standard correlation coefficient between drug a and drug b using the following formula:

$$r_{a,b} = \frac{\sum a_c b_d}{\sqrt{\sum a_c^2 \sum b_d^2}},$$

where r denotes the correlation of drug a and drug b based on their antitumor activity, a_c represents the activity of drug a in xenograft c, and b_d represents the activity of drug b in xenograft d.

Genechip

Automated processing of the image scans for the absolute expression analysis was done using Microarray Suite version 5.0 (Affymetrix). The software provided each transcript with a 'detection call', which predicted whether the gene was present at a level detectable by the array. The call specifies whether the transcript is detectable (P, present), undetectable (A, absent), or at the limit of detection (M, marginal). These data were then imported into GeneSpring software (Agilent, Palo Alto, CA, USA). We carried out a per-chip (the expression of each probe set in each chip divided by the median of the chip) and a per-gene (each gene divided by the mean of all the samples) normalization using the GeneSpring software. The normalized gene expression values were transformed logarithmically ($\log_2$). Genes for which the number of 'present' calls was less than half of the number of samples were dropped from the analysis. Furthermore, to prevent outlier values from biasing the correlation coefficient, we calculated the entropy, H, using the following formula:

$$H = -\sum_{x=1}^{10} p(x)\log_2(p(x)),$$

where $p(x)$ is the probability that a value was within decile x of that gene expression profile. Genes whose entropy values were within the lowest 10% were dropped from further analysis. Finally, we selected 4144 genes for subsequent analysis.

Correlation analysis between gene expression and drug sensitivity

To investigate the correlation between gene expression and drug sensitivity, we calculated the Pearson correlation coefficients according to the following formula:

$$r = \frac{\sum (x_k - x_{mean})(y_k - y_{mean})}{\sqrt{\sum (x_k - x_{mean})^2 (y_k - y_{mean})^2}},$$

where x_κ represents the log-transformed expression value of gene x in the xenograft k, y_k is the sensitivity to drug y in the xenograft k, and x_{mean} represents the mean expression value. For this analysis, the difference between maximum and minimum drug sensitivity was fixed as 1. We selected genes with a significant correlation ($P < 0.05$) and whose absolute value of the slope of the regression line was larger than 1.5, where the difference in drug sensitivity between the most and the least sensitive xenograft was fixed as one.

Real-time RT-PCR

Real-time RT-PCR analysis was carried out using the Micro-Fluidic Cards™ system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. In brief, 2.5 μg of total RNA was reverse transcribed using the High Capacity cDNA Archives Kit (Applied Biosystems) and MultiScribe™ reverse transcriptase. The reaction mixtures were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (TaqMan™) assays were carried out using the Micro-Fluidic Cards system incorporating Assays-On-Demand™ (Applied Biosystems), a prevalidated library, into 384-well Micro-Fluidic Cards. cDNA samples (100 ng), along with 50 μL of 2 × PCR master mix, were loaded into each channel on the Micro-Fluidic Card followed by a brief centrifugation (300 × g for 2 min at room temperature). The card was then sealed, and real-time PCR and relative quantification were carried out using an ABI PRISM 7900 Sequence Detection System. The expression of each gene was normalized using β-actin as a reference, and the relative expression levels were qualified using the ΔCt method (Applied Biosystems).[26]

DPD activity

The DPD enzymatic activity was measured using a method described by Takechi *et al*.[27] Briefly, tumor tissues were sonicated in four volumes of homogenization buffer (20 mM potassium phosphate [pH 8.0] containing 1 mM EDTA and 1 mM β-mercaptoethanol). Each homogenate was centrifuged at 105 000g for 1 h at 4°C, and its supernatant (cytosol) was collected. The enzyme reaction mixture, which contained 10 mM potassium phosphate (pH 8.0), 0.5 mM EDTA, 0.5 mM β-mercaptoethanol, 2 mM dithiothreitol, 5 mM magnesium chloride, 20 μM [6-^{14}C] 5-FU (American Radiolabeled Chemicals, St Louis, MO, USA), 100 μM NADPH, and 25 μL of the cytosol fraction in a final volume of 50 μL, was incubated at 37°C for 30 min. DPD activity was then determined by measuring the sum of the dihydrofluorouracil and 2-fluoro-β-alanine produced from [6-^{14}C] 5-FU. Supernatant aliquots (5 μL) were applied to thin-layer chromatography plates (Silica gel 60_{F254}; Merck, Darmstadt, Germany) and developed with a mixture of ethanol and 1 M ammonium acetate (5 : 1, v/v) according to a method described previously.[28]

TS contents

The TS content was determined as the quantity of [6-^{3}H]-FdUMP binding activity in the cytosol of tumor tissue homogenates, based on the method described by Spears and colleagues,[29] with minor modifications.

Results

Relationship of drug sensitivities

To evaluate the characteristics of each drug, we first carried out a hierarchical clustering analysis on seven drugs based on their antitumor effects on the 30 xenografts. The clustering analysis showed a weak negative correlation between the antitumor activities of the fluoropyrimidines and paclitaxel. Moreover, roughly two clusters were generated (Fig. 1). 5-FU based drugs occupied the upper cluster, whereas the lower cluster consisted of non-5-FU derivatives. For the

doi: 10.1111/j.1349-7006.2006.00204.x

Fig. 1. Two-dimensional hierarchical clustering of each antitumor effect in the 30 xenografts. The inhibition rate of the relative tumor volume was regarded as representing antitumor activity. *Red*, represents a high sensitivity to the drug; *green*, resistant.

upper one, the dendrogram was divided into two groups: an 'UFT/S-1 cluster' and a '5′-DFUR/capecitabine cluster'. These results indicate that even among fluorinated pyrimidines, the pattern of antitumor activity differs between DIF and non-DIF drugs.

Correlations between gene expression and drug sensitivity

To screen for genes that may be associated with drug sensitivity, we carried out a correlation analysis based on the Pearson correlation coefficient between the expression profiles of the 4144 genes and the antitumor activities of the anticancer drugs in the 30 xenografts. We used all xenografts to screen for genes that would account for differences in the efficacy of the drugs against various types of tumors. The correlation analysis showed that the expression profiles of 684 genes showed significant association with sensitivity to at least one of the drugs. Furthermore, as shown in Table 1, various genes whose expression profiles were significantly correlated with tumor sensitivity to each drug were extracted by the correlation analysis. Table 2 summarizes the top 20 genes with the highest Pearson's correlation coefficients. These genes have various functions, as shown in the gene lists. Although some genes exhibited a broad negative correlation with fluoropyrimidines, many genes that showed specific correlations with each drug were also seen. These results indicate that each of the 5-FU-based drugs has distinctive characteristics, despite having a common cytocidal mechanism, and suggests that unidentified factors other than DPD are likely involved in the regulation of drug efficacy.

Analysis of functional genes affecting antitumor activity

To screen the genes that may serve as predictive markers of antitumor activity, we examined genes that were associated with multiple drug sensitivities, and classified them ontologically into key pathways suggested or previously shown to play a role in drug metabolism or resistance. First, we selected 179 genes whose expression profiles were correlated with sensitivity to more than two 5-FU-based drugs. Next, we selected 25 genes that were closely

Table 1. Number of genes significantly correlated with antitumor activity to seven anticancer drugs

	Number of genes		
	Positive correlation	Negative correlation	Total
UFT	72	59	131
S-1	84	31	115
Capecitabine	128	64	192
5′-DFUR	110	85	195
CDDP	25	88	113
CPT-11	99	90	189
Paclitaxel	30	35	65

Correlation analysis between 4144 probe sets and antitumor activity for seven anticancer drugs across 30 xenografts were carried out to screen the genes that correlated with sensitivity to the drug sensitivity. Genes were selected that represented the significant correlation that satisfied the following criteria: $P < 0.05$, and the absolute value of the slope of the regression line was >1.5 where the difference of the drug sensitivity between the most and the least xenografts was fixed as 1. CDDP, cisplatin; CPT-11, irinotecan hydrochloride; 5′-DFUR, 5′-deoxy-5-fluorouridine; S-1, 1 M tegafur–0.4 M 5-chloro-2,4-dihydroxypyridine–1 M potassium oxonate; UFT, 1 M tegafur–4 M uracil.

correlated with more than two drugs other than fluoropyrimidines. We found three genes that were common to these gene lists, and selected 210 genes by combining these lists. We then selected genes that could be ontologically classified into key pathways or functions thought to be related to drug efficacy. The Kyoto Encyclopedia of Genes and Genomes, Simplified Gene Ontology (GeneSpring version 6.1), and Gene Ontology Consortium were used to investigate the biological processes and important pathways (such as transforming growth factor-α/MAPK/Wnt signaling, cell proliferation, cell adhesion, oncogenes, nucleotide sugars metabolism, and pyrimidine metabolism). Finally, we identified 39 genes using an ontological approach (Fig. 2). In addition to the genes that have been shown previously to play important roles in drug resistance, we found many genes that have not been reported to be associated with drug sensitivity and that may serve as putative predictive markers of chemosensitivity. The expression level of *DPD*, a well-known predictor of resistance to 5-FU, showed a broad negative correlation with the 5-FU-based drugs, except S-1. Furthermore, a significant negative correlation between *TS* expression and sensitivity to non-DIF (5′-DFUR and capecitabine) was seen, but *TS* expression was not correlated with sensitivity to DIF. The expression level of *AKR1B1* has been reported to be associated with multiple drug sensitivity.[20,30] In the present study, *AKR1B1* expression was correlated with four of the seven drugs. *CYR61*, which is involved in angiogenesis and mediates diverse roles in cellular development,[31] showed significant associations with six drugs, including all of the 5-FU-based drugs. Finally, we visualized the relationships between gene expression and drug sensitivity (Fig. 3). In this network, the connection between drugs and genes whose association was not previously known can be seen. Some genes associated with sensitivity to 5-FU derivatives showed a significant inverse correlation with sensitivity to paclitaxel.

Table 2. Summary of the top 20 genes significantly correlated with sensitivity to each drug

Drug	Index†	Gene name	Slope‡	*r*§	*P*-value	GenBank no.	Product
S-1	202922_at	*GCLC*	1.69	0.627	<0.001	BF676980	Glutamate-cysteine ligase, catalytic subunit
	207463_x_at	*PRSS3*	4.70	0.631	<0.001	NM_002771	Mesotrypsin preproprotein
	202609_at	*EPS8*	3.74	0.617	<0.001	NM_004447	Epidermal growth factor receptor pathway substrate 8
	202831_at	*GPX2*	5.75	0.614	<0.001	NM_002083	Gastrointestinal glutathione peroxidase 2
	218854_at	*SART2*	–3.47	–0.602	<0.001	NM_013352	Squamous cell carcinoma antigen recognized by T cells 2
	203476_at	*TPBG*	–3.78	–0.593	0.001	NM_006670	5T4 oncofetal trophoblast glycoprotein
	217794_at	*DKFZp564J157*	1.94	0.593	0.001	NM_018457	DKFZp564J157 protein isoform 1
	201425_at	*ALDH2*	3.64	0.586	0.001	NM_000690	Mitochondrial aldehyde dehydrogenase 2 precursor
	208453_s_at	*XPNPEP1*	1.57	0.581	0.001	NM_006523	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble
	205402_x_at	*PRSS2*	4.48	0.557	0.001	NM_002770	Protease, serine, 2 preproprotein
	219115_s_at	*IL20RA*	2.35	0.555	0.001	NM_014432	Interleukin 20 receptor, alpha
	221016_s_at	*TCF7L1*	–2.08	–0.557	0.001	NM_031283	HMG-box transcription factor TCF-3
	202794_at	*INPP1*	1.83	0.552	0.002	NM_002194	Inositol polyphosphate-1-phosphatase
	203832_at	*SNRPF*	1.64	0.551	0.002	NM_003095	Small nuclear ribonucleoprotein polypeptide F
	201829_at	*NET1*	1.71	0.545	0.002	AW263232	Neuroepithelial cell transforming gene 1
	204608_at	*ASL*	1.86	0.546	0.002	NM_000048	Argininosuccinate lyase
	220189_s_at	*MGAT4B*	1.51	0.546	0.002	NM_014275	Mannosyl-glycoprotein β-1,4-*N*-acetylglucosaminyltransferase, isoenzyme B isoform 1
	209605_at	*TST*	5.09	0.544	0.002	D87292	Rhodanese
	211184_s_at	*aie-75*	3.17	0.541	0.002	AB006955	AIE-75
	202674_s_at	*LMO7*	1.97	0.540	0.002	NM_005358	LIM domain only 7
UFT	205395_s_at	*MRE11A*	–3.68	–0.749	<0.001	NM_005590	Meiotic recombination 11 homolog A isoform 2
	201312_s_at	*SH3BGRL*	–5.76	–0.639	<0.001	NM_003022	SH3 domain binding glutamic acid-rich protein like
	203752_s_at	*JUND*	1.50	0.633	<0.001	NM_005354	*Jun-D* proto-oncogene
	204333_s_at	*AGA*	–1.91	–0.632	<0.001	NM_000027	Aspartylglucosaminidase precursor
	217788_s_at	*GALNT2*	–2.21	–0.616	<0.001	NM_004481	Polypeptide *N*-acetylgalactosaminyltransferase 2
	218854_at	*SART2*	–4.22	–0.620	<0.001	NM_013352	Squamous cell carcinoma antigen recognized by T cells 2
	209526_s_at	*HRP-3*	–4.47	–0.581	0.001	AB029156	HRP-3
	214600_at	*TEAD1*	–2.17	–0.580	0.001	AW771935	TEA domain family member 1
	205187_at	*Smad5*	–2.34	–0.567	0.001	AF010601	SMAD5
	212983_at	*HRAS*	–2.05	–0.564	0.001	NM_005343	v-Ha-ras Harvey rat sarcoma viral oncogene homolog isofrom 1
	217759_at	*TRIM44*	–1.89	–0.554	0.002	NM_017583	DIPB protein
	201481_s_at	*PYGB*	6.03	0.552	0.002	NM_002862	Brain glycogen phosphorylase
	203874_s_at	*SMARCA1*	–3.09	–0.540	0.002	NM_003069	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a1 isoform a
	201177_s_at	*UBA2*	–1.68	–0.538	0.002	NM_005499	SUMO-1 activating enzyme subunit 2
	201540_at	*FHL1*	–4.53	–0.530	0.003	NM_001449	Four and a half LIM domains 1
	202602_s_at	*HTATSF1*	–1.67	–0.530	0.003	NM_014500	HIV TAT specific factor 1
	200821_at	*LAMP2*	–1.78	–0.524	0.003	NM_013995	Lysosomal-associated membrane protein 2 precursor
	214257_s_at	*SEC22L1*	–1.93	–0.524	0.003	AA890010	Hypothetical protein
	202082_s_at	*SEC14L1*	–1.74	–0.518	0.003	AV748469	SEC14 (S. cerevisiae)-like 1
	203953_s_at	*CLDN3*	8.32	0.517	0.003	BE791251	Claudin 3
5′-DFUR	201481_s_at	*PYGB*	7.46	0.686	<0.001	NM_002862	Brain glycogen phosphorylase
	203586_s_at	*ARF4L*	–1.97	–0.669	<0.001	NM_001661	ADP-ribosylation factor 4-like
	205395_s_at	*MRE11A*	–3.30	–0.674	<0.001	NM_005590	Meiotic recombination 11 homolog A isoform 2
	202620_s_at	*PLOD2*	–5.49	–0.644	<0.001	NM_000935	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 isoform b
	214600_at	*TEAD1*	–2.33	–0.626	<0.001	AW771935	TEA domain family member 1
	218854_at	*SART2*	–4.30	–0.634	<0.001	NM_013352	Squamous cell carcinoma antigen recognized by T cells 2
	222065_s_at	*FLII*	–1.73	–0.632	<0.001	AI830227	Flightless I homolog (*Drosophila*)
	204201_s_at	*PTPN13*	–3.53	–0.610	<0.001	NM_006264	Protein tyrosine phosphatase, non-receptor type 13 isoform 2
	204646_at	*DPYD*	–4.86	–0.603	<0.001	NM_000110	Dihydropyrimidine dehydrogenase
	219255_x_at	*IL17RB*	4.16	0.603	<0.001	NM_018725	Interleukin 17B receptor isoform 1 precursor
	210827_s_at	*ESE-1*	3.79	0.599	0.001	U73844	ESE-1a
	200989_at	*HIF1A*	–1.61	–0.592	0.001	NM_001530	Hypoxia-inducible factor 1, α subunit isoform 1
	220147_s_at	*C12orf14*	1.69	0.592	0.001	NM_021238	Chromosome 12 open reading frame 14
	201528_at	*RPA1*	–1.89	–0.580	0.001	BG398414	Replication protein A1, 70 kDa
	201540_at	*FHL1*	–4.94	–0.578	0.001	NM_001449	Four and a half LIM domains 1
	212983_at	*HRAS*	–2.09	–0.577	0.001	NM_005343	v-Ha-ras Harvey rat sarcoma viral oncogene homolog isofrom 1

doi: 10.1111/j.1349-7006.2006.00204.x

Table 2. *Continued.*

Drug	Index[†]	Gene name	Slope[‡]	$r^{§}$	*P*-value	GenBank no.	Product
	219553_at	*NME7*	−1.94	−0.580	0.001	NM_013330	Nucleoside-diphosphate kinase 7 isoform a
	213330_s_at	*STIP1*	−1.64	−0.576	0.001	BE886580	Stress-induced-phosphoprotein 1
	201289_at	*CYR61*	−5.85	−0.572	0.001	NM_001554	Cysteine-rich, angiogenic inducer, 61
	203953_s_at	*CLDN3*	9.18	0.572	0.001	BE791251	Claudin 3
Capecitabine	201481_s_at	*PYGB*	7.79	0.742	<0.001	NM_002862	Brain glycogen phosphorylase
	218059_at	*LOC51123*	1.71	0.660	<0.001	NM_016096	HSPC038 protein
	218854_at	*SART2*	−4.56	−0.696	<0.001	NM_013352	Squamous cell carcinoma antigen recognized by T cells 2
	201528_at	*RPA1*	−2.01	−0.641	<0.001	BG398414	Replication protein A1, 70 kDa
	200989_at	*HIF1A*	−1.68	−0.637	<0.001	NM_001530	Hypoxia-inducible factor 1, α subunit isoform 1
	203586_s_at	*ARF4L*	−1.80	−0.632	<0.001	NM_001661	ADP-ribosylation factor 4-like
	204073_s_at	*C11orf9*	3.24	0.618	<0.001	NM_013279	Chromosome 11 open reading frame 9
	205395_s_at	*MRE11A*	−2.93	−0.620	<0.001	NM_005590	Meiotic recombination 11 homolog A isoform 2
	204201_s_at	*PTPN13*	−3.38	−0.605	<0.001	NM_006264	Protein tyrosine phosphatase, non-receptor type 13 isoform 2
	209620_s_at	*ABCB7*	−2.09	−0.605	<0.001	AB005289	ATP-binding cassette, subfamily B (MDR/TAP), member 7
	222065_s_at	*FLII*	−1.60	−0.606	<0.001	AI830227	Flightless I homolog (*Drosophila*)
	213330_s_at	*STIP1*	−1.62	−0.590	0.001	BE886580	Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)
	214600_at	*TEAD1*	−2.12	−0.590	0.001	AW771935	TEA domain family member 1 (SV40 transcriptional enhancer factor)
	217848_s_at	*PP*	1.96	0.592	0.001	NM_021129	Inorganic pyrophosphatase
	32837_at	*AGPAT2*	1.96	0.589	0.001	U56418	Lysophosphatidic acid acyltransferase-beta
	204351_at	*S100P*	8.87	0.581	0.001	NM_005980	S100 calcium binding protein P
	205403_at	*IL1R2*	6.30	0.579	0.001	NM_004633	Interleukin 1 receptor, type II precursor
	209160_at	*c-hluPGFS*	7.40	0.578	0.001	AB018580	HluPGFS
	212983_at	*HRAS*	−2.02	−0.578	0.001	NM_005343	v-Ha-ras Harvey rat sarcoma viral oncogene homolog isofrom 1
	36742_at	*ZNFB7*	2.67	0.577	0.001	U34249	Zinc finger protein
CDDP	208012_x_at	*SP110*	−2.77	−0.662	<0.001	NM_004509	SP110 nuclear body protein isoform a
	201278_at	*DAB2*	−2.45	−0.636	<0.001	N21202	Disabled homolog 2, mitogen-responsive phosphoprotein
	218070_s_at	*GMPPA*	−1.66	−0.593	0.001	NM_013335	GDP-mannose pyrophosphorylase A
	201661_s_at	*ACSL3*	−1.51	−0.592	0.001	NM_004457	Acyl-CoA synthetase long-chain family member 3
	203423_at	*RBP1*	4.07	0.584	0.001	NM_002899	Retinol binding protein 1, cellular
	202659_at	*PSMB10*	−3.07	−0.581	0.001	NM_002801	Proteasome beta 10 subunit proprotein
	211429_s_at	*MYCPBP*	−7.04	−0.577	0.001	AF119873	PRO2275
	217844_at	*CTDSP1*	−1.74	−0.575	0.001	NM_021198	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1
	201482_at	*QSCN6*	−3.49	−0.569	0.001	NM_002826	quiescin Q6 isoform a
	202100_at	*RALB*	−2.02	−0.561	0.001	BG169673	*v-ral* simian leukemia viral oncogene homolog B
	203228_at	*PAFAH1B3*	1.92	0.558	0.001	NM_002573	Platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29 kDa
	204306_s_at	*CD151*	−1.94	−0.556	0.001	NM_004357	CD151 antigen
	209761_s_at	*SP110*	−1.91	−0.555	0.001	AA969194	SP110 nuclear body protein
	203964_at	*NMI*	−2.38	−0.544	0.002	NM_004688	*N-myc* and STAT interactor
	1729_at	*TRADD*	−1.68	−0.533	0.002	NM_003789	Tumor necrosis factor receptor type 1 associated protein
	202733_at	*P4HA2*	−1.58	−0.533	0.002	NM_004199	Procollagen-proline, 2-oxoglutarate 4-dioxygenase,alpha polypeptide II
	204001_at	*SNAPC3*	1.52	0.531	0.003	NM_003084	Small nuclear RNA activating complex, polypeptide 3, 50 kDa
	221523_s_at	*RAGD*	2.92	0.519	0.003	AL138717	Ras-related GTP binding D
	201887_at	*IL13RA1*	−2.13	−0.515	0.004	NM_001560	Interleukin 13 receptor, alpha 1 precursor
	202863_at	*SP100*	−2.50	−0.514	0.004	NM_003113	Nuclear antigen Sp100
CPT11	202870_s_at	*CDC20*	2.08	0.694	<0.001	NM_001255	Cell division cycle 20
	203832_at	*SNRPF*	2.26	0.657	<0.001	NM_003095	Small nuclear ribonucleoprotein polypeptide F
	205085_at	*ORC1L*	1.64	0.751	<0.001	NM_004153	Origin recognition complex, subunit 1
	205412_at	*ACAT1*	3.01	0.730	<0.001	NM_000019	Acetyl-coenzyme A acetyltransferase 1 precursor
	206653_at	*POLR3G*	2.60	0.664	<0.001	BF062139	Polymerase (RNA) III (DNA directed) polypeptide G (32 kDa)
	208967_s_at	*adk2*	1.60	0.723	<0.001	U39945	Adenylate kinase 2
	210567_s_at	*SKP2*	2.13	0.661	<0.001	BC001441	S-phase kinase-associated protein 2, isoform 2
	212136_at	*ATP2B4*	−3.26	−0.744	<0.001	AW517686	ATPase, Ca++ transporting, plasma membrane 4

Table 2. *Continued.*

Drug	Index†	Gene name	Slope‡	*r*§	*P*-value	GenBank no.	Product
	214177_s_at	*PBXIP1*	–1.68	–0.714	<0.001	AI935162	Pre-B-cell leukemia transcription factor interacting protein 1
	215127_s_at	*RBMS1*	–2.43	–0.663	<0.001	AL517946	RNA binding motif, single stranded interacting protein 1
	217988_at	*CCNB1IP1*	2.50	0.680	<0.001	NM_021178	Cyclin B1 interacting protein 1 isoform a
	220892_s_at	*PSAT1*	3.14	0.697	<0.001	NM_021154	Phosphoserine aminotransferase isoform 2
	204127_at	*RFC3*	1.62	0.640	<0.001	BC000149	Replication factor C 3, isoform 1
	205909_at	*POLE2*	1.64	0.640	<0.001	NM_002692	DNA polymerase epsilon subunit 2
	200078_s_at	*ATP6V0B*	1.86	0.634	<0.001	BC005876	ATPase, H+ transporting, lysosomal 21 kDa, V0 subunit c″
	202705_at	*CCNB2*	1.55	0.622	<0.001	NM_004701	Cyclin B2
	204244_s_at	*ASK*	1.80	0.624	<0.001	NM_006716	Activator of S phase kinase
	204559_s_at	*LSM7*	1.91	0.632	<0.001	NM_016199	U6 snRNA-associated Sm-like protein LSm7
	206752_s_at	*DFFB*	2.58	0.631	<0.001	NM_004402	DNA fragmentation factor, 40 kDa, beta polypeptide isoform 1
	216321_s_at	*NR3C1*	–6.46	–0.624	<0.001	X03348	β-Glucocorticoid receptor
Paclitaxel	201272_at	*AKR1B1*	3.88	0.581	0.001	NM_001628	Aldo-keto reductase family 1, member B1
	205659_at	*HDAC9*	3.14	0.577	0.001	NM_014707	Histone deacetylase 9 isoform 3
	204867_at	*GCHFR*	–2.41	–0.566	0.001	NM_005258	GTP cyclohydrolase I feedback regulatory protein
	206247_at	*MICB*	2.72	0.532	0.003	NM_005931	MHC class I polypeptide-related sequence B
	204981_at	*SLC22A18*	–2.99	–0.530	0.003	NM_002555	Tumor suppressing subtransferable candidate 5
	201012_at	*ANXA1*	3.48	0.516	0.004	NM_000700	Annexin I
	201564_s_at	*FSCN1*	3.95	0.517	0.004	NM_003088	Fascin 1
	207717_s_at	*PKP2*	–2.32	–0.515	0.004	NM_004572	Plakophilin 2 isoform 2b
	210264_at	*GPR35*	–1.98	–0.515	0.004	AF089087	G protein-coupled receptor
	202722_s_at	*GFPT1*	–1.75	–0.514	0.004	NM_002056	Glucosamine-fructose-6-phosphate aminotransferase
	205443_at	*SNAPC1*	1.79	0.512	0.004	NM_003082	Small nuclear RNA activating complex, polypeptide 1, 43 kDa
	201746_at	*TP53*	–1.67	–0.506	0.004	NM_000546	Tumor protein p53
	204527_at	*MYO5A*	1.97	0.504	0.005	NM_000259	Myosin VA (heavy polypeptide 12, myoxin)
	201540_at	*FHL1*	3.43	0.503	0.005	NM_001449	Four and a half LIM domains 1
	203423_at	*RBP1*	2.66	0.498	0.005	NM_002899	Retinol binding protein 1, cellular
	204404_at	*SLC12A2*	–2.63	–0.497	0.005	NM_001046	Solute carrier family 12, member 2
	213757_at	*EIF5A*	2.92	0.492	0.006	AA393940	Eukaryotic translation initiation factor 5 A
	209016_s_at	*KRT7*	4.56	0.488	0.006	BC002700	Keratin 7
	203953_s_at	*CLDN3*	–6.15	–0.480	0.007	BE791251	Claudin 3
	35148_at	*TJP3*	–2.87	–0.478	0.008	AC005954	Tight junction protein 3 (zona occludens 3)

†Index: Affimetrix probe set ID, a unique identifier that can be used for GenBank accession numbers and consensus gene sequences. ‡Slope: slope of the regression line between gene expression level and drug sensitivity. §*r*: Pearson's correlation coefficient. CDDP, cisplatin; CPT-11, irinotecan hydrochloride; 5′-DFUR, 5′-deoxy-5-fluorouridine; S-1, 1 M tegafur–0.4 M 5-chloro-2,4-dihydroxypyridine–1 M potassium oxonate; UFT, 1 M tegafur–4 M uracil.

Validation of genechip experiments using real-time RT-PCR

To verify the genechip expression data more quantitatively, we carried out real-time RT-PCR using the same RNA as that used in the genechip analysis. To validate the genechip data, we verified the mRNA level of topic genes mainly associated with pyrimidine metabolism, folate metabolism, and some genes selected by the correlation analysis carried out in this study (*CYR61*, *MYB*). As shown in Table 3, although the expression data for most of the genes (57 out of 65 genes) that we examined by real-time RT-PCR were significantly correlated with the expression data obtained by the genechip analysis, the data for eight genes were not significantly correlated. Next, we checked the number of 'detection calls' in the genechip data in all of the genes whose expression was verified by real-time RT-PCR. Six of seven genes (*AK5*, *ENTPD1*, *FOLR2*, *TNSF6*, *GPR44* and *MTHFR*) whose expression was not correlated had less than 1 or 0 'present' calls. This result indicates that the expression of these genes may be difficult to detect using the genechip system. The remaining gene (*UMPS*) had a 'present' call in each of the xenografts. Although the reason for the discrepancy in the expression data is uncertain, the observation that the coefficient of variance of *UMPS* among 30 xenografts was the lowest among all of the genes examined by real-time RT-PCR may be related to the discrepancy. Overall, these results indicate that the majority of the gene expression data obtained using the genechip system was reliable.

Correlation between gene expression level and enzymatic activity

Among the genes that were screened in the global gene expression analysis, we focused on *DPD* and *TS* because these genes are associated with the molecular mechanism of 5-FU and many reports have examined the relationship between the expression of these genes and the antitumor activity of fluoropyrimidines. We examined the enzymatic activity of *DPD* and the protein level of *TS* in 30 xenografts. As shown in Fig. 4, the *DPD* mRNA level was significantly correlated with activity. As for *TS*, a positive

doi: 10.1111/j.1349-7006.2006.00204.x

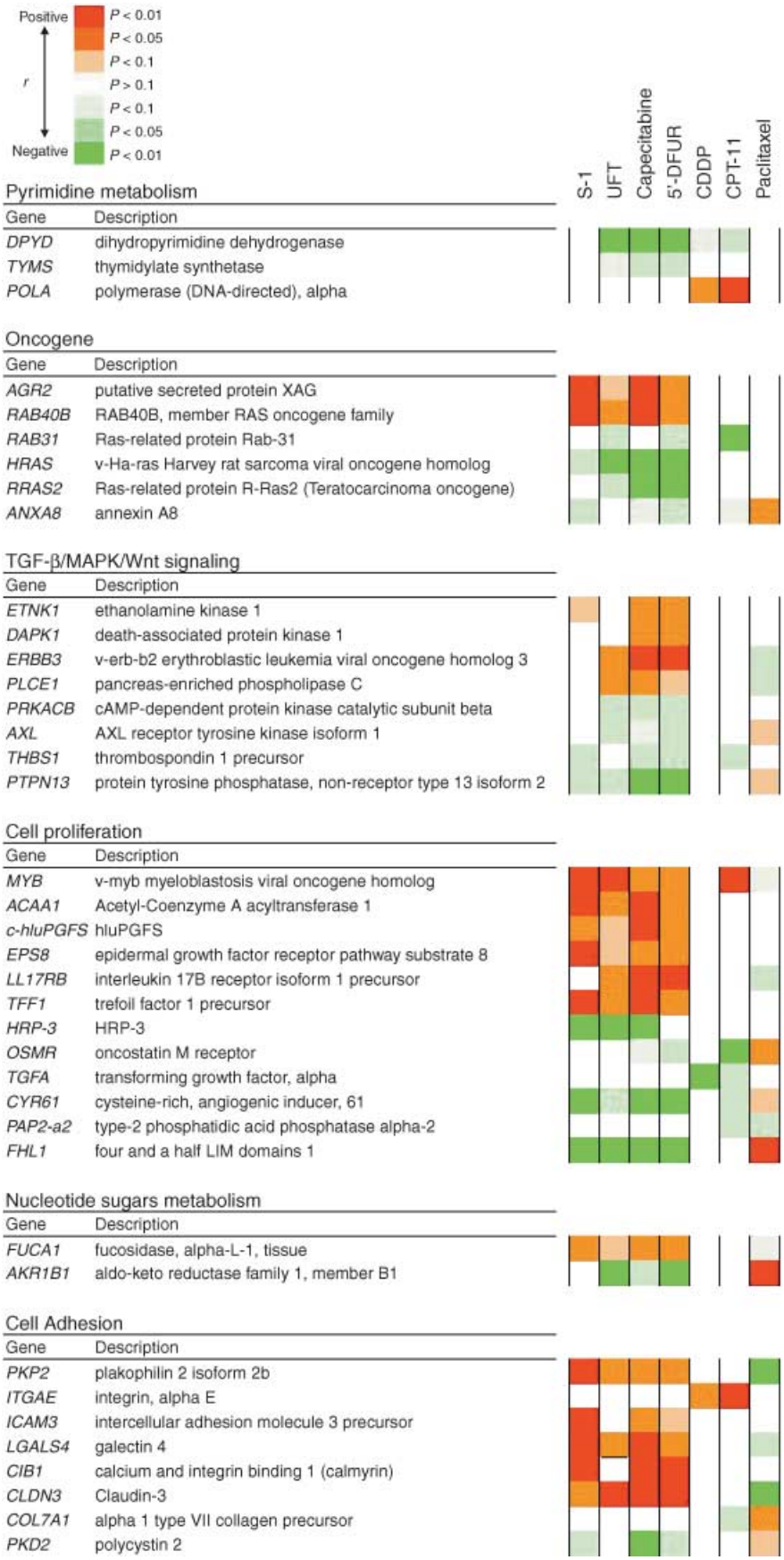

Fig. 2. Correlation between drug sensitivity and the expression profiles of 39 genes that were significantly correlated with multidrug sensitivity, and classified into key pathways. *Red*, a positive correlation; *green*, a negative correlation.

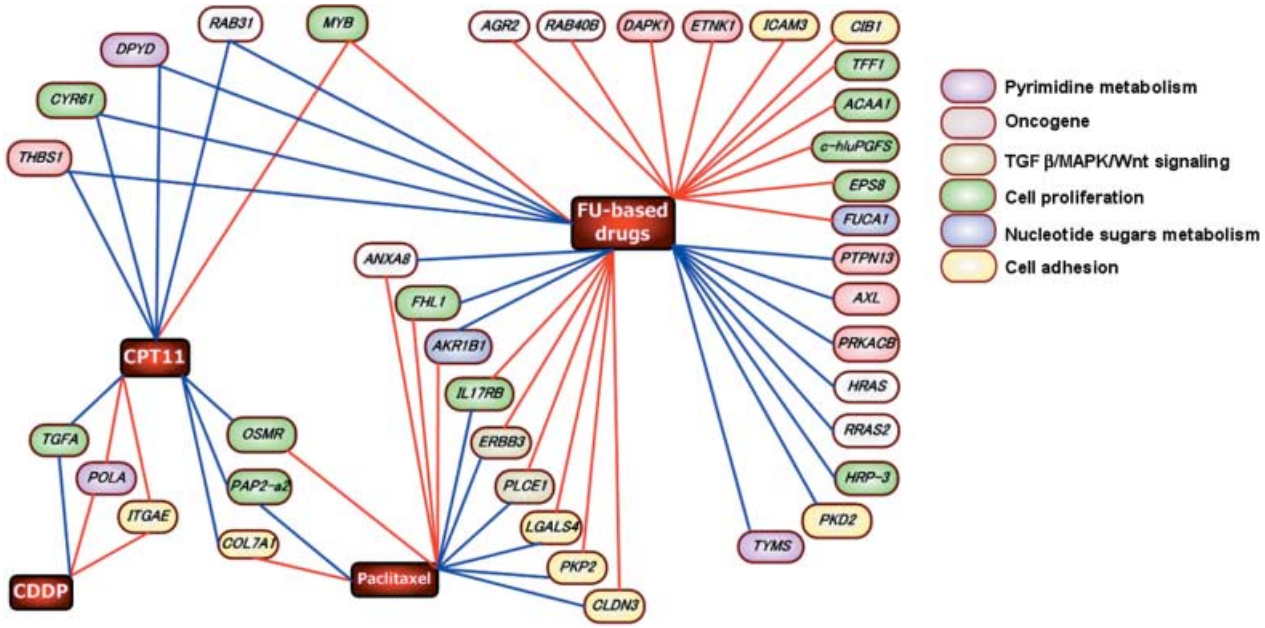

Fig. 3. The association between gene expression profiles correlated with antitumor acticity of multiple drugs. The nodes represent genes whose expression profiles were significantly correlated with drug sensitivity. The *red* connecting lines indicate a positive correlation, and the *blue* connecting lines indicate a negative correlation. The nodes between genes and FU-based drugs means whose expression profile were correlated with sensitivity to more than two fluoropyrimidine drugs. The color of each node represents the ontology or pathway based on the KEGG and GOC. Detailed information on the selected ge is presented in Fig. 2.

correlation was also confirmed between the level of mRNA and protein.

Discussion

Microarray technology has enabled us to determine the expression levels of thousands of genes in a single experiment. This technology is a very powerful tool for screening new target genes that have not been previously reported to be associated with drug resistance,[32,33] or for investigating the global expression patterns of various tissues or cell lines. This technology has been successfully applied to shed light into complex phenomena, such as invasion and metastasis,[34,35] and has provided novel insights into the mechanism of drug resistance and sensitivity.[36]

One of our goals is to establish a method for predicting the chemosensitivity of various types of tumors. In the present study, we carried out gene expression analysis to screen for genes whose expression profiles were significantly correlated with the sensitivity of 30 human tumor xenografts to seven anticancer drugs. There are some disadvantages to in evaluating antitumor activities using nude mouse models. One is that the characteristics of tumors in nude mouse transplants may not reflect the original tissues because the tumors are grown subcutaneously in nude mouse that may differ from the original environment. This may lead to some concern regarding the compatibility of the transplants to the parent tumor. However, this may not be such a concern in our study as we were investigating the correlation between gene expression in the tumors and chemosensitivity. There are possible differences in drug metabolism among species. The metabolic enzymes against each drug are unclear in the mouse model. However, *cyp2a5* is the mouse homolog of human *CYP2A6*, the main enzyme responsible for the metabolism of tegafur, and these enzymes are highly expressed in the liver. We demonstrated previously that FT is metabolized to 5-FU when it is incubated in mouse liver microsomes (Nagayama *et al*., unpublished data), and these data suggest the possibility that tegafur is metabolized by *cyp2a5* in mouse liver. For other drugs we have no idea about the mouse internal systems. Even so, there are enormous differences in drug metabolism ability between each mouse, as is the case in humans. Drug delivery and metabolism are vastly subject to the effects of various factors, including sex, age and polymorphisms. However, the antitumor effect of each drug may reflect different gene expression profiles in each tumor as it could be assumed that drug metabolism capacity is approximately uniform in each mouse model. Furthermore, it was reported that antitumor activity in human tumor xenograft models tends to coincide well with clinical effects, although drug metabolism in the mouse may be different from that in humans.[37] We therefore assume chemosensitivities in xenografts can be correlated with clinical effects.

At first, we focused on the drug sensitivity profiles of the 30 xenografts. A clustering analysis based on drug sensitivity revealed that the 5-FU-based drugs could be divided into two clusters: DIF and non-DIF drugs (Fig. 1). In addition to the DPD inhibitory effect, DIF drugs differ from capecitabine and 5′-DFUR in terms of the mechanism of 5-FU activation. Whereas capecitabine and 5′-DFUR are converted to 5-FU by an enzyme (TP), DIF drugs are activated to 5-FU by CYP2A6

doi: 10.1111/j.1349-7006.2006.00204.x

Table 3. Validation of gene expression data obtained using the genechip system

Gene	No. 'present'	$r^{\dagger}$	*P*-value
ABCC1	30	0.727	<0.001***
NT5E	26	0.933	<0.001***
THBS1	22	0.917	<0.001***
PTGS2	17	0.909	<0.001***
MYB	20	0.887	<0.001***
UPP1	16	0.875	<0.001***
CDA	12	0.866	<0.001***
ABCC3	17	0.861	<0.001***
CYR61	20	0.859	<0.001***
CNN3	26	0.858	<0.001***
TP53	27	0.854	<0.001***
ABCC4	18	0.846	<0.001***
ECGF1	28	0.842	<0.001***
GSTP1	30	0.826	<0.001***
ABCC2	2	0.817	<0.001***
GSTT1	7	0.807	<0.001***
DPYD	21	0.795	<0.001***
GGH	30	0.763	<0.001***
BRCA1	26	0.750	<0.001***
TYMS	30	0.748	<0.001***
ATP7B	28	0.733	<0.001***
SLCO2B1	6	0.729	<0.001***
SLC19A3	2	0.687	<0.001***
RPLP0	30	0.678	<0.001***
MTHFD2	30	0.660	<0.001***
E2F1	30	0.658	<0.001***
POLA	24	0.654	<0.001***
PCNA	30	0.639	<0.001***
SHMT2	30	0.628	<0.001***
POLB	26	0.626	<0.001***
FOLR1	14	0.610	<0.001***
SHMT1	2	0.599	<0.001***
DCTD	30	0.591	<0.001***
UNG	30	0.584	<0.001***
DCK	30	0.563	0.001**
RRM1	30	0.559	0.001**
TOP2A	30	0.555	0.001**
VEGFB	19	0.550	0.002**
GCLC	30	0.539	0.002**
RRM2	30	0.519	0.003**
DUT	30	0.483	0.007**
MTR	29	0.476	0.008**
MFTC	30	0.466	0.009**
GART	30	0.462	0.010*
AMT	8	0.457	0.011*
NME1	30	0.457	0.011*
CAD	29	0.456	0.011*
DTYMK	24	0.452	0.012*
TOP1	30	0.450	0.012*
ERCC1	20	0.450	0.013*
ATIC	30	0.443	0.014*
LIG3	17	0.438	0.015*
ITPA	25	0.423	0.020*
DHFR	29	0.421	0.020*
CTPS	30	0.417	0.022*
CTPS2	30	0.413	0.023*
MTHFD1	30	0.394	0.031*
POLD1	22	0.329	0.076
AK5	1	0.314	0.091
ENTPD1	0	0.180	0.341
FOLR2	1	0.128	0.499
TNFSF6	1	0.128	0.500
GPR44	0	0.113	0.552
UMPS	30	0.078	0.680
MTHFR	1	−0.026	0.892

$^{\dagger}r$: Pearson's correlation coefficient. ***$P < 0.001$. **$P < 0.01$. * $P < 0.05$.

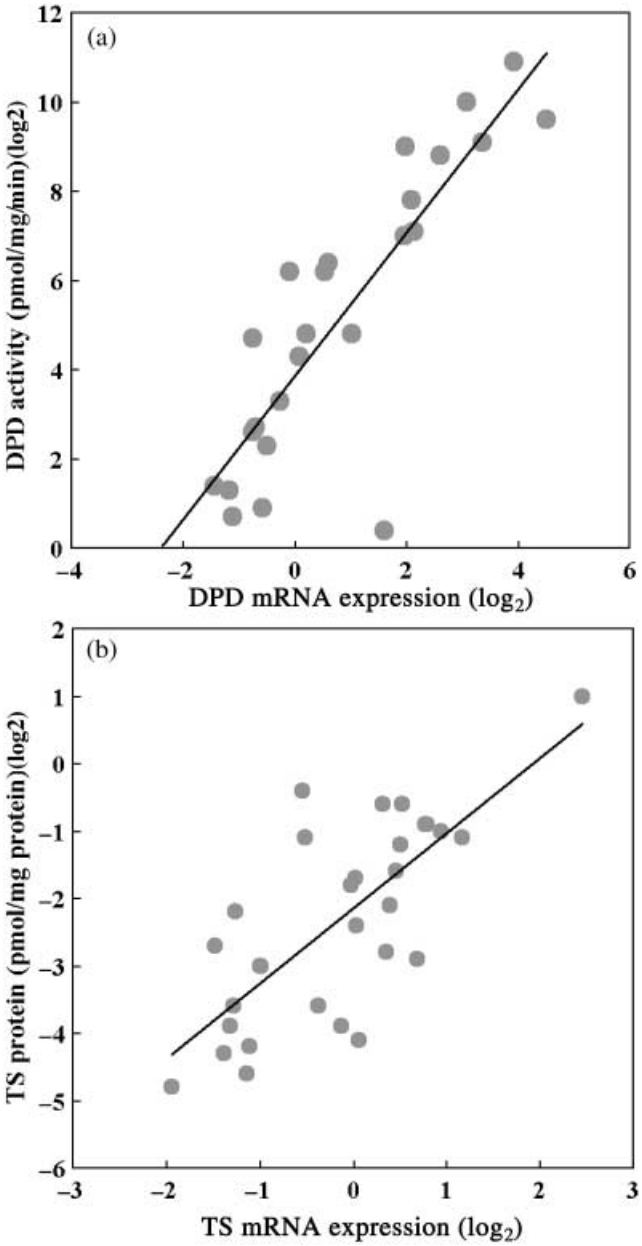

Fig. 4. Correlation between mRNA expression and protein level. Scatter plots of protein expression levels against mRNA expression levels obtained by GeneChip system. Each symbol represents a xenograft. The Pearson correlation coefficients (*r*) and *P* value (*P*) are shown.

in the liver. These differences may reflect differences in chemosensitivity profiles. In candidate genes, the DPD mRNA expression profiles of the tumors were negatively correlated with chemosensitivity to UFT, 5′-DFUR and capecitabine, except for S-1 (Fig. 2). In the present study, sensitivity to UFT was correlated with DPD regardless of DIF. This discrepancy was thought to originate from the difference in DPD inhibitory activity between uracil and gemeracil. Gemeracil, which is present in S-1, is 200-fold more potent as a DPD inhibitor than uracil.[38] The combination of 5-FU and gimeracil for the treatment of tumors with high

DPD has led to greater antitumor activity than treatment with 5-FU alone.[39] The superior antitumor activity of S-1 in tumors with high DPD activity has been reported *in vivo*,[40] and in a clinical study.[41]

In the present study, we also identified some genes that showed a significant correlation with sensitivity to a specific drug. We applied an ontological approach to further characterize these gene lists as it would be difficult to examine the biological function of all of the genes in the list, Finally, we identified 39 genes that were correlated with sensitivity to either two or six drugs; these associations seem to be more important than those of other genes that showed a correlation to a specific drug. As shown in Fig. 2, among the 39 genes, some have already been to shown to be associated with drug sensitivity. *TGF*α activates the epidermal growth factor receptor.[42] An antisense oligonucleotide against *TGF*α has been reported to enhance the effects of some anticancer drugs, including CDDP.[43] Our results suggest that *TGF*α may contribute to the drug resistance of CDDP and CPT-11. The expression of *TS* was associated with sensitivity to some fluoropyrimidines. TS is a key enzyme in the synthesis of DNA and is the target enzyme of 5-FU. The relationship between overexpression of *TS* and 5-FU resistance has been well characterized.[44] The Ras-related protein *RAB40B*, which is a member of the RAS oncogene family, showed a significant correlation with sensitivity to all fluoropyrimidines. In agreement with our study, the expression of *RAB40B* was downregulated in 5-FU-resistant colorectal cell lines.[45] Caudin-3 (*CLDN3*) can mediate cell adhesion and play a major role in tight junction-specific obliteration of the intracellular surface. A low level of *CLDN3* was associated with poor patient outcome.[46] Galectin-4 (*LGALS4*) is an S-type lectin that is strongly underexpressed in colorectal cancer.[47] Expression of *LGALS4* was associated with multidrug sensitivities in a previous report.[19] The role of these genes in drug sensitivities should be clarified. In addition, some genes ontologically categorized as being involved in cell adhesion, cell proliferation, or Wnt signaling, such as death-associated protein kinase (*DAPK1*), v-erb-b2 erythroblastic leukemia vial oncogene (*ERBB3*), and intracellular adhesion molecule 3 (*ITGAE3*) may be a candidate target of the development of a new drug. *CYR61*, from the *CCN* gene family, is a secreted and matrix-associated protein, which is known as an angiogenic inducer that can promote tumor growth vascularization.[48] Its expression level is regulated by *HIF1A* under hypoxic conditions.[31] *CYR61* plays an important role in resistance to chemotherapeutic agent-induced apoptosis by a mechanism involving the activation of the integrin/NF-κB/XIAP signaling pathways.[49] It has also been suggested that the expression level of *CYR61* is associated with sensitivity to multiple drugs, including 5-FU.[20] In our study, the chemosensitivities of six drugs showed a significant association with the expression of *CYR61*. Furthermore, *HIF1A* expression exhibited a significant negative correlation with 5-FU-based drugs for S-1 ($r = 0.42$, $P = 0.021$), UFT ($r = 0.51$, $P = 0.004$), 5′-DFUR ($r = 0.59$, $P = 0.001$), and capecitabine ($r = 0.64$, $P < 0.001$). These results support the hypothesis that angiogenic factors might play an important role in resistance to fluoropyrimidines, suggesting that these genes might be useful as common predictive markers for sensitivity to 5-FU-based therapy. In addition, the development of drugs that disrupt the *HIF1A* pathway may lead to additional antitumor effects by targeting tumor-infiltrating stromal cells, including tumor-associated fibroblasts and endothelial cells. The fact that our analysis could pick up some genes that have been previously known to be related to drug sensitivity or resistance appears to support the validity of our results, and other genes, which have not been reported previously, may become novel target genes for therapeutic strategies. A validation study for some of these genes is ongoing.

Interestingly, some genes that showed a significant association with sensitivity to 5-FU-based drugs were inversely correlated with paclitaxel (Fig. 3). This result suggests that tumors resistant to 5-FU-based drugs may respond to paclitaxel therapy. In fact, the combination of S-1 and paclitaxel has been shown to have potent antitumor and antimetastatic effects on refractory human breast cancer.[23] This combination was also evaluated in a clinical trial and appeared to be tolerated well.[50]

Next, we carried out real-time RT-PCR for some of the genes and measured the TS and DPD protein levels to validate the expression data obtained by the genechip analysis. The expression data obtained using these two methods were closely correlated for most of the genes that were examined. As for these genes, we also confirmed strong correlations between protein level and mRNA levels. Collectively, these results indicate that the expression profiles obtained using the genechip system are reliable and underscore the importance of the identified genes in drug sensitivity. Our next step will be *in vivo* validation of the identified genes.

Most previous studies on drug resistance have focused on a limited number of genes with proven functional significance to specific drug sensitivity. Although an evaluation of the genome-wide gene expression profile may be necessary to identify novel targets, it is difficult to interpret the biological importance of all of the selected genes using various statistical methods alone. In a typical microarray experiment, we are also faced with an extreme multiple testing situation and the possibility of statistical errors. Therefore, we applied an ontological approach to a list of genes that were statistically associated with drug sensitivity to eliminate genes that were unlikely to be related to drug efficacy, despite their statistical significance. The combination of a global gene expression analysis and an ontology analysis provided useful information on possible new gene candidates involved in drug resistance. These results will provide comprehensive genetic information linked to drug sensitivity and serve as a foundation for subsequent functional studies. The results may also enhance the prediction of tumor response to anticancer drugs and contribute to the development of tailor-made chemotherapy. It has become apparent that tumor response to an anticancer drug cannot be predicted by a single factor and may be determined by a critical balance of various factors. In addition to gene expression profiling, a combination of various approaches, including analyses of polymorphisms, proteomes, metaboromes and genomics, may be applied to achieve a precise diagnosis of future cancer patients.

doi: 10.1111/j.1349-7006.2006.00204.x

References

1 Golub TR, Slonim DK, Tamayo P *et al*. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 1999; **286**: 531–7.
2 Golub TR. Genome-wide views of cancer. *N Engl J Med* 2001; **8**: 601–2.
3 Ulrich CM, Robien K, McLeod HL. Cancer pharmacogenetics: polymorphisms, pathways and beyond. *Nat Rev Cancer* 2003; **12**: 912–20.
4 Mayer F, Hartmann JT, von Pawel J *et al*. A phase I study of oral uracil–ftorafur plus folinic acid in combination with weekly paclitaxel in patients with solid tumors. *Ann Oncol* 2002; **5**: 755–9.
5 Takahashi N, Suzuki H, Iwabuchi S, Yamazaki Y, Yanaga K. Third-line chemotherapy with paclitaxel, irinotecan hydrochloride and cisplatin for recurrent gastric cancer: a case report. *Hepatogastroenterology* 2005; **61**: 326–8.
6 Iwase H, Shimada M, Tsuzuki T *et al*. A phase II multicentric trial of S-1 combined with 24 h infusion of cisplatin in patients with advanced gastric cancer. *Anticancer Res* 2005; **25**: 1297–301.
7 Schipper DL, Wagener DJ. Chemotherapy of gastric cancer. *Anticancer Drugs* 1996; **7**: 137–49.
8 Langenbach RJ, Danenberg PV, Heidelberger C. Thymidylate synthetase: mechanism of inhibition by 5-fluoro-2′-deoxyuridylate. *Biochem Biophys Res Commun* 1972; **6**: 1565–71.
9 Matsuoka H, Ueo H, Sugimachi K, Akiyoshi T. Preliminary evidence that incorporation of 5-fluorouracil into RNA correlates with antitumor response. *Cancer Invest* 1992; **4**: 265–9.
10 Diasio RB. Clinical implications of dihydropyrimidine dehydrogenase inhibition. *Oncology* 1999; **13**: 17–21.
11 Malet-Martino M, Martino R. Clinical studies of three oral prodrugs of 5-fluorouracil (capecitabine, UFT, S-1): a review. *Oncologist* 2002; **7**: 288–323.
12 Fukushima M, Shimamoto Y, Kato T *et al*. Anticancer activity and toxicity of S-1, an oral combination of tegafur and two biochemical modulators, compared with continuous i.v. infusion of 5-fluorouracil. *Anticancer Drugs* 1998; **9**: 817–23.
13 Shirasaka T, Taguchi T. Preclinical and clinical practice of S-1 in Japan. In: Rustum YH, ed. *Fluoropyrimidines in Cancer Therapy*. Totowa, NJ: Humana Press, 2002; 285–302.
14 Kato H, Ichinose Y, Ohta M *et al*. A randomized trial of adjuvant chemotherapy with uracil–tegafur for adenocarcinoma of the lung. *N Engl J Med* 2004; **350**: 1713–21.
15 Sakata Y, Ohtsu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T. Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1 M tegafur–0.4 M gimestat–1 M otastat potassium) in advanced gastric cancer patients. *Eur J Cancer* 1998; **34**: 1715–20.
16 Shirao K, Ohtsu A, Takada H *et al*. Phase II study of oral S-1 for treatment of metastatic colorectal carcinoma. *Cancer* 2004; **100**: 2355–61.
17 Saeki T, Takashima S, Sano M *et al*. A phase II study of S-1 in patients with metastatic breast cancer – a Japanese trial by the S-1 Cooperative Study Group, Breast Cancer Working Group. *Breast Cancer* 2004; **11**: 194–202.
18 Furuse K, Kawahara M, Hasegawa K *et al*. Early phase II study of S-1, a new oral fluoropyrimidine, for advanced non-small-cell lung cancer. *Int J Clin Oncol* 2001; **5**: 236–41.
19 Zembutsu H, Ohnishi Y, Tsunoda T *et al*. Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. *Cancer Res* 2002; **62**: 518–27.
20 Dan S, Tsunoda T, Kitahara O *et al*. An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* 2002; **62**: 1139–47.
21 Park JS, Young Yoon S, Kim JM, Yeom YI, Kim YS, Kim NS. Identification of novel genes associated with the response to 5-FU treatment in gastric cancer cell lines using a cDNA microarray. *Cancer Letter* 2004; **214**: 19–33.
22 Fukushima M, Fujioka A, Uchida J, Nakagawa F, Takechi T. Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistance to 5-fluorouracil (5-FU) in human cancer xenografts *in vivo*. *Eur J Cancer* 2001; **37**: 1681–7.
23 Nukatsuka M, Fujioka A, Nakagawa F *et al*. Antimetastatic and anticancer activity of S-1, a new oral dihydropyrimidine-dehydrogenase-inhibiting fluoropyrimidine, alone and in combination with paclitaxel in an orthotopically implanted human breast cancer model. *Int J Oncol* 2004; **25**: 1531–6.
24 Takechi T, Okabe H, Ikeda K *et al*. Correlations between antitumor activities of fluoropyrimidines and DPD activity in lung tumor xenografts. *Oncol Report* 2005; **14**: 33–9.
25 United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). *Br J Cancer* 1998; 77: 1–10.
26 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–8.
27 Takechi T, Okabe H, Fujioka A, Murakami Y, Fukushima M. Relationship between protein levels and gene expression of dihydropyrimidine dehydrogenase in human tumor cells during growth in culture and in nude mice. *Jpn J Cancer Res* 1998; **89**: 1144–53.
28 Ikenaka K, Shirasaka T, Kitano S, Fujii S. Effect of uracil on metabolism of 5-fluorouracil *in vitro*. *Gann* 1979; **70**: 353–9.
29 Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. *In vivo* kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res* 1982; **42**: 450–6.
30 Dan S, Shirakawa M, Mukai Y *et al*. Identification of candidate predictive markers of anticancer drug sensitivity using a panel of human cancer cell lines. *Cancer Sci* 2003; **94**: 1074–82.
31 Kunz M, Moeller S, Koczan D *et al*. Mechanisms of hypoxic gene regulation of angiogenesis factor Cyr61 in melanoma cells. *J Biol Chem* 2003; **278**: 45 651–60.
32 Scherf U, Ross DT, Waltham M *et al*. A gene expression database for the molecular pharmacology of cancer. *Nat Genet* 2000; **24**: 236–44.
33 Zhou Y, Gwadry FG, Reinhold WC. Transcriptional regulation of mitotic genes by camptothecin-induced DNA damage: microarray analysis of dose- and time-dependent effects. *Cancer Res* 2002; **62**: 1688–95.
34 Pusztai L, Sotiriou C, Buchholz TA. Molecular profiles of invasive mucinous and ductal carcinomas of the breast: a molecular case study. *Cancer Genet Cytogenet* 2003; **141**: 148–53.
35 De Lange R, Burtscher H, Jarsch M, Weidle UH. Identification of metastasis-associated genes by transcriptional profiling of metastatic versus non-metastatic colon cancer cell lines. *Anticancer Res* 2001; **21**: 2329–39.
36 Ross DT, Scherf U, Eisen MB *et al*. Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* 2000; **24**: 227–35.
37 Tashiro T, Inaba M, Kobayashi T *et al*. Responsiveness of human lung cancer/nude mouse to antitumor agents in a model using clinically equivalent doses. *Cancer Chemother Pharmacol* 1989; **24**: 187–92.
38 Tatsumi K, Fukushima M, Shirasaka T, Fujii S. Inhibitory effects of pyrimidine, barbituric acid and pyridine derivatives on 5-fluorouracil degradation in rat liver extracts. *Jpn J Cancer Res* 1987; **78**: 748–55.
39 Takechi T, Fujioka A, Matsushima E, Fukushima M. Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2,4-dihydroxypyridine (CDHP) in human tumour cells. *Eur J Cancer* 2002; **38**: 1271–7.
40 Fujiwara H, Terashima M, Irinoda T *et al*. Superior antitumor activity of S-1 in tumours with a high dihydropyrimidine dehydrogenase activity. *Eur J Cancer* 2003; **39**: 2387–94.
41 Usuki H, Ishimura K, Yachida S *et al*. Dihydropyrimidine dehydrogenase (DPD) activity in gastric cancer tissue and effect of DPD inhibitory fluoropyrimidines. *Gastric Cancer* 2003; **6**: 66–70.
42 Massague J. Transforming growth factor-α. A model for membrane-anchored growth factors. *J Biol Chem* 1990; **15**: 21 393–6.
43 De Luca A, Selvam MP, Sandomenico C *et al*. Anti-sense oligonucleotides directed against EGF-related growth factors enhance anti-proliferative effect of conventional anti-tumor drugs in human colon-cancer cells. *Int J Cancer* 1997; **73**: 277–82.
44 Johnston PG, Lenz HJ, Leichman CG. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 1995; **55**: 1407–12.
45 Mariadason JM, Arango D, Shi Q *et al*. Gene expression profiling-based prediction of response of colon carcinoma cells to 5-fluorouracil and camptothecin. *Cancer Res* 2003; **63**: 8791–812.
46 Heinzelmann-Schwarz VA, Gardiner-Garden M, Henshall SM *et al*. Overexpression of the cell adhesion molecules DDR1, Claudin 3, and Ep-CAM in metaplastic ovarian epithelium and ovarian cancer. *Clin Cancer Res* 2004; **10**: 4427–36.
47 Rechreche H, Mallo GV, Montalto G, Dagorn JC, Iovanna JL. Cloning and expression of the mRNA of human galectin-4, an S-type lectin

down-regulated in colorectal cancer. *Eur J Biochem* 1997; **248**: 225–30.
48 Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 1998; **95**: 6355–60.
49 Lin MT, Chang CC, Chen ST *et al*. Cyr61 expression confers resistance to apoptosis in breast cancer MCF-7 cells by a mechanism of NF-κB-dependent XIAP up-regulation. *J Biol Chem* 2004; **279**: 24 015–23.
50 Villalona-Calero MA, Weiss GR, Burris HA *et al*. Phase I and pharmacokinetic study of the oral fluoropyrimidine capecitabine in combination with paclitaxel in patients with advanced solid malignancies. *J Clin Oncol* 1999; **17**: 1915–25.

doi: 10.1111/j.1349-7006.2006.00204.x