Genome-wide cDNA Microarray Screening to Correlate Gene Expression Profiles with Sensitivity of 85 Human Cancer Xenografts to Anticancer Drugs

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ABSTRACT

One of the most critical issues to be solved in regard to cancer chemotherapy is the need to establish a method for predicting efficacy or toxicity of anticancer drugs for individual patients. To identify genes that might be associated with chemosensitivity, we used a cDNA microarray representing 23,040 genes to analyze expression profiles in a panel of 85 cancer xenografts derived from nine human organs. The xenografts, implanted into nude mice, were examined for sensitivity to nine anticancer drugs (5-fluorouracil, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride, adriamycin, vincristine, cyclophosphamide, cisplatin, mitomycin C, methotrexate, and vinblastine). Comparison of the gene expression profiles of the tumors with sensitivities to each drug identified 1,578 genes whose expression levels correlated significantly with chemosensitivity; 333 of those genes showed significant correlation with two or more drugs, and 32 correlated with six or seven drugs. These data should contribute useful information for identifying predictive markers for drug sensitivity that may eventually provide "personalized chemotherapy" for individual patients, as well as for development of novel drugs to overcome acquired resistance of tumor cells to chemical agents.

INTRODUCTION

The efficacy of anticancer drugs varies widely among individual patients. A large proportion of cancer patients suffer adverse effects of chemotherapy while showing no effective response in terms of tumor regression. No prediction of effectiveness before treatment can be done at present, with some exceptions, such as tamoxifen treatment for patients with estrogen receptor positive breast cancer. Numerous investigators have attempted to establish a diagnostic method for predicting chemosensitivity, and a few markers have been identified (1–4). However, properties of cancer cells are determined by complicated interactions among all gene products expressed in cancer cells, and it is certain that many proteins, including enzymes involved in apoptosis, DNA repair, and metabolism and detoxification of drugs, affect individual responses. Hence, to distinguish responders from nonresponders before starting treatment, i.e., to offer a "personalized" program of more effective chemotherapy and also to relieve patients from unnecessary side effects, a larger set of genes must be identified to serve as accurate predictive markers.

Development of cDNA microarray technology has facilitated analysis of genome-wide expression profiles that can generate a large body of information concerning genetic networks related to response to various drugs and to identify genes involved in pathological conditions. Thus, the cDNA microarray is a promising method for identifying genes associated with sensitivity of tumors to various anticancer drugs using amplified RNA extracted from a very small piece of sample (5, 6).

Using a cDNA microarray consisting of 23,040 genes, we obtained gene expression profiles of 85 cancer xenografts in mice that had been established from nine different human organs. Here we report identification of genes in these tumors that revealed significant associations with sensitivity to one or more of nine anticancer drugs examined.

MATERIALS AND METHODS

Xenografts. A total of 85 human cancer xenografts were transplanted to athymic BALB/c-nu/nu mice. The xenografts were maintained by serial s.c. transplantation of 2×2×2-mm fragments into the right subaxillary region (Clea Japan, Inc., Tokyo, Japan) approximately once a month.

Anticancer Drugs. VLB, VCR, and CPM (Shionogi & Co., Osaka, Japan); 5FU (Sigma Chemical Co., St. Louis, MO); MTX (Lederle Japan, Ltd., Tokyo, Japan); MMC and ADR (Kyowa Hakko Kogyo Co., Tokyo, Japan); Vinblastine (VBL; Bristol-Myers Research Institute, Ltd., Tokyo, Japan) was dissolved in sterile 0.85% NaCl just before use. DDP (Bristol-Myers Research Institute, Ltd., Tokyo, Japan) was dissolved in sterile 0.85% NaCl containing 1% mannitol. The maximum tolerated dose for each of these drugs was determined as described previously (7).

Examination of Xenografts for Chemosensitivity. Each anticancer drug was administered individually, at the maximum tolerated dose, to nude mice bearing human cancer xenografts. Treatment with a given drug commenced when the tumor volume reached 100–300 mm3. Drugs were administered by daily i.v. injection for 5 days in the case of 5FU and MTX or as a single administration for all other drugs. The average T/C (%) of six treated mice for each drug with respect to six untreated controls for each xenograft was measured on any given experimental day. Chemosensitivity was represented by a T/C (%) value on day 14, as shown in Fig. 1 (8).

Extraction and Purification of RNA and Preparation of Probes. Total RNA was extracted from each xenograft using TRIZOL reagent (Life Technologies, Inc.) and purified using an RNA Purification Kit (Amersham Pharmacia Biotech) according to the supplier’s protocols. Poly(A) RNAs from xenografts and a mixture of pooled mRNA from four normal human tissues (brain, lung, liver, and kidney) purchased from Clontech were amplified using Ampliscribe T7 Transcription Kits (Epigenome Technologies) and labeled with Cy5-dCTP and Cy3-dCTP, respectively, as described previously (9).

cDNA Microarrays. We established a "genome-wide" cDNA microarray system containing 23,040 cDNAs selected from the UniGene database of the National Center for Biotechnology Information. Fabrication of the microarray, hybridization, washing, and detection of signal intensities were described previously (9). To normalize the amount of mRNA between tumors and controls, the Cy5:Cy3 ratio for each gene’s expression was adjusted so that the averaged Cy5:Cy3 ratio of 52 housekeeping genes was 1. (9). We assigned a cutoff value, using a variance analysis, to each microarray slide. Genes whose Cy5 or Cy3 signal intensities were lower than the cutoff values were excluded.
from further investigation. We also excluded data from genes where the signal-to-noise ratio was less than 3.

Cluster Analysis of Expression Profiles. We carried out hierarchical clustering analysis for both genes and tumor samples using Web-available software ("Cluster" and "TreeView") written by M. Eisen. To obtain reproducible clusters, we selected only genes that passed a filter protocol designed to exclude: (a) genes where both Cy3 and Cy5 signal intensities were lower than the cutoff value; (b) genes where values were obtained in <50% of the samples tested; or (c) genes with SDs in observed values of <2. Before the clustering algorithm was applied, the fluorescence ratio for each spot was first log transformed, and then the data for each sample were centered to remove experimental biases.

Identification of Genes Associated with Anticancer Drugs. To estimate correlation between the expression ratio (log2 Cy5:Cy3) and sensitivity to each drug, we calculated a Pearson correlation coefficient by the following formula:

\[ r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2}(\sum (y_i - \bar{y})^2)} \]

where \(x_i\) represented the log expression ratio of gene \(x\) in xenograft \(i\), whereas \(y_i\) represented sensitivity (1-T/C) of xenograft \(i\) to drug \(y\). \(\bar{x}\) represented the mean of the log expression ratio of gene \(x\), and \(\bar{y}\) represented the mean sensitivity (1-T/C) of the drug. We selected genes showing significant correlation (\(P < 0.01\)) and the absolute value of the slope of the regression line >1.5, when the difference of the T/C values between the most and the least sensitive samples was fixed as one.

Table 1. Numbers of genes showing significant correlation with sensitivity to nine anticancer drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>All xenografts (85)</th>
<th>NSCLC (11)</th>
<th>Breast cancer (14)</th>
<th>Gastric cancer (13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU</td>
<td>240</td>
<td>184</td>
<td>69</td>
<td>72</td>
</tr>
<tr>
<td>ACNU</td>
<td>290</td>
<td>151</td>
<td>78</td>
<td>101</td>
</tr>
<tr>
<td>ADR</td>
<td>283</td>
<td>72</td>
<td>94</td>
<td>93</td>
</tr>
<tr>
<td>CPM</td>
<td>459</td>
<td>168</td>
<td>103</td>
<td>92</td>
</tr>
<tr>
<td>DDP</td>
<td>217</td>
<td>166</td>
<td>76</td>
<td>100</td>
</tr>
<tr>
<td>MMC</td>
<td>321</td>
<td>137</td>
<td>199</td>
<td>102</td>
</tr>
<tr>
<td>MTX</td>
<td>219</td>
<td>92</td>
<td>75</td>
<td>171</td>
</tr>
<tr>
<td>MTX</td>
<td>244</td>
<td>200</td>
<td>107</td>
<td>112</td>
</tr>
<tr>
<td>VLB</td>
<td>210</td>
<td>201</td>
<td>109</td>
<td>60</td>
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</tbody>
</table>

These genes were identified not only in 85 xenografts but also in xenografts from NSCLC, breast cancer, and gastric cancer in order to explore the genes related to tissue-dependent chemosensitivity. These genes satisfied the following criteria: \(P < 0.01\), and the absolute value of the slope of the regression line was larger than 1.5 between the 100-T/C range (see “Materials and Methods”).

**Fig. 1.** Nine anticancer drugs and respective sensitivities in each of the 85 xenografts, evaluated by T/C. Red, high sensitivity to the drug; green, resistance; gray, combinations that were not tested.
Drug Sensitivity Score. Using values of relative expression ratio of the genes associated with anticancer drugs (Table 1), we established the algorithm to calculate “drug sensitivity score.” We calculated the sum of $\log_2(Cy5:Cy3)$ of genes multiplied by the value of Pearson’s correlation coefficient for each of the 85 xenografts, as described previously (10).

RESULTS

Cluster Analysis of Gene Expression Profiles of 85 Xenografts. We subjected the expression profiles of all 85 xenografts to a hierarchical clustering analysis to investigate similarities among them. Reproducible clusters were obtained with 961 genes (see “Materials and Methods”); their expression patterns across the 85 xenografts are shown in Fig. 2a. The expression profiles of xenograft MC9, analyzed in duplicate independently, were clustered into the closest branch among the xenografts in the sample axis (Fig. 2b). Xenografts derived from glioblastoma, neuroblastoma, small-cell lung carcinoma, and choriocarcinoma were correctly categorized into single tissue-specific branches. However, xenografts established from carcinomas of the breast, colon, pancreas, stomach, and ovary, as well as non-small cell lung carcinomas, were not clustered into single branches, suggesting that those tumors had heterogeneous expression profiles that reflected wider differences in their histological and/or biological natures.
Identification of Genes Associated with Chemosensitivity. To identify genes having significant associations with efficacy of one or more of the nine anticancer drugs (5FU, ACNU, ADR, CPM, DDP, MMC, MTX, VCR, and VLB) examined in our nude mice system, we analyzed expression profiles of the genes filtered according to criteria described in “Materials and Methods.” Pearson correlation coefficients between the expression level of each filtered gene and chemosensitivity to each drug across the 85 cancer xenografts were calculated. As shown in Table 1, when 85 xenografts were analyzed together, a cluster of more than 200 genes appeared to show significant correlation with sensitivity to all nine drugs. Expression levels of 1578 genes were associated with sensitivity to at least one of the drugs we examined. When we analyzed the data separately on the basis of the specific organs from which the xenografts had originated, the statistical power to identify significant associations was lower because the data that discriminated between sensitive and resistant phenotypes were limited. However, because xenografts of NSCLC, breast cancer, and gastric cancer showed more variable responses to the panel of drugs than xenografts derived from other organs, we still obtained significant association data for xenografts derived from those three tissues. Although the number of genes selected was smaller, we found more significant correlation of expression levels of some genes with chemosensitivity in these three tissues than the correlation obtained by analysis using all 85 xenografts together, e.g., among the 1578 genes mentioned above, the correlation value between the expression level of GPX2 and sensitivity to CPM in the 11 xenografts from NSCLC (r = −0.94) was more significant than that of all 85 xenografts (r = −0.59; Fig. 3, a and b). Similarly, MAP2K3 revealed a stronger correlation between expression level and sensitivity to VCR in the 14 xenografts from breast cancer (r = −0.91, P < 0.001) than in all xenografts combined (r = −0.29, P < 0.01; Fig. 3, c and d). Therefore, these two genes seem to be closely involved in tissue-specific efficacy of CPM or VCR, especially as regards NSCLCs or breast cancers.

Identification of Genes Correlated with Two or More Anticancer Drugs. Table 2 summarizes data for 20 genes showing the most significant positive or negative Pearson correlation coefficients to each of the drugs and corresponding P values calculated on the basis of expression profile data from all 85 xenografts. Because the plasma concentrations of 5FU and MTX, both of which are metabolic antag-
<table>
<thead>
<tr>
<th>Drug</th>
<th>Unigene</th>
<th>r</th>
<th>slope</th>
<th>P value</th>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>SFU</td>
<td>Hs.169355</td>
<td>0.84</td>
<td>7.4</td>
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<td>ETS</td>
<td>expression profile for sensitivity to anticancer drugs</td>
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<td>Hs.120196</td>
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<td>3.4</td>
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<td>Hs.120350</td>
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<td>3.15</td>
<td>0.001</td>
<td>ADAMTS2</td>
<td>expression profile for sensitivity to anticancer drugs</td>
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<tr>
<td></td>
<td>Hs.128050</td>
<td>0.60</td>
<td>0.99</td>
<td>0.001</td>
<td>MAG2</td>
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<td></td>
<td>Hs.68658</td>
<td>0.57</td>
<td>-2.72</td>
<td>0.001</td>
<td>RP160981</td>
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<td>Hs.243218</td>
<td>0.57</td>
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<td>Hs.160869</td>
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<td>2.46</td>
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<td>Hs.111739</td>
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<td>Hs.197710</td>
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<td>Hs.72712</td>
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<td>Hs.127169</td>
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<td></td>
<td>Hs.90025</td>
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<td>-1.89</td>
<td>0.001</td>
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<td></td>
<td>Hs.79707</td>
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<td>0.30</td>
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<td></td>
<td>Hs.23229</td>
<td>0.02</td>
<td>0.77</td>
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<td>Hs.80825</td>
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<td>Hs.7567</td>
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<td>0.001</td>
<td>Hsp70</td>
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<td></td>
<td>Hs.104</td>
<td>0.02</td>
<td>-1.81</td>
<td>0.001</td>
<td>HGFAC</td>
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<td></td>
<td>Hs.49943</td>
<td>0.01</td>
<td>2.36</td>
<td>0.001</td>
<td>ETS</td>
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</tr>
</tbody>
</table>

### Table 2

Genes closely related with each drug, as identified among 85 xenografts; the 20 genes showing the highest positive or negative Pearson correlation coefficients are included.

EXPRESSION PROFILES FOR SENSITIVITY TO ANTICANCER DRUGS

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DISCUSSION

To identify molecular markers that might predict chemosensitivity of individual tumors, we analyzed gene expression profiles of 85 human cancer xenografts in nude mice after treating each mouse with one of nine anticancer drugs (7, 11–13). Disadvantages of the approach to chemosensitivity using an in vivo animal model are: (a) the internal system for drug metabolism and/or delivery in the mouse may be different from that of humans; therefore, drug efficacy in the model may not always correctly reflect what occurs in the human (14); and (b) because xenografts are grown s.c. in nude mice, the local environment is different from that in the original tissues or metastatic organs. Hence, some selective pressure may encourage a subpopulation of tumors. However, the latter problem seemed to be of minimal concern as long as we focused on expression levels of genes associated with differences in chemosensitivity. Regarding the former problem, it may be better to analyze clinical materials. However, drug-metabolizing or -transporting conditions can differ enormously among individual patients anyway, because of genetic variations, age, sex, and physical condition. Hence, although the metabolism and transport of anticancer drugs are not exactly the same in both species, in the murine model, one can investigate efficacy of a drug at similar concentrations in all of the mice examined. Furthermore, repeated and
reproducible experiments can be made with xenograft models, an impossibility in clinical cases; e.g., we can obtain information about the efficacy of a particular drug by comparing the same xenografted tumors with or without drug treatment. In clinical cases on the other hand, we have to speculate the efficacy of anticancer drugs on the basis of changes in tumor size, but that is sometimes very hard to judge because the growth rates of tumors vary significantly from one patient to another. Because accumulated evidence does suggest that chemosensitivities in xenografts tend to accord well with clinical results (7, 13), we believe that to obtain information regarding chemosensitivity vis-a-vis gene expression, an animal model has great advantages.

As shown in Fig. 2, xenografts derived from NSCLCs and gastric cancers were distributed in multiple branches. As to clinicopathological features, our 11 xenografts derived from non-small cell lung carcinomas consisted of seven large cell carcinomas, two squamous cell carcinomas (QG56 and Lu61), and two papillary adenocarcinomas (LC11 and LC17). When the dendrogram was constructed on the basis of gene expression, all xenografts derived from papillary adenocarcinomas and squamous cell carcinomas of the lung were clustered in the same branch (Fig. 2b), whereas four of the seven xenografts from large cell lung carcinomas were clustered in the same branch as non-small cell lung carcinomas, and the others were scattered. Likewise, xenografts derived from gastric cancers fell into six different branches, suggesting that the clinicopathological features of large cell lung cancer and gastric cancer are relatively heterogeneous (15, 16). Clustering analysis of tumor samples on the basis of gene expression might open the possibility of detailed diagnosis of individual cancers. Moreover, we can now recognize a novel type of classification based on gene expression by exploring a large number of cancerous tissues, and current methods of cancer therapy might be improved if this novel classification could be implemented (17).
**Fig. 4.** Panel of 333 genes commonly correlated with sensitivity or resistance to two or more anticancer drugs, not including 5FU and MTX. The number of genes and drugs that commonly correlate with each other are indicated. Green, a positive correlation (sensitive) between drug sensitivity and expression level of the gene; red, a negative correlation (resistance). Gradation of color patterns corresponded to the degree of correlation coefficient.

**Fig. 5.** Differences in the relationship between expression levels of TNFRSF14 and sensitivities to ACNU in all versus tissue-specific xenografts. In a, in all 85 xenografts, the correlation coefficient between expression level of TNFRSF14 and sensitivity of the tumor cells to ACNU (100-T/C) was weak ($r = -0.42$, $P < 0.001$). In b, in nine xenografts derived from NSCLCs, excluding two samples whose values for this gene were below cutoff, the correlation between expression level of TNFRSF14 and sensitivity to ACNU was strong ($r = -0.87$, $P < 0.001$). In c and d, in nine xenografts from breast cancers and 13 from gastric cancers, the correlation between expression level of TNFRSF14 and sensitivity to ACNU was far weaker than that obtained in xenografts from NSCLCs (breast cancer: $r = 0.14$, $P > 0.1$; gastric cancer: $r = -0.15$, $P > 0.1$).
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ciated with sensitivity to anticancer drugs in 85 xenografts. Some of the genes listed in Table 2 have also been indicated their relations to chemosensitivity by others, supporting the reliability of our analysis, e.g., another study showed that expression levels of topoisomerase II-α, a molecular target of ADR, correlate positively with sensitivity to that drug (19, 20); our data also indicated a positive correlation (r = 0.44, P < 0.001) between expression of topoisomerase II-α and sensitivity to ADR among the 85 xenografts. Similarly, abundant expression of thymidylate synthetase, which inactivates 5FU to 5-fluoro-dUMP, was reported to correlate with resistance of colon cancer cells to 5FU (21). Our own data consistently revealed a negative correlation (r = −0.29, P < 0.01) between expression levels of thymidylate synthetase and sensitivity to 5FU. In addition, aldehyde dehydrogenase 1, which is involved in the resistance of tumor cells to CPM, was negatively correlated with sensitivity to CPM (r = −0.5, P < 0.001) in accord with a previous report (22). We also identified genes that were associated with chemosensitivity in cancers of specific organs (Table 1). Although those correlations were not conclusive because the number of samples used for each analysis was small, expression of the genes involved may reflect differences related to tissue specificity. This observation should be verified in a larger panel of xenografts.

Among the genes showing correlation with two or more anticancer drugs (Fig. 4), 32 were correlated with sensitivity to either six or seven drugs; such multiple associations are likely to be more meaningful than those of other genes that showed correlation to one to five drugs, and these 32 genes or their products could be more useful molecules for diagnosis of chemosensitivity, as well as good targets for development of novel drugs to overcome acquired resistance of tumor cells to chemical agents. Of the 32 genes on the list, some have already been shown to be associated with chemosensitivity, e.g., major vault protein, a negative regulator for all of the drugs tested here except VLB, is essential for normal vault structure. In accord with our data, Kitazono et al. (23) demonstrated that major vault protein was involved in resistance to ADR, VCR, VP-16, Taxol, and gramicidin D; this protein appears to have an important role in the transport of ADR between nucleus and cytoplasm in the SW-620 human colon carcinoma cell line (23–25). CYP3A5, a member of the cytochrome P450 subfamily 3A (hemicholate monoxygenases), is involved in an NADPH-dependent electron transport pathway. The product of this gene oxidizes a variety of xenobiotics and is involved in their detoxification (26, 27); it also plays some role in the metabolism of CPT-11 (28). Hence, increased expression of CYP3A5 may enhance detoxification and inactivation of anticancer drugs and confer drug resistance on cancer cells. Regarding UGT1A1, its significance in the CPT-11 detoxification pathway is already well known (29). Although we have no data for CPT-11, our data support an important role for UGT1A1 in the metabolic pathway of other anticancer drugs. Galectin 4 (LGALS4) is involved in the assembly of adherent junctions and increases malignant potential in some cancers of the digestive system (30, 31). Expression of LGALS4 is more highly upregulated in metastatic foci than in corresponding primary tumors (31), although its role in chemosensitivity should be clarified. In addition, genes encoding cell-surface receptors or cell-adhesion molecules, such as TNFRSF14, laminin β 3, gap-junction protein, β 1 (GJB1), and CD74 antigen (CD74), may be good targets for development of novel drugs for overcoming drug resistance. It has been suggested that MDR proteins are associated with resistance to a wide variety of anticancer drugs (32). Our cDNA microarray includes seven genes belonging to the ATP binding cassette, subfamily B (MDR transporter associated with antigen processing). Among them, the expression level of MDR4 was significantly correlated to the sensitivity to 5FU and VLB (P < 0.01), that of MDR3 was correlated to the sensitivity to 5FU (P < 0.01), and also that of MDR9 was correlated to the sensitivity to MMC (P < 0.05).

Our experiments selected cyclin B1 and budding uninhibited by benzimazolide 1 β as two genes whose reduced expression is likely to induce chemoresistance in cancer cells. Expression of both genes is cell cycle dependent and detectable at the G2-M phase (33, 34). Because many anticancer drugs induce arrest at G2-M (35, 36), we suspect that abrogation of cell cycle arrest because of decreased expression of these two genes may allow cancer cells to survive the stress caused by anticancer drugs. The list of genes whose level of expression showed a significant relationship to sensitivity to two or more drugs also includes biologically and medically interesting ones. GPX2, which showed significant associations with five drugs in our experiments, has been reported to protect cancer cells from chlorambucil and melphalan (37). Genes associated with DNA repair, Mut-S homologue 6 and BRCA1-associated RING domain 1, or those involved in cell cycle regulation, cyclins D1 and E1, have also been associated with response to DNA damage or drug sensitivity (38, 39).

As shown in Fig. 6, we established the algorithm to calculate drug sensitivity score using the value of expression levels of the hundreds of sensitivity-related genes to each anticancer drug (Table 1). Certainly it is still uncertain whether our data exactly reflect clinical responsiveness of human cancer patients and whether all of the hundreds of genes listed in this study may in fact play significant roles in the chemosensitivity of cancer cells. However, the data presented here should provide an initial set of candidate genes that may be applicable to diagnosis of chemosensitivity. As the next step, we need to evaluate clinical materials to determine which of the selected genes are truly important and potentially useful for prediction of chemosensitivity. Using a small amount of biopsy materials, we assume to be able to further select a subset of genes from these candidate genes associated with chemosensitivity of in vivo animal model. This could lead to an appropriate treatment to each individual cancerous case. Clinical investigations might also define molecular targets for overcoming drug resistance in tumors. Accumulation of such data should eventually lead to “personalized chemotherapy” with more effective and less harmful anticancer drugs and improvements in therapeutic effectiveness if genes associated with drug resistance can be identified and modulated.

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