

Prediction of Sensitivity of Esophageal Tumors to Adjuvant Chemotherapy by cDNA Microarray Analysis of Gene-Expression Profiles¹

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ABSTRACT

We applied cDNA microarray analyses of 9216 genes to establish a genetic method for predicting the outcome of adjuvant chemotherapy to esophageal cancers. We analyzed expression profiles of 20 esophageal cancer tissues from patients who were treated with the same adjuvant chemotherapy after removal of tumor by operation, and we attempted to find genes associated with the duration of survival after surgery. By comparing expression profiles of those cancer tissues, we identified by statistical analysis 52 genes that were likely to be correlated with prognosis and possibly with sensitivity/resistance to the anticancer drugs. We also developed a drug response score based on the differential expression of these genes, and we found a significant correlation between the drug response score and individual patients' prognoses. Our results indicated that this scoring system, based on microarray analysis of selected genes, is likely to have great potential for predicting the prognosis of individual cancer patients with the adjuvant chemotherapy.

INTRODUCTION

In clinical trials, adjuvant and neoadjuvant chemotherapies for patients with SCCs⁴ of esophageal cancers have seemed to improve survival times (1, 2). However, a large number of patients in those trials suffered from severe side effects, and, in some cases, the drugs showed very poor or no effect on cancer cells anyway. Despite recent progress in understanding the molecular genetics and biology of cancer, the prediction of sensitivity to anticancer drugs and clinical outcomes of chemotherapy still remains very difficult. To avoid unnecessarily subjecting a patient to the side effects of anticancer drugs, it is a matter of urgency to establish a diagnostic method to determine the precise biological properties of each cancer and its sensitivity to chemotherapy. Investigators using various molecular approaches to this problem have suggested that genetic and epigenetic changes in tumor tissues might correlate with prognoses of cancer patients (3–5); however, only a few markers have yet proven useful for predicting sensitivity to chemotherapy. Because the properties of cancer cells can vary enormously from one patient to another, it is not possible to characterize individual tumors by means of a single, or even several, molecular markers. The properties of cancer cells are likely to reflect the functions of all gene products. Therefore, expression profiles of individual cancer cells may represent their biological properties and significant determinants for the patient's response to anticancer drugs. Hence, we hypothesized that identification of such

genes might permit us to establish a novel diagnostic approach for the personalized treatment of each cancer patient. The development of microarray technology has enabled us to analyze the expression of thousands of genes in a single experiment and holds great promise for overcoming the therapeutic difficulties that we have faced for many years (6–8). To investigate this new paradigm, we set up a cDNA microarray containing 9216 genes and examined the expression profiles of normal tissues *versus* esophageal cancer specimens from 26 patients who had received adjuvant chemotherapy after surgery. Statistical analysis of the expression profiles of these materials extracted 52 genes that may be correlated with patient prognosis (*i.e.*, may reflect drug sensitivity or effectiveness of chemotherapy). We report here a possible scoring system for predicting a patient's prognosis with adjuvant chemotherapy before treatment.

MATERIALS AND METHODS

Clinical Samples. Twenty-six cases operated upon at Kurume University, School of Medicine (Kurume, Japan) were treated with CDDP (70 mg/m²) and 5-FU (700 mg/m²) for 2 or 3 weeks (administered 5 days and discontinued 2 days/week) after removal of the tumor by operation. Comprehensive informed consent was obtained from each of the patients. All specimens had been snap-frozen in liquid nitrogen just after resection.

Construction and Analysis of cDNA Microarray. The fabrication of cDNA microarray slides have been described elsewhere (9, 10). A set of cDNA microarray slides containing a duplicate set of 9216 cDNA spots were used for each analysis of expression profiles to reduce the experimental fluctuation. Total RNA was extracted from the frozen samples using TRIzol reagent (Life Technologies, Inc., Rockville, MD) and digested with RNase-free DNase I (Nippon Gene Co., Tokyo, Japan) according to the recommendations of the manufacturers. T7-based RNA amplifications and preparations of cDNA probes were carried out, as described elsewhere, using 5 µg each of the total RNA (11, 12). Amplified RNA from cancer tissues (2.5 µg of each) was labeled with Cy5-dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden), and an equal amount of amplified RNA from a pool of total RNA of normal esophagus (Invitrogen, Carlsbad, CA) was labeled with Cy3-dCTP (Amersham Pharmacia Biotech). Hybridization, washing, and scanning were performed as described previously (9, 10). The intensity of each duplicated signal was evaluated photometrically using the ArrayVision computer program (Imaging Research, Inc., St. Catharines, Canada). To normalize the amount of mRNA of tumor and normal cells from each patient, the Cy5: Cy3 ratio of each gene expression was adjusted so that the averaged Cy5: Cy3 ratio of 60 housekeeping genes was 1.0. Subsequently, the duplicated spots on each slide were averaged (9, 10, 12). In addition, a cutoff value for each expression level was automatically calculated using a variance analysis, and data with low signal intensities were excluded from additional investigation.

Selection of Separating Genes. To select the genes that can contribute to separating the drug-sensitive group from the drug-nonsensitive group, we calculated for each gene the *U* values of the Mann-Whitney test, which measures a difference of distribution in groups 1 and 3. The distribution of the Cy5: Cy3 ratio of a gene was measured for the two groups. Mann-Whitney *U* value is defined as the number of combinations of overlapping patients between the two distributions. If the two groups are completely separated by the value of the Cy5: Cy3 ratio (an ideal gene), the *U* value becomes 0, because there is no overlap between these two groups according to the Cy5: Cy3 ratio of the gene. Therefore, the less the two groups overlap, the smaller the *U* value.

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⁴ The abbreviations used are: SCC, squamous cell carcinoma; CDDP, cisplatin; 5-FU, 5-fluorouracil; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; DRS, drug response score; MDR, multidrug resistance.

Table 1 Clinicopathological features of 26 esophageal cancer patients

ID no.	Gender	Age (yr)	Outcome ^a	Survival period (mo) ^b	TNM stage	Histopathological grading ^c	Residual tumor ^d	Lymph node dissection ^e	Classification ^f	DRS
A. Learning cases										
91-21	M	62	Survival	>98	IV	G2	R1	Three-field	Group 1	80
92-36	M	68	Survival	>82	IV	G3	R0	Three-field	Group 1	138
94-23	M	61	Survival	>62	IV	G1	R1	Three-field	Group 1	37
96-08	M	67	Survival	>40	III	G1	R0	Two-field	Group 1	251
97-06	F	71	Survival	>34	III	G3	R0	Two-field	Group 1	269
97-07	M	50	Survival	>34	IV	G1	R1	Three-field	Group 1	14
97-20	M	67	Survival	>30	IV	G2	R1	Three-field	Group 1	215
97-21	M	67	Survival	>30	IV	G2	R1	Three-field	Group 1	35
94-12	M	43	Deceased	24	IV	G2	R1	Three-field	Group 2	-195
98-09	M	55	Deceased	20	IV	G2	R1	Three-field	Group 2	77
91-35	M	58	Deceased	19	IV	G2	R1	Three-field	Group 2	-134
94-22	M	65	Deceased	18	IV	G3	R2	Three-field	Group 2	-306
91-25	M	60	Deceased	16	IV	G2	R1	Three-field	Group 2	16
91-41	M	55	Deceased	16	IV	G1	R1	Three-field	Group 2	-296
90-31	M	56	Deceased	12	IV	G2	R1	Three-field	Group 3	-343
98-07	M	63	Deceased	11	IV	G2	R1	Three-field	Group 3	-349
97-33	M	64	Deceased	9	III	G1	R0	Three-field	Group 3	-373
92-03	M	45	Deceased	7	IV	G1	R2	Three-field	Group 3	-345
92-30	M	64	Deceased	6	III	G2	R0	Three-field	Group 3	-444
91-26	M	60	Deceased	4	IV	G1	R1	Three-field	Group 3	-370
B. Test cases										
89-22	M	54	Survival	>122	III	G2	R1	Three-field	Test (Group 1)	217
98-21	M	57	Survival	>30	IV	G2	R1	Three-field	Test (Group 1)	143
91-07	M	60	Deceased	24	III	G3	R0	Three-field	Test (Group 2)	-48
95-37	M	69	Deceased	24	IV	G2	R2	Three-field	Test (Group 2)	83
1571	F	64	Deceased	28	IV	G3	R0	Three-field	Test (Group 2)	-228
1781	M	53	Deceased	16	III	G2	R0	Two-field	Test (Group 2)	-158

^a Sixteen patients were deceased of causes related to esophageal cancer.

^b Follow-up months were established as the time between surgery and either death or last follow up date (December 28, 1999).

^c G1, G2, and G3, well, moderately, or poorly differentiated SCC, respectively.

^d R0, no residual tumor; R1, microscopic residual tumor; R2, macroscopic residual tumor.

^e Operation procedure, total esophagectomy with regional lymphnode dissection.

^f Group 1, survived >30 mo; Group 2, deceased, 12–24 months; Group 3, deceased within 12 months.

We selected 52 genes that had $P < 0.1$ (U values of ≤ 11 for eight plus six learning samples) as biologically significant.

Calculation of DRS Using Separating Genes. The DRS of each patient is defined as the sum of the weighted log ratio of the gene expression profile:

$$X_i = 10 \sum_k S_k \log_2(r_{ik})$$

where r_{ik} is the expression ratio (Cy5: Cy3) of gene k of patient i , and S_k is the sign for each gene k , which is determined as follows. For each gene, we first calculated the average log ratio of each gene expression of patients within each group: $ave_{group A} = \sum_{i \text{ in group A}} \log_2(r_{ik})/n_A$ and $ave_{group B} = \sum_{i \text{ in group B}} \log_2(r_{ik})/n_B$. Then, we determined the sign for each gene: $S_k = +1$ if $ave_{group A} \geq ave_{group B}$, and $S_k = -1$ if $ave_{group A} < ave_{group B}$. Applying a set of S_k to the expression profiles of new patients, we can calculate the DRS for each patient.

Semiquantitative RT-PCR. A 2- μ g aliquot of total RNA from each tissue sample was reverse-transcribed for single-stranded cDNAs using oligo(dT)₁₂₋₁₈ primer and Superscript II (Life Technologies, Inc.). Each cDNA mixture was diluted for subsequent PCR amplification with the same primer sets that were prepared for construction of the target DNA- or *G3PDH*-specific primer set (5'-GACAACAGCCTCAAGATCATCA-3' and 5'-GGTCCACCACTGACACTGTG-3'). Expression of *G3PDH* served as an internal control. The PCR reactions were optimized for number of cycles to ensure product intensity within the linear phase of amplification.

RESULTS

Classification of Patients. Clinicopathological characteristics of 20 patients who underwent surgery for esophageal cancer at Kurume University School of Medicine between 1989 and 1998 are summarized in Table 1A. All cases were diagnosed as SCC, and all patients were treated according to the same adjuvant chemotherapy protocol: CDDP (70 mg/m²) and 5-FU (700 mg/m²) after removal of tumor. Microscopic or macroscopic residual tumors were confirmed in 15 of

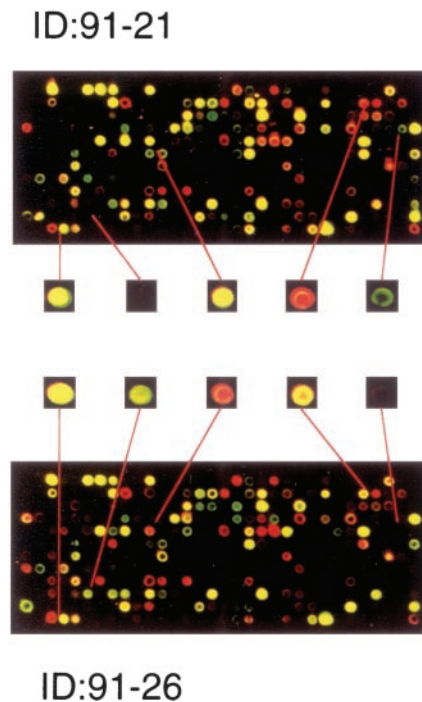


Fig. 1. Image of microarray. Example of the microarray images of the same area (including 384 genes) from two patients. Top, a patient who survived >98 months; bottom, one who died 4 months after surgery. Magnified spots, examples of genes that indicate different expression-changes between the patients.

Gene ID	Group1 (8 patients)	Group3 (6 patients)	Group2 (6 patients)	unseen (6 patients)	u-value	Accession / Hs. No	Definition	sign
B0647					11	Hs.116412	EST	
A8958					10	Hs.153260	C-Cbl-interacting protein	
B4008					3	Hs.66762	H4/j gene	
A6447					10	Hs.7857	Protein 4.1-G	
B6773					8	Hs.74566	Dihydropyrimidinase related protein-3	
B4413					6.5	Hs.15871	EST	
A9564					11	Hs.106070	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	
A4786					11	AB002386	KIAA0388	
B8795					11	Hs.72444	EST	
B0557					9	Hs.115880	EST	
A2744					3	U28963	Gps2 (GPS2)	
B7553					7	Hs.12866	EST	-1
A1731					11	U41635	OS-9 precursor	
A7244					10	Hs.102402	Mad4 homolog (Mad4)	
A9451					9.5	Hs.110309	Major histocompatibility complex, class I, F	
A8884					11	Hs.12151	EST	
B4895					11	Hs.3452	EST	
A0366					11	A29103	TNF-binding polypeptide	
A3349					7	M98343	Amplixin (EMS1)	
A2264					8	HSU41654	adenovirus protein E3-14.7k interacting protein 1 (FIP-1)	
B4272					10	Hs.8839	EST	
A0903					11	L37368	Human (clone E5.1) RNA-binding protein	
A5131					9	AF022654	Homeodomain protein (OG12)	
A2668					9	X54232	Heparan sulfate proteoglycan (glypican).	
A2746					9	U54777	hMSH6 protein (MSH6)	
A2324					6.5	M16650	Ornithine decarboxylase (ODC1)	
A4146					10	AA586974	EST	
B2430					6	Hs.118966	EST	
B7933					11	Hs.22599	KIAA0705	
B3985					8	Hs.63788	Propionyl Coenzyme A carboxylase, beta polypeptide	
A7265					7	Hs.102484	Glutathione S-transferase A3	
C0670					9	Hs.31655	EST	
A3828					9	AB006202	Cytochrome b small subunit of complex	
A1043					10	U09813	Mitochondrial ATP synthase subunit 9, P3 gene copy	
A1571					11	U75362	Isopeptidase T-3 (ISOT-3)	
A0649					11	AF053551	Metaxin 2 (MTX2)	
A2849					10	U91618	Human proneurotensin/proneuromedin N	1
A3709					11	U91985	DNA fragmentation factor-45	
A2378					10	X68836	S-adenosylmethionine synthetase.	
A3196					11	U57627	Fetal brain oculocerebrorenal syndrome (OCRL1)	
A1356					9	AF079564	Ubiquitin-specific protease UBP41 (UBP41)	
A0043					10	AF028840	Kruppel-associated box protein	
A1854					10	M31899	DNA repair helicase (ERCC3)	
A0510					10	U10689	MAGE-5a antigen (MAGE5a)	
A4009					9.5	D17793	KIAA0119	
A8952					11	Hs.109253	EST	
A1019					10	M29065	hnRNP A2 protein	
A9262					11	Hs.109059	Mrp17	
B0829					10	Hs.107884	EST	
A6528					9	Hs.8215	EST	
A6811					8	Hs.100734	EST	
B2316					10	Hs.117381	EST	

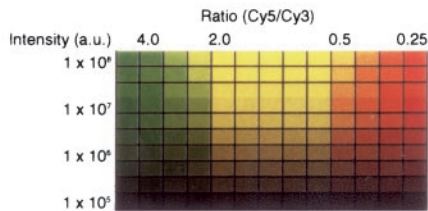
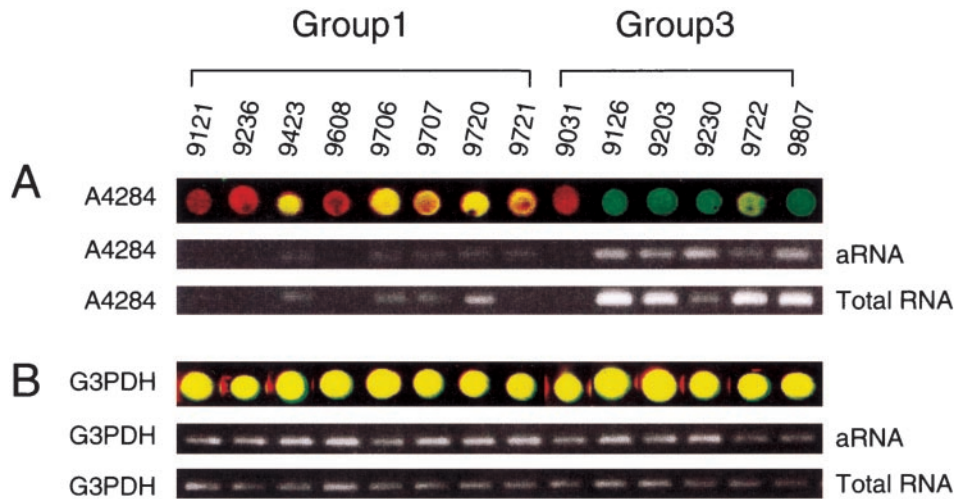


Fig. 2. Schema of 52 selected genes. The color scale was constructed from expression ratios (Cy5: Cy3) and the levels of signal intensity. The degree of contribution of each gene to distinguishing group 1 from group 3 is exhibited as a Mann-Whitney *U* value. Values ≤ 11 were considered to be adequate for the purposes of discrimination. The sign for each gene was determined according to the direction to separate the drug-sensitive group from the nonsensitive group (plus for contributing to longer survival and minus for contributing to shorter survival).

the patients. The 20 patients were classified into three groups on the basis of the duration of postoperative survival, a parameter that was likely to reflect responsiveness to the anticancer drugs: group 1, >30 months (8 cases); group 2, 13–30 months (6 cases); and group 3, ≤ 12 months (6 cases; Table 1A). The three groups showed no significant differences with respect to clinicopathological features.

Analysis of Expression Profiles. Although each of the 20 primary SCCs had originated from the same organ, the expression patterns of the genes analyzed in our microarray were enormously different (Fig. 1). We compared expression profiles of groups 1 and 3; patients in group 1 were considered to respond well to anticancer treatment, and those in group 3 responded very poorly. Furthermore, we analyzed the

Fig. 3. Confirmation of reliability of the microarray data. A, signals of A4284 in the microarray. Expression of A4284 in each tumor was examined by semiquantitative RT-PCR using cDNA prepared from amplified or preamplified total RNA. B, expression of G3PDH detected by the microarray and semiquantitative RT-PCR. Signals of the microarray were compatible with the results of semiquantitative RT-PCR experiments.



array results to identify genes that were commonly down-regulated or up-regulated in either group 1 or group 3. For example, the expression of B4008 (GenBank, accession no. Z80787) was significantly decreased in seven of eight tumors belonging to group 1 and unchanged in all six tumors in group 3 (Fig. 2). According to our selection algorithm, described in "Materials and Methods," a total of 52 genes were selected, on the basis of Mann-Whitney *U* values of ≤ 11 , as elements likely to be associated with patient survival. The expression pattern of all 52 genes according to their characteristics in each tumor is summarized in Fig. 2.

Evaluation of Microarray Data. To verify differences in expression indicated by microarray analysis, we performed semiquantitative RT-PCR using preamplified total RNAs as templates, and we confirmed that the results for the A4284 gene corresponded to those of the microarray analysis in 13 of 14 cases constituting groups 1 and 3 (Fig. 3). Comparing the ratios of the expression levels of the 17 randomly selected genes in normal *versus* cancerous materials from those 14 patients (a total of 238 examples), we found that the ratios were well maintained in the great majority of the tested cases (data not shown).

Calculation of DRS According to Expression Levels of Selected Genes. To investigate a possibility that the altered expression of the 52 genes might be clinically applicable for predicting drug sensitivity, we introduced a numerical scoring system, termed DRS, as described in "Materials and Methods." The calculated DRSs for each patient are summarized in Table 1A. Cancer tissues from group 1, the longer survivors, revealed high scores (14 to 269), and those from group 3, the shorter survivors, revealed low scores (-444 to -343), indicating that these scores correlate significantly with the outcome of each patient (Fig. 4). We applied this scoring system to the patients belonging to group 2 whose survival periods were intermediate (12–30 months) and calculated their DRSs. Although the scores of two (their survival durations were 20 and 16 months) of six patients overlapped with those in group 1, DRSs of groups 2 and 3 were well separated.

To evaluate this scoring system further, we obtained 6 additional cases that were completely independent from the 14 samples used for selection of the 52 genes, performed cDNA microarray analysis, and then calculated DRSs. Clinicopathological characteristics of these six patients are summarized in Table 1B. As shown in Table 1B and in Fig. 4, the DRSs of two patients whose survival periods were >122 and 30 months after operation were as high as 217 and 143, respectively; whereas, those of four patients whose survival periods were 28, 24, 24, and 16 months were -228, 83, -48, and -158, respectively. DRSs for these six patients correlate well to their prognoses.

DISCUSSION

The recent development of cDNA microarray or cDNA chip technology, a high-throughput method of monitoring gene expression, has made it possible to analyze the expression of thousands of genes at once (6, 13, 14, 15). Consequently, new classifications of cancer types can now be proposed on the basis of the altered expression of multiple genes in tumor tissues (8, 16). In addition, Scherf *et al.* (17) have reported evidence that gene expression profiles may reflect drug sensitivity of the cancer cells. In the study reported here, we analyzed clinical samples and indicated that the use of microarray may have a great potential to solve one of the most serious dilemmas that clinicians have faced in treatment of cancer patients: an inability to predict an individual patient's responsiveness to anticancer drugs.

All 26 esophageal cancer patients in our panel were at clinically advanced stages (Tumor-Node-Metastasis stages III–IV). Such patients usually have poor prognoses; only a small percentage achieves more than a 5-year survival after surgery. For the nine patients of our panel who survived for more than 30 months (group 1), we assume that postoperative anticancer treatment with CDDP and 5-FU was effective in killing residual cancer cells. Hence, we considered the

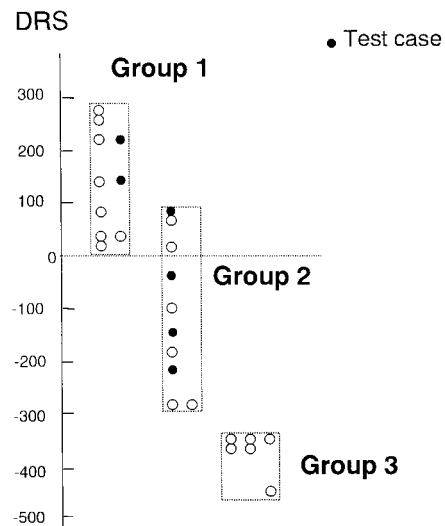


Fig. 4. Schematic distinction of groups 1–3 and verified cases on the basis of DRS. ○, esophageal cancer patients used to select genes for the establishment of DRS; ●, newly obtained patients used for validation of DRS.

nine patients (group 1) as drug-sensitive patients and the six with short survival (group 3) as nonsensitive patients. Notably, the patients analyzed were treated with anticancer drugs after the surgical resection; the survival period is the only parameter that could estimate drug sensitivity.

Our data from the patients analyzed in this study allowed us to identify not only consistent patterns of gene expression in esophageal cancers but also a set of genes discriminating the outcome of adjuvant chemotherapy after surgical treatment. We detected elevated expression of *c-erbB2* in 3 of 26 cases and that of *epidermal growth factor receptor*, in 8 of 26 (data not shown), which were almost similar to the data previously reported (18, 19). These data, together with the consistent results of semiquantitative RT-PCR, corroborated the reliability of our data.

The most advantageous point of the algorithm we have described for establishing the DRS is that, after selecting genes that may influence the drug response, the algorithm sums up the log ratios of genes, each of which is multiplied by the sign determined according to the contributing direction to the group separation. The selection of genes depends only on a difference in expression patterns between drug-sensitive and -nonsensitive individuals. However, this process only selects genes according to their individual behaviors in a given sample set. Because the actual mechanism that leads to each type of cancer implies a rather complicated network of genetic events, differences of phenotype depend not on single genes but on a global expression pattern. We tried several multivariate statistical analyses, e.g., linear discriminant analysis and qualification theory II, and found this simple method of summing up the log ratio of each gene with each sign separated more efficiently the drug-sensitive group from the drug-nonsensitive group. The ability to assess the effectiveness of anticancer drugs in resected cancer materials before treatment would avoid unnecessary treatment as well as the side effects that would add to the patient's suffering in the absence of any benefit.

It is apparent that survival periods reflect not only drug sensitivity but also the malignant properties of the tumor cells (the potentiality of invasion or metastasis), the histopathological grade of the tumors, the surgical procedures, and their curativity. However, because clinicopathological characteristics such as Tumor-Node-Metastasis stage, histopathological grade, lymph node dissection, and the condition of residual tumor in the group-1 and group-3 patients were similar, we assume that the former factors seem to contribute largely to the survival of the patients analyzed. Although it may be overstated that DRS predicts only the sensitivity to anticancer drugs, it is a clinically valuable predictive score reflecting the outcome of the adjuvant chemotherapy after surgical resection for patients with advanced esophageal cancer.

Our data from the patients analyzed in this study allowed us to identify consistent patterns of gene expression in esophageal cancers. Some of the 52 genes were indicated to be associated in some aspects with sensitivity to anticancer drugs or with esophageal carcinogenesis. For example, *glutathione S-transferase π* and S-adenosylmethionine were shown to be related to drug resistance (20, 21). Overexpression of heterogeneous nuclear ribonucleoprotein A2 in lung cancers was reported (22). *EMS1*, mapped at chromosome 11q13, was sometimes amplified in breast cancers (23). The activity of *ornithine decarboxylase*, a rate-limiting enzyme in the synthesis of polyamines, which are essential for cellular proliferation, was found to be elevated in colorectal tumors and polyps (24). The human multidrug resistance-associated protein family currently consists of seven members and has the ability to transport a wide range of anticancer drugs out of cells (25). Particularly, *MDR1/P-glycoprotein* expression was suggested to be a predictor of response and survival in advanced ovarian cancer patients (26). In our microarray experiment, significant reduction of

MDR1 expression was observed in 3 of 20 patients in groups 1 and 2, although down-regulation was observed in 0 of 6 patients belonging to group 3, suggesting that *MDR1* might affect the sensitivity of 5FU and/or CDDP in the adjuvant chemotherapy for esophageal cancers. However, this gene was not included in the 52 genes because the correlation between the expression and the sensitivity was moderate. The usefulness of the prediction of the outcome of adjuvant chemotherapy using the DRS system with the 52 genes selected from 9216 genes on our cDNA microarray raises a possibility that extended analyses of expression profiles with an increased number of genes using a larger number of samples will help in the development of a more accurate DRS system.

In conclusion, the strategy outlined here should shed light on freeing cancer patients from suffering the side effects of ineffective adjuvant chemotherapy, and, in the near future, would be useful for clinicians to select an optimal, personalized therapy for each patient. It is also certain that products of these currently unidentified genes as well as genes of known functions may become targets of novel anticancer drugs in the future.

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