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DNA microarray analysis of the correlation between gene expression patterns and acquired resistance to 5-FU/cisplatin in gastric cancer☆

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Abstract

The mechanisms of intrinsic and/or acquired anti-cancer drug resistance have been described in in vitro resistance models, but the clinical relevance has remained undefined. We undertook a prospective study to identify correlations between gene expression and clinical resistance to 5-FU/cisplatin. We compared expression profiles from gastric cancer endoscopic biopsy specimens obtained at a chemosensitive state (partial remission after 5-FU/cisplatin) with those obtained at a refractory state (disease progression), using Affymetrix oligonucleotide microarray technology (U133A). Using 119 discriminating probes and a cross-validation approach, we were able to correctly identify the chemo-responsiveness of 7 pairs of training samples and 1 independent test pair. These exploratory data demonstrate that the gene expression profiles differ between chemosensitive and refractory state gastric cancer biopsy samples.

Keywords: Resistance; Microarray; Expression; Profile; Gastric; Stomach; Cancer

One of the major obstacles to the successful treatment of cancer with cisplatin-based chemotherapy is the emergence of drug-resistant clones. The underlying mechanisms of platinum resistance, either intrinsic or acquired, are classified into two major groups: (1) those that limit the formation of cytotoxic platinum–DNA adducts, and (2) those that prevent cell death following platinum–DNA adduct formation, i.e., increased DNA adduct repair or platinum–DNA damage tolerance [1]. These mechanisms have previously been described in in vitro resistance models, but their clinical relevance has not been previously defined [2]. Resistance to fluoropyrimidines is a similarly multifactorial event that includes transport mechanisms, metabolism, molecular mechanisms, protection from apoptosis, and resistance via cell cycle kinetics [3]. Patients who develop clinical resistance may harbor tumor cells that have adopted multiple mechanisms for protecting themselves against chemotherapeutic agents. Accordingly, a correlative study focusing on a single factor may be less informative than a comprehensive, genome-wide investigation. The relatively recent development of DNA microarray technology now allows us to simultaneously monitor the expression levels of tens of thousands of genes in clinical samples, and allows researchers to investigate whether tumor expression profiles can be used to predict clinical responses to chemotherapy [4]. Indeed, several investigators have reported that gene expression profiling of biopsy specimens could enhance the accurate risk stratification of a subset of leukemia and lymphoma patients who have received chemotherapy [4].

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Combination chemotherapy with 5-FU/cisplatin is one of the most widely used regimens for treating metastatic gastric cancer patients [5]. About 20–50% of metastatic gastric cancer patients enter into remission after treatment with 5-FU/cisplatin, but during the course of treatment, all patients eventually develop acquired resistance [5–7]. To our knowledge, no clinically relevant mechanism for acquired resistance to 5-FU/cisplatin has previously been studied on a genome-wide scale. Hence, we undertook a prospective study to investigate whether and how gene expression profiles differ in endoscopic biopsy samples taken from gastric cancer patients at a chemosensitive state versus those taken at a refractory state.

Materials and methods

Tissue sampling. We have maintained a prospective database for diagnostic biopsy tissue specimens and clinicopathological information in metastatic gastric cancer patients treated with a 5-FU/cisplatin regimen at the National Cancer Center Hospital in Korea, since 2001. Eligibility criteria were as follows: (1) >18 years of age, (2) Eastern Cooperative Oncology Group performance status 0–2, (3) chemonaive, and the patients (4) had adequate organ functions, and (5) signed an institutional review board-approved informed consent form. The treatment consisted of 5-FU 1000 mg/m^2 IV on days 1–5 and cisplatin 60 mg/m^2 IV on day 1 of a 3-week schedule, given until disease progression. Patient responses were assessed every 3 cycles mainly by computed tomography (CT) according to WHO criteria [8]. Partial remission (PR) was defined as a decrease of 50% or more in the sum of the products of the largest perpendicular diameters of the bidimensionally measurable disease per CT. Progressive disease (PD) was defined as a 25% or more increase in the sum of the products of the largest perpendicular diameters of bidimensionally measurable disease, or the appearance of new lesions. Among the patients enrolled in our database, those who entered into clinical remission (PR) after 5-FU/cisplatin were eligible for the current molecular study. In these patients, biopsy specimens were obtained at the time of remission via endoscopy. Ten pieces of fresh tissue were obtained at each endoscopy. When these patients ultimately developed resistance, i.e., showed progression of disease (PD) during continued chemotherapy, endoscopic biopsies were repeated at the time of initial PD, i.e., before second-line chemotherapy. Seven patients met these criteria

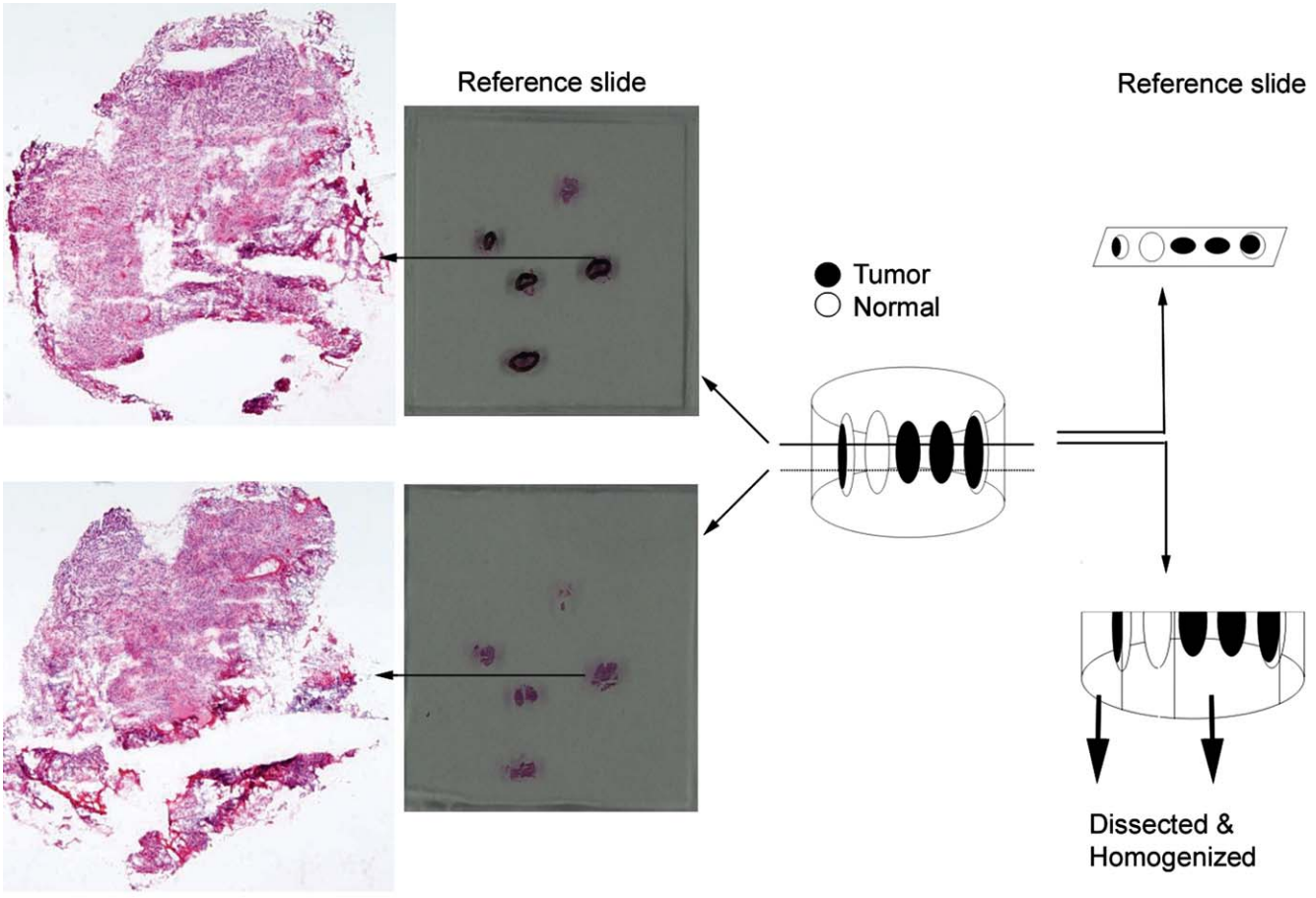

Fig. 1. Tissue processing. The harvested biopsy tissues were cryomolded together in OCT compound and cryosectioned until a representative section containing the most part of embedded tissue could be obtained. This representative cryostat section was placed on a reference slide, which was hematoxylin/eosin (H/E)-stained to evaluate tumor cell volume. Tumor-rich area of this reference slide was marked with a pen by a pathologist. Preliminary tests with three independent gastric cancer biopsy samples of our tissue database demonstrated largely consistent distributions of tumor cells between a representative slide and the other slides sectioned at lower levels in each sample (a representative result shown left). Guided by marked reference slides, corresponding tumor-rich areas of the study samples were manually dissected out of the remaining OCT blocks in a cryostat, and then crushed, and homogenized (right).

during the study period and became the training set cases of the current study. The fresh biopsy tissue samples were immersed in isopentane on ice during the endoscopy procedure and were transferred and stored in liquid nitrogen immediately after the completion of endoscopy (within 15 min of the first biopsy harvest).

Tissue processing (Fig. 1). The harvested biopsy tissues were cryomolded together in OCT compound and cryosectioned until a representative section containing the most part of embedded tissue could be obtained. This representative cryostat section was placed on a reference slide, which was hematoxylin/eosin (H/E)-stained to evaluate tumor cell volume. Tumor-rich area of this reference slide was marked with a pen by a pathologist (H.S.K). Guided by a marked reference slide, corresponding tumor-rich area was manually dissected out of the remaining OCT blocks in a cryostat, with care taken to avoid contamination of nonneoplastic epithelium in the tumor samples. Preliminary tests with three independent gastric cancer biopsy samples of our tissue database had demonstrated largely consistent distributions of tumor cells between a representative slide and the other slides sectioned at lower levels in each sample (Fig. 1). The excised samples were immediately crushed into a fine powder under liquid nitrogen and transferred to a tube containing 5 ml TRI Reagent (Molecular Research Center, Cincinnati, OH). Samples were then subjected to mechanical homogenization and RNA isolation as recommended by the manufacturer. We assessed total RNA integrity by electrophoresis using the Agilent Bioanalyzer (Agilent, Palo Alto, CA).

Gene expression profiling. We prepared biotin-labeled cRNA from 1.5 to 3.3 μg of total RNA using T7-$(dT)_{24}$ primers, the SuperScript Choice Kit (Invitrogen Life Technologies, Carlsbad, CA) and the BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostic, Farmingdale, NY), according to the recommendations of the DNA chip manufacturer (Affymetrix, Santa Clara, CA). Labeled cRNA was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA), fragmented, and hybridized to a DNA oligonucleotide expression array (HG-U133A, Affymetrix) containing 22,283 probe sets.

To assess array reproducibility and to rule out the possibility of bias from topographic heterogeneity in expression profile related to the biopsy site, we performed duplicate microarray experiments for the biopsy samples taken from a gastric cancer patient not included among the study patients. Tissue samples were collected via endoscopy at the time of initial progression after 5-FU/cisplatin, when care was taken to perform the biopsy evenly at the margin of a 3 cm-sized ulceration. Instead of being analyzed as a whole (as in study patients), these 10 endoscopic biopsy specimens collected during this single procedure were divided equally into 2 tubes according to the biopsy order (i.e., the first 5 pieces into the first tube), and the sample in each tube was independently processed for RNA isolation and DNA microarray analysis, as described above. The percentage of tumor cells in the OCT blocks was estimated as 25% and 40%, respectively. These 2 expression profile data correlated very highly, when Affymetrix Microarray Analysis Suite (MAS, Version 5.0) signals of the 22,283 probes were compared overall (Pearson's correlation, 0.99; $R^2 = 0.98$), indicating that our experimental approach generated highly reproducible data without significant heterogeneity-associated bias.

Statistical analysis. Scanned data (.cel files) were normalized using invariant set normalization with the Affy R package of Bioconductor. The preprocessed data were then subjected to unsupervised clustering and supervised classification analyses using the BRB-ArrayTools version 3.0 (Molecular Statistics and Bioinformatics Section, National Cancer Institute, Bethesda, MD). PR samples were labeled 'chemosensitive' and PD samples were labeled 'refractory.' The most discriminating probes were selected using paired t tests between data from serially obtained biopsy samples from the same patients ('chemosensitive' versus 'refractory'), for supervised classification analyses. Supervised classification with 'leave-one-out' cross-validation (LOOCV) was performed by different classifiers: compound covariate predictor, linear discriminant analysis, support vector machine, k-nearest neighbors ($k = 1$ and 3), and nearest centroid classifiers. The permutation P value for the cross-validated misclassification rate was calculated for each class prediction method requested. For each random permutation of class labels, the entire cross-validation procedure was repeated to determine the cross-validated misclassification rate obtained from developing a multivariate predictor with two random classes. The final P value was the proportion of 2000 permuted experiments that gave as small a cross-validated misclassification rate as was obtained with the real class labels. All of the class prediction methods were used to predict the class labels of 1 independent test pair.

Table 1
Patient characteristics

No.	Sex/age	Primary tumor				Time to progression[b] (week)	Interval between biopsies (week)	Chemotherapy-free interval[c]	% Tumor cell
		Pathology (WHO[a]/Laruen)	Borrmann	Location	Diameter (cm)			First/second biopsy (week)	First/second biopsy
Training set									
1	F/62	Adeno[d]/diffuse	III	Antrum	6	22.2	8.4[e]	3.3/3.1	60/90
2	M/58	Neuroendocrine[f]	III	Cardia	4	34.3	23.1	3.4/7.6	70/30
3	M/68	Adeno/diffuse	III	Body	>7	35.0	24.6	3.0/2.6	70/70
4	M/61	Adeno/intestinal	III	Antrum	6	22.9	12.3	3.1/2.7	90/70
5	M/42	Adeno/intestinal	IV	Diffuse	>7	33.9	22.7	3.4/3.1	90/70
6	M/63	Adeno/diffuse	III	Antrum	>7	25.7	13.1	3.1/4.7	20/60
7	M/66	Adeno/diffuse	III	Body	3	54.1	43.9	2.6/3.0	60/70
Test case									
8	M/51	Adeno/diffuse	III	Cardia	>7	55.0	55.0		80/50

[a] World Health Organization.
[b] From the beginning of treatment until the disease progression documented according to WHO criteria.
[c] From the administration of the last dose of chemotherapeutic agents to the biopsy.
[d] Adenocarcinoma.
[e] In this patent, the first biopsy specimen was obtained after 4 cycles, instead of after 3 cycles (as in all other patients), of 5-FU/cisplatin.
[f] Neuroendocrine carcinoma.

Table 2
The status of chemo-responsiveness according to the WHO criteria and endoscopy finding of study patients at the time of the first and the second biopsies

No.	At the time of the first biopsy		At the time of the second biopsy	
	WHO criteria[a]	Endoscopy	WHO criteria (lesion[b])	Endoscopy
Training set				
1	PR[c]	Improved[d]	PD[e] (LN[f], peritoneum)	Aggravated[g]
2	PR	Improved[d]	PD (LN, liver)	Aggravated[g]
3	PR	Improved[d]	PD (liver)	Aggravated[g]
4	PR	Improved[d]	PD (LN, liver)	Aggravated[g]
5	PR	Unchanged[h]	PD (LN, liver)	Aggravated[g]
6	PR	Unchanged[h]	PD (LN, liver)	Aggravated[g]
7	PR	Unchanged[h]	PD (peritoneum)	Aggravated[g]
Test case				
1	PR	Improved[d]	PD (liver)	Aggravated[g]

[a] World Health Organization criteria based on the bidimensional CT measurement.
[b] Metastatic organ site of lesions that showed progression according to CT findings.
[c] Partial remission.
[d] PR according to the clinical criteria proposed by Japanese Classification of Gastric Carcinoma.
[e] Progressive disease.
[f] Abdominal lymph nodes.
[g] PD according to Japanese Classification.
[h] NC according to Japanese Classification.

Results

During the period of Aug 2001–Nov 2002, seven patients met the selection criteria for the current study, i.e., they entered clinical remission (PR) after 5-FU/cisplatin treatment, developed resistance (PD) during continued chemotherapy, and gave informed consent (Table 1). The median number of chemotherapy cycles was 8 (range; 6–15) per patient, and median relative dose intensities of 5-FU and cisplatin were 74.4% (range; 65.9–84.1) and 81.5% (range; 77.5–86.5), respectively. Endoscopies were performed at chemosensitive state (PR according to WHO criteria) and refractory state (PD) of each patient. As shown in Table 2, endoscopy findings demonstrated the aggravation of primary tumors at the time of second biopsy (PD) in all study patients. The endoscopic biopsy samples taken at the refractory state (PR) were compared pair-wise with those taken at the chemosensitive state (PD). The median time interval between the 2 serial biopsies (i.e., from PR to PD) was 22.7 weeks (Table 1). The chemotherapy-free interval, defined as the time interval between the administration of the last dose of chemotherapeutic agents and the biopsy, did not differ between chemosensitive (median 3.3 weeks) and refractory (median 3.1 weeks) state samples. The median tumor cell proportion, estimated by light microscopy examination of H/E-stained cryosectioned frozen tissue slides, was 70%, and did not differ between chemosensitive and refractory state samples. The median percent present call among the 14 microarray datasets was 22.5% (range; 17.7–34.7) when analyzed by MAS 5.0, and no array images showed grossly visible artifacts.

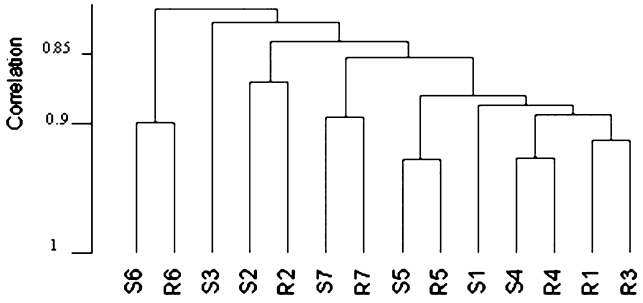

Fig. 2. Unsupervised hierarchical clustering of the 14 gastric cancer endoscopic biopsy specimens by comparison of their expression profiles across all 22,283 probes. Letters refer to the status of chemo-responsiveness (S; sensitive (PR), R; refractory (PD)) and numbers specify patients; thus, S1 refers to 'the sample obtained from patient 1 at a sensitive state (PR),' and R1 refers to 'the sample obtained from patient 1 at a refractory state (PD).' Using a matrix of standard Pearson's correlation coefficients from the complete pair-wise comparison of all experiments, the 14 training set samples are displayed as a hierarchical clustering dendrogram. The average linkage cluster shows that serial biopsy samples obtained from the same patient tended to cluster together, indicating that on a genome-wide scale the change in expression profile during disease progression is less prominent than individual variation (robustness index, 0.996).

First, we performed unsupervised hierarchical clustering of all genes to compare the composite expression profiles of the 14 training set samples. Generally, serial biopsy samples obtained from the same patient tended to cluster together, indicating that the change in expression profile during disease progression was less prominent than individual variation, at least on a genome-wide scale (Fig. 2). Supervised classification methods were then applied to identify gene expression

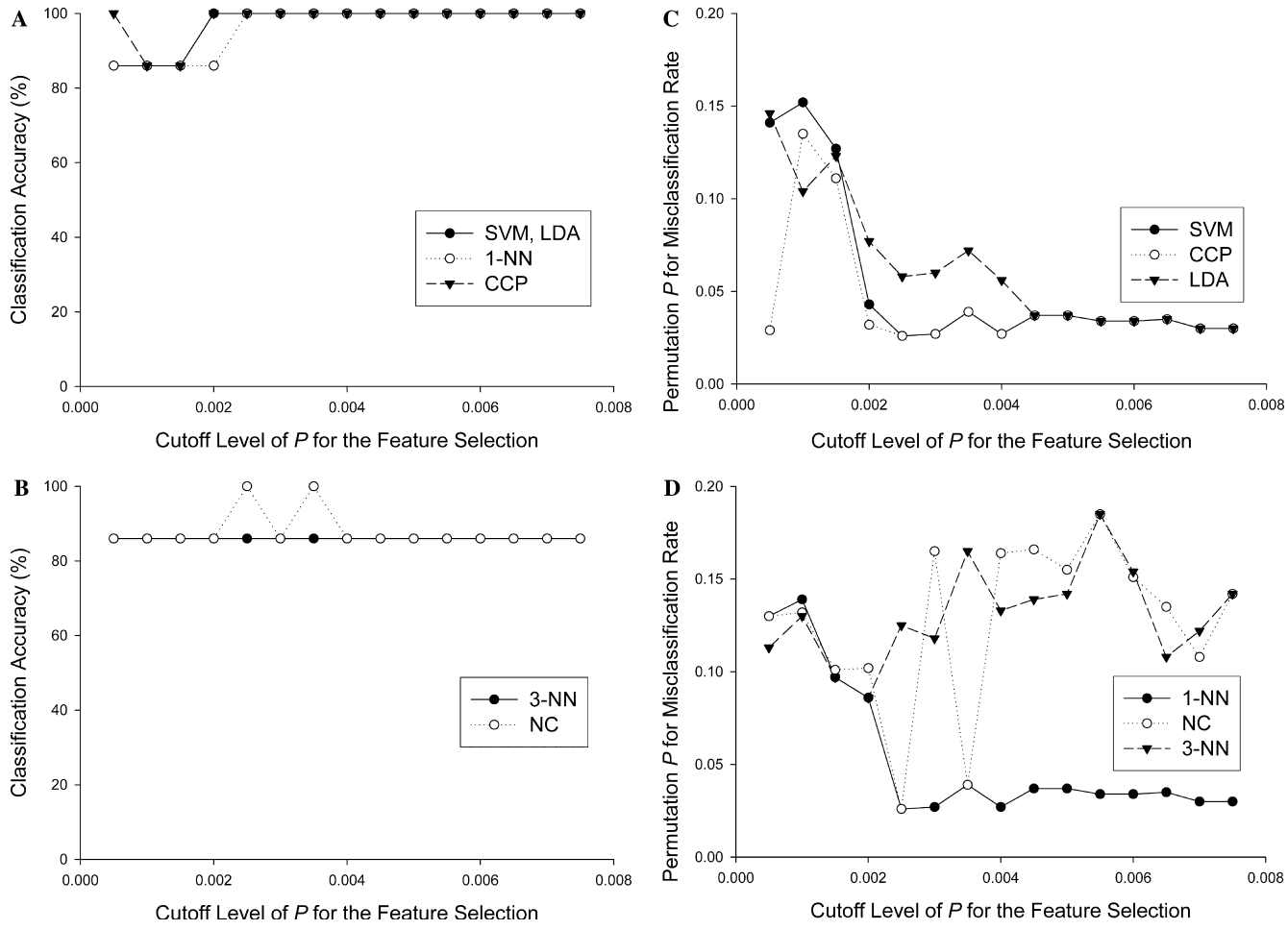

Fig. 3. Cross-validation experiments. Classification accuracy (A,B) and permutation P value for the cross-validated misclassification rate (C,D) were plotted against the cutoff level of P for the feature selection. Classifiers were constructed using top-ranked discriminating probes selected by paired t test, and 'leave-one-out' cross-validation was performed using BRB-ArrayTools version 3.0. We achieved 100% classification accuracy and significant ($P < 0.05$) permutation test results with SVM, CCP, 1-NN, and LDA, at cutoff levels of $P \geqslant 0.002$, 0.002, 0.0025, and 0.0045, respectively. Thus, gene expression signatures that differentiated chemosensitive (PR) from refractory state (PD) samples were clearly identified (SVM, support vector machine; CCP, compound covariate predictor; LDA, linear discriminant analysis; k-NN, k-nearest neighbors ($k = 1$ and 3); and NC, nearest centroid).

signatures that differed between chemosensitive and refractory state samples. Using top-ranked discriminating probes selected by paired t test, supervised classification with LOOCV was performed with various classifiers: compound covariate predictor, linear discriminant analysis, support vector machines, k-nearest neighbors ($k = 1$ and 3), and nearest centroid classifiers. The classification accuracy and empirical P values were obtained using different cutoff levels of P (from 0.0005 to 0.0075) for the feature selection (Fig. 3). The use of a support vector machine with various subsets of discriminating probes (P cutoff $\geqslant 0.002$) resulted in 100% classification accuracy (7 out of 7 pairs) between 'chechemosensitive' (PR) and 'refractory' (PD) (Fig. 3A). The permutation test showed that empirical P values for the cross-validated misclassification rate were less than 0.05 in these subsets (Fig. 3C). We also achieved 100% classification accuracy (Figs. 3A and B) and significant ($P < 0.05$) permutation test results (Figs. 3C and D), with compound covariate predictor, 1-nearest neighbors, and linear discriminant analysis, at cutoff levels of $P \geqslant 0.002$, 0.0025, and 0.0045, respectively. Thus, we could clearly identify gene expression signatures that differentiated chemosensitive (PR) from refractory state (PD) samples. The permutation P value for the misclassification rate tended to fall into the significant (<0.05) range as the cutoff level of P for the feature selection increased, except for nearest centroid and 3-nearest neighbors (Figs. 3C and D). The support vector machine and the compound covariate predictor gave significant permutation P values for the misclassification rate when the cutoff level of P for the feature selection reached 0.002 (Fig. 3C).

Fig. 4 shows the change in expression levels of the 119 probes (86 known genes and 33 expressed sequence tags/hypothetical proteins) that were selected by a paired

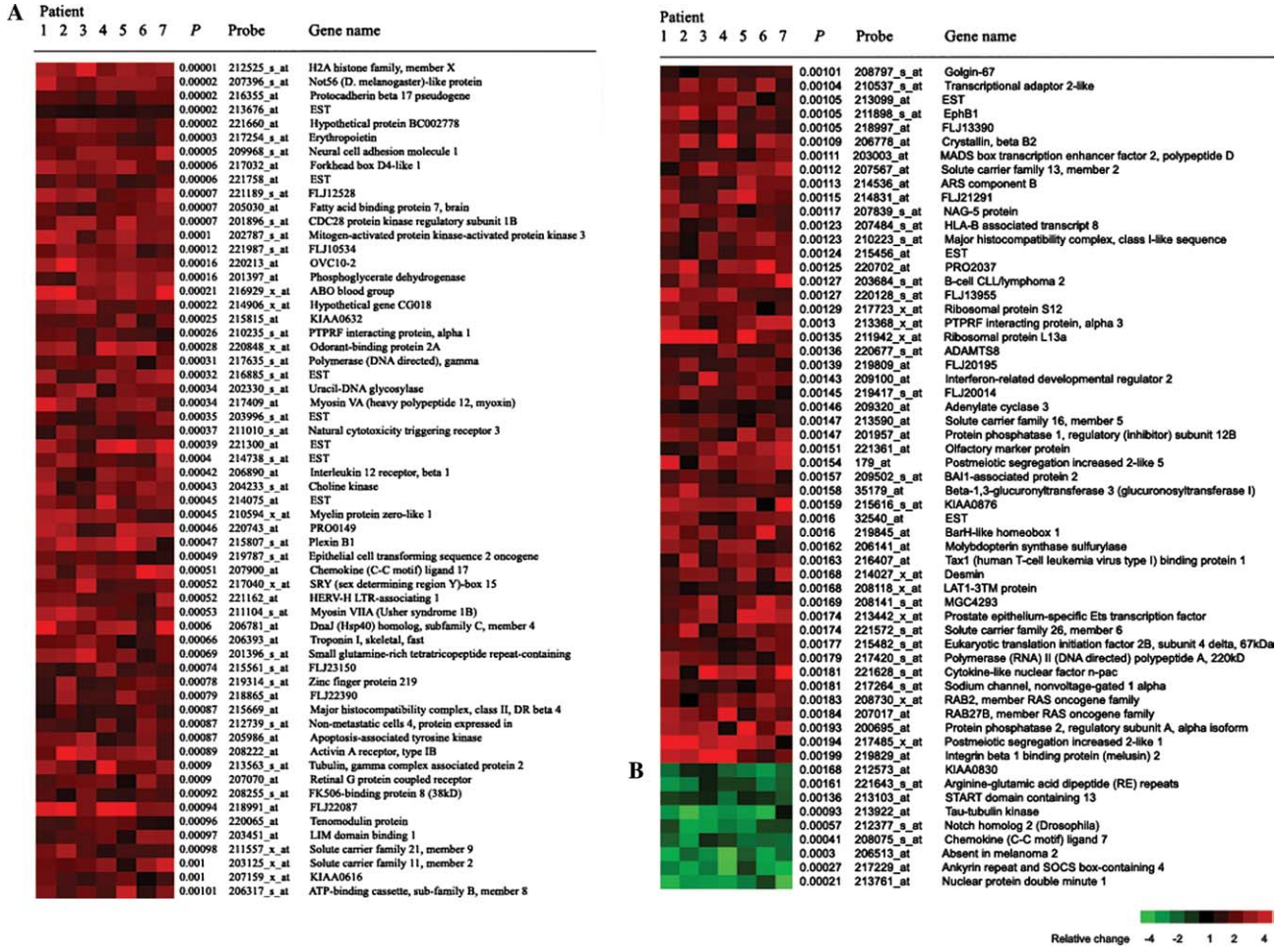

Fig. 4. The 119 probes capable of discriminating chemosensitive state (PR) from refractory state (PD) samples. Of these, 110 probes were upregulated in refractory state samples (A) and 9 were downregulated in refractory-state samples (B). Each column represents an individual study patient in the training set. Each row represents a discriminating probe selected by the paired *t* test ($P < 0.002$). Red and green represent up and downregulation during disease progression, respectively. Parametric *P* value for paired *t* test, probe set ID, and Unigene nomenclature are also presented for each probe. Graphic display was performed using Cluster and TreeView software.

t test $P < 0.002$. These 86 known genes had various functions, including signal transduction ($n = 23$ (27%), such as *FK506-binding protein 8*), DNA or RNA metabolism ($n = 16$ (19%), such as *uracil DNA glycosylase*), transport ($n = 11$ (13%), such as *ABCB8*), metabolism ($n = 8$ (9%), such as *glucuronosyltransferase I*), immune response ($n = 6$ (7%), such as *AIM2*), apoptosis ($n = 3$ (4%), such as *Bcl-2*), stress response ($n = 2$ (2%), such as *MAPKAPK3* and *DnaJ* (*Hsp40*) *homolog*, *subfamily C*, *member 4*), and others ($n = 17$ (19%)). Of the 119 probes, 110 were upregulated in refractory state samples, including the well-known anti-apoptotic gene *Bcl-2* and several DNA repair enzymes. Previous reports have suggested that *Bcl-2* transfection conferred cisplatin resistance on various types of cancer cells [9,10], and that *Bcl-2* antisense oligonucleotides chemosensitized human gastric cancer in a SCID mouse xenotransplantation model [11]. DNA repair enzymes upregulated in the refractory state included uracil DNA glycosylase and DNA polymerase γ. Uracil DNA glycosylase excises 5-FU-incorporated promutagenic DNA, thereby contributing to 5-FU resistance [12,13]. DNA polymerase γ, along with DNA polymerases β and ζ, catalyzes the translesion DNA synthesis past Pt–DNA adducts, leading to enhanced adduct tolerance, which has been recognized as one of the mechanisms of cisplatin resistance [14]. Interestingly, the upregulated probes also include two members of an hPMS2-related gene family (*PMS2L1* and *PMS2L5*), both of which are located on chromosome 7 and share a high degree of identity with the mismatch repair gene *hPMS2*. Given a previous report that lack of the *hPMS2* gene was associated with an increased sensitivity to cisplatin [15], further studies are warranted to determine whether *PMS2L1* and *PMS2L5* are associated with cisplatin resistance as well.

Of the 9 probes that were downregulated in refractory state samples, two genes (*absent in melanoma 2* (*AIM2*) and *arginine–glutamic acid dipeptide repeats* (*RERE*)) have been previously reported to have proapoptotic

functions. *AIM2* overexpression increased the susceptibility of murine fibroblasts to cell death under reduced serum conditions [16], and *RERE* was shown to enhance apoptosis of neuroblastoma cell lines by recruiting a fraction of the proapoptotic protein Bax to promyelocytic leukemia oncogenic domains [17]. Concurrent with upregulation of *Bcl-2*, downregulation of these 2 proapoptotic genes in our refractory state samples suggests that transcriptional changes in apoptosis regulators could be correlated with the development of clinical drug resistance, although the direct association of *AIM2* and *RERE* with drug-induced apoptosis in gastric cancer cells has not been previously reported. Overall, these data are consistent with accumulating evidence that activators or inhibitors of known signal transduction pathways related to apoptosis can influence chemosensitivity [18,19].

For further confirmation of the microarray data, we performed real-time RT-PCR for *MAPKAPK3*, *DNAJC4*, and *RERE* (primers/probes purchased from Applied Biosystems, Foster City, CA). In all 7 refractory state samples, the invariant set-normalized microarray signals for *MAPKAPK3* and *DNAJC4* were higher than for the corresponding chemosensitive state samples, and those for *RERE* were consistently lower in refractory state samples than in chemosensitive state samples (Fig. 4). The β-actin-normalized RT-PCR expression levels of *MAPKAPK3* and *DNAJC4* were higher in the refractory-state samples of 6/7 and 4/7 training set cases, respectively (median 1.4- and 1.0-fold increases, respectively). Likewise, the β-actin-normalized RT-PCR expression level of *RERE* was lower in 5/7 refractory-state training set cases (median 0.8-fold decrease), indicating a moderate concordance between the two methods of assessment.

Following its construction and cross-validation, our predictive model was tested in a pair of samples from a test case: a 51-year-old male who also entered partial remission and later progressed despite continued treatment with 5-FU/cisplatin. Samples from this patient were not used in the model building process due to a technical failure in obtaining adequate RNA from his PR biopsy sample. For the application of our predictive models to this patient, we substituted his pre-treatment biopsy sample for the PR sample, assuming that the expression profile of his pre-treatment biopsy sample should resemble that of the PR sample, i.e., the profile of a chemosensitive tumor. Using discriminating probes selected at P cutoff levels ranging from 0.001 to 0.0075, the support vector machine correctly identified the chemo-responsiveness of paired samples from this test case, i.e., his pre-treatment sample was identified as 'chemosensitive' and his PD sample as 'refractory' (Table 3) in all cases. The other 5 class prediction methods also correctly predicted the class labels of the paired samples in this test case, using discriminating probes selected at P cutoff levels ranging from 0.001 to 0.0075 (except at 0.002–0.0025, where 1 out of 5 classifiers gave an incorrect prediction).

Table 3
Performance of support vector machine for the prediction of an independent test case, according to P value for the feature selection

P[a] cutoff for feature selection	No. of genes selected	Prediction result
0.0005	40	Incorrect
0.001	64	Correct
0.0015	94	Correct
0.002	119	Correct
0.0025	138	Correct
0.003	161	Correct
0.0035	185	Correct
0.004	211	Correct
0.0045	235	Correct
0.005	259	Correct
0.0055	283	Correct
0.006	312	Correct
0.0065	327	Correct
0.007	342	Correct
0.0075	358	Correct

[a] P for paired t test.

Discussion

Our results demonstrate that we were able to identify a gene expression signature that correlated with disease progression in this particular group of gastric cancer patients treated with 5-FU/cisplatin. Moreover, the specific expression signature appears to be a predictor of the development of chemoresistance, although these data will need to be validated in larger studies. Admittedly, the current data are still preliminary, but the rarity of such kind of clinical samples makes the larger study very difficult to perform, especially in the single-institution setting. While the emergence of the resistant phenotype may in part be a function of the selection pressure exerted by treatment, certain determinants of chemoresistance may be caused by genetic changes accompanying disease progression. Although the possible in vivo relationship of several gene products to 5-FU/cisplatin resistance in gastric cancer was indicated by our data, further experiments will be required to determine whether or to what extent the individual discriminating genes are related to chemoresistance.

There are several issues to be discussed regarding our study design. First, to correlate gene expression to drug resistance, we compared samples obtained at a partial remission state with those obtained at a refractory state. This was intended to minimize (although not completely eliminate [20]) the confounding influences of treatment effect regardless of the development of drug resistance, which can become the source of bias when pre-treatment samples are compared with refractory state samples. Notably, the current analysis focuses on the relative

change in gene expression during disease progression, based on the assumption that the proportion of chemoresistant clones to chemosensitive ones should be higher in samples taken at the time of progression than in corresponding samples taken at the time of PR, regardless of the absolute fraction of chemosensitive clones in each sample. The validity of this assumption was supported by the CT-documentation of further tumor shrinkage occurring after PR in 5 out of 8 study patients (4 out of 7 training set patients), suggesting that most samples taken at the time of PR had an appreciable fraction of chemosensitive clones. Second, we wish to note that the gene expression signature we identified is unlikely to be biased by chemotherapy-free interval and tumor cell proportion, given that the chemosensitive state and refractory state samples did not differ in these parameters. Third, bulk tumor samples, rather than microdissected samples, were used in the current study, given the considerations that expression signatures from nonmalignant cells might also be informative and that use of microdissected samples would entail the higher degree of bias in RNA amplification [21,22].

Taken together, our results show that the gene expression profiles differ between chemosensitive and refractory state samples obtained from this particular subset of 5-FU/cisplatin-treated gastric cancer patients. There have been no previous prospective genome-wide studies examining whether and how gene expression profiles at refractory state differ from those at chemosensitive state. This study suggests that the expression profiling of endoscopic biopsy can be a feasible approach to the research on the mechanism of anti-cancer drug resistance. The current exploratory data can be validated by larger data sets and compared with gene expression correlates of *intrinsic* 5-FU/cisplatin resistance in the future, which then may provide more comprehensive insights into the clinically relevant mechanism of anti-cancer drug resistance.

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