

Available online at www.sciencedirect.com



Biochemical and Biophysical Research Communications 316 (2004) 781-789

www.elsevier.com/locate/ybbrc

DNA microarray analysis of the correlation between gene expression patterns and acquired resistance to 5-FU/cisplatin in gastric cancer^{\ddagger}

Hark Kyun Kim,^a Il Ju Choi,^a Hee Sung Kim,^a Ju Han Kim,^b Eugene Kim,^a In Sook Park,^a Jong Ho Chun,^a In-Hoo Kim,^a Il-Jin Kim,^c Hio Chung Kang,^c Jae-Hyun Park,^c Jae-Moon Bae,^a Jin Soo Lee,^a and Jae-Gahb Park^{a,*}

^a Research Institute and Hospital, National Cancer Center, Goyang, Gyeonggi, Republic of Korea ^b Seoul National University Biomedical Informatics, Department of Preventive Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea

^c Korean Hereditary Tumor Registry, Laboratory of Cell Biology, Cancer Research Center and Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea

Received 9 February 2004

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.

© 2004 Elsevier Inc. All rights reserved.

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].

^{*} Supported by National Cancer Center Grant 0110180.

^{*} Corresponding author. Fax: +82-31-920-1511.

E-mail address: park@ncc.re.kr (J.-G. Park).

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/m² IV on days 1-5 and cisplatin 60 mg/m² 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 $3.3 \,\mu$ g 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 'chemomosensitive' 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, knearest 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	Interval between	Chemotherapy- free interval ^c	% Tumor cell
		Pathology (WHO ^a /Laruen)	Borrmann	Location	Diameter (cm)	(week)	biopsies (week)	First/second biopsy (week)	First/second biopsy
Train	ing set								
1	F/62	Adeno ^d /diffuse	III	Antrum	6	22.2	8.4 ^e	3.3/3.1	60/90
2	M/58	Neuroendocrinef	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 a	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. ^fNeuroendocrine 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°	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 ()rganization criteria based on t	he bidimensional CT measur	rement			

world Health Organization chieffa 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 $P \ge 0.002$, 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 ≥ 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 $P \ge 0.002$, 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 3nearest 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

0.0001 2125 0.0002 2733 0.0002 2163 0.0002 2163 0.0002 2163 0.0002 2164 0.0002 2164 0.0002 2164 0.0005 2179 0.0005 2179 0.0007 2118 0.0007 2118 0.0007 2118 0.0007 2118 0.0007 2118 0.0007 2108 0.0001 2270 0.00012 2199 0.00012 2199 0.00012 2199 0.00012 2199 0.0002 2149 0.0002 2168 0.0002 2168 0.0002 2169 0.0002 2169 0.0002 2169 0.0002 2169 0.0002 2169 0.0002 2169 0.0003 2170 0.0003 2170 0.0003 2170 0.0005 2169 0.0003 2170 0.0005 2169 0.0005 2170 0.0005 2170 0.	525_s_st II2A bistone family, member X 596_s_st Not56 (D. melanogaster)-like protein 596_st H. Protocadberin beta 17 pseudogene 576_st EST 576_st ENT 57_st F1/10534 77_st ENT 77_st ENT 78_st <t< th=""><th></th><th></th><th></th><th>0.00101 0.00104 0.00105 0.00105 0.00105 0.00105 0.00112 0.00112 0.00112 0.00115 0.00117 0.001123 0.00123 0.00124</th><th>208797_s_st 210537_s_at 2110537_s_at 211989_at 2118987_at 206776_at 206776_at 207567_at 214536_at 214535_at 214831_at 207844_s_at 207844_s_at 210223_s_at</th><th>Golgin-67 Transcriptional adaptor 2-like EST EphB1 FuL13300 Crystallin, bet B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein HLA-6 associated transcript 8 HLA-6 associated transcript 8 HLA-6 associated transcript 8</th></t<>				0.00101 0.00104 0.00105 0.00105 0.00105 0.00105 0.00112 0.00112 0.00112 0.00115 0.00117 0.001123 0.00123 0.00124	208797_s_st 210537_s_at 2110537_s_at 211989_at 2118987_at 206776_at 206776_at 207567_at 214536_at 214535_at 214831_at 207844_s_at 207844_s_at 210223_s_at	Golgin-67 Transcriptional adaptor 2-like EST EphB1 FuL13300 Crystallin, bet B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein HLA-6 associated transcript 8 HLA-6 associated transcript 8 HLA-6 associated transcript 8
0.0002 2133 0.0002 2163 0.00002 2163 0.00002 2163 0.00002 2164 0.00003 2125 0.00005 2079 0.00005 2170 0.00007 2018 0.00007 2018 0.00007 2018 0.0001 2077 0.0001 2018 0.0001 2018 0.0001 2018 0.0001 2018 0.0001 2018 0.0001 2018 0.00015 2018 0.0002 2168 0.0002 2168 0.0002 2168 0.0002 2168 0.0002 2168 0.0002 2168 0.0003 2118 0.0003 2118 0.0003 2118 0.0003 2118 0.0003 2118 0.0003 2139 0.0003 2118 0.0003 2139 0.0003 2118 0.0003 2139 0.0003 2110 0.0003 2110 0.0003 2110 0.0003 21100 0.0003	956_s_at Not56 (D. melanogaster)-like protein 955_at Protocadherin beta 17 pseudogene 976_at EST 976_st EST 975_st EST </td <td></td> <td></td> <td></td> <td>0.00104 0.00105 0.00105 0.00105 0.00109 0.00112 0.00112 0.00113 0.00115 0.00115 0.00117 0.00123 0.00123 0.00124</td> <td>210537-s_at 210537-s_at 218995_at 218995_at 206776_at 207567_at 214536_at 214536_at 214535_at 207839_s_at 207845_s_at 207845_s_at</td> <td>Transcriptional adaptor 2-like EST EphB1 FuL13300 Crystallin, bet B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLI21201 NAG-5 protein HLA-6 passociated transcript 8 HLA-6 passociated transcript 8 HLA-6 passociated transcript 8</td>				0.00104 0.00105 0.00105 0.00105 0.00109 0.00112 0.00112 0.00113 0.00115 0.00115 0.00117 0.00123 0.00123 0.00124	210537-s_at 210537-s_at 218995_at 218995_at 206776_at 207567_at 214536_at 214536_at 214535_at 207839_s_at 207845_s_at 207845_s_at	Transcriptional adaptor 2-like EST EphB1 FuL13300 Crystallin, bet B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLI21201 NAG-5 protein HLA-6 passociated transcript 8 HLA-6 passociated transcript 8 HLA-6 passociated transcript 8
0.0002 2135 0.0002 2136 0.0002 2136 0.0003 2172 0.0003 2172 0.0005 2099 0.0006 2170 0.0006 2170 0.0007 22118 0.0007 2218 0.0001 2278 0.0001 2278 0.0001 2219 0.0001 2219 0.0002 219 0.0002 219 0.0001 219 0.0002 219 0.0002 219 0.0003 219 0.0005 21	355_st Protocadberin beta 17 pseudogene 355_st EST 560_st IST 561_st IST 562_st IST 563_st IST 563_st IST 563_st IST 563_st IST 563_st IST 564_st IST 575_st IST 575_st IST 575_st IST 575_st INDobalyscent edulydrogenase 575_st INDobalyscent dolydrogenase 575_st <td></td> <td></td> <td></td> <td>0.00105 0.00105 0.00105 0.00109 0.00111 0.00112 0.00113 0.00115 0.00115 0.00117 0.00123 0.00123 0.00124</td> <td>213099_at 211898_s_at 218997_at 206778_at 203003_at 207567_at 214831_at 207389_s_at 207484_s_at 215456_at</td> <td>EST EphB1 FLJ13390 Crystallin, beta B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein FLA8 associated transcript 8 HLA6 associated transcript 8 HLA6 associated transcript 8</td>				0.00105 0.00105 0.00105 0.00109 0.00111 0.00112 0.00113 0.00115 0.00115 0.00117 0.00123 0.00123 0.00124	213099_at 211898_s_at 218997_at 206778_at 203003_at 207567_at 214831_at 207389_s_at 207484_s_at 215456_at	EST EphB1 FLJ13390 Crystallin, beta B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein FLA8 associated transcript 8 HLA6 associated transcript 8 HLA6 associated transcript 8
0.00002 2136 0.00002 2136 0.00005 2213 0.00005 2173 0.00007 2118 0.00007 2018 0.0001 2273 0.0001 2273 0.0001 2274 0.0001 2274 0.0001 2274 0.00015 2135 0.0002 2145 0.00022 2145 0.00023 2145 0.0003 2110 0.0003 2110 0.0004 2147 0.0004 2147 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2150 0.0007 2155 0.0005 2150 0.0005 215	576_att EST 56_att Hypothetical protein BC002778 54_a_att Erydtropoietin 54_a_att Erydtropoietin 554_att Erydtropoietin 57a_att EST 189_a_att Full>200_att 57a_att EST 57a_att ESC28 protein kinase regulatory subunit 1B 57a_att OVC10-2 57a_att OVS10534 57a_att PSDobpolgiveersite dehydrogenase 57a_att KIAA0652 57a_att KIAA0652 57a_att PTP interacting protein alpha 1 57a_att OVOrant-binding protein 2A 57a_att ENT protein 2A 57a_att ENT protein 2A				0.00105 0.00105 0.00109 0.00111 0.00112 0.00113 0.00115 0.00115 0.00123 0.00123 0.00124	211898_s_at 218997_at 206778_at 203703_at 207567_at 214536_at 214536_at 207839_s_at 207484_s_at 215456_at	Eph81 FuLi13300 Crystallin, beta B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component FLI21201 NAG-5 protein FLIA-6 associated manacript 8 HLA-6 associated manacript 8 HLA-6 associated manacript 8
0.00002 2216 0.00003 2172 0.00005 2099 0.00006 2170 0.00006 2170 0.00006 2170 0.00007 2018 0.00007 2018 0.00012 2219 0.00012 2219 0.00016 2203 0.00012 2219 0.00016 2203 0.00012 2199 0.00012 2199 0.00022 2189 0.00022 2189 0.00023 2180 0.00023 2180 0.00032 2180 0.00032 2180 0.00032 2180 0.00032 219 0.00032 219 0.00052 2110 0.00052 210 0.00052 21	560_st Hypothetical protein BC002778 554_st Evydropoletin 565_st Evydropoletin 565_st Evydropoletin 565_st Evydropoletin 565_st EVXhead box D4-like 1 575_st EST 575_st EST 575_st EST 575_st EST 575_st EST 575_st EST 575_st FO228 portein kinase-regulatory suburit 1B 575_st OVC10-2 575_st OVC10-2 577_st ABO blood group 575_st FO20 bedicid group 57_st FO20 bedicid group 55_st FO20 bedicid group 55_st FO20 bedicid group 55_st FO20 bedicid group 55_st FO20 bedicid group <				0.00105 0.00109 0.00111 0.00112 0.00113 0.00115 0.00115 0.00123 0.00123 0.00124	218997_at 206778_at 203003_at 207567_at 214536_at 214536_at 207839_s_at 207484_s_at 215456_at	FLJ13390 Crystallin, beta B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein HLA-8 associated manscript 8 March bitrocomsthillity complex class Lillia accurate
0.0003 2173 0.00005 2173 0.00005 2173 0.00007 218 0.00007 218 0.00012 2199 0.00016 2007 0.00016 2007 0.00016 2007 0.00016 2007 0.00016 213 0.00016 213 0.00016 213 0.000016 213 0.000012 2199 0.000012 2199 0.000012 2199 0.000012 213 0.00001 2176 0.00001 2135 0.00001 2135 0.00005 2135 0.00007 2155 0.00007	254_s_s Erythropoietin 965_s_st Neural cell adbesion molecule 1 932_st Forkhead box D4-like 1 932_st FL12528 930_st Fatty scid binding protein 7, brain 986_s_st CDC28 protein kinase regulatory subunit 1B 987_s_st CDC28 protein kinase-activated protein kinase-activated protein kinase 3 987_s_st CDC28 protein kinase-activated protein kinase-activated protein kinase 3 987_s_st CDC28 protein dehydrogenase 927_st AD0 blood group 96_s_st Hydrothetical gene CG018 815_st KIAA0652 25_s_st PTPR interacting protein alpha 1 845_s_st COArast-binding protein 2A 95_s_st EST 85_st EST 85_st EST 85_st EST				0.00109 0.00111 0.00112 0.00113 0.00115 0.00115 0.00123 0.00123 0.00124	206778_at 203003_at 207567_at 214536_at 214831_at 207839_s_at 207484_s_at 210223_s_at	Crystallin, beta B2 MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein FLJA-6 associated transcript 8 HLA-6 associated transcript 8 HLA-6 associated transcript 8
0.00005 2099 0.00006 2170 0.00006 2170 0.00007 2018 0.00007 2018 0.00012 2219 0.00012 2219 0.00012 2219 0.00012 2219 0.00012 2219 0.00012 2219 0.00022 2189 0.00022 2189 0.00022 2189 0.00028 2208 0.00028 2208 0.00028 2208 0.00028 2208 0.00032 2163 0.00032 2163 0.00032 2163 0.00032 2163 0.00032 2163 0.00032 2170 0.0004 2173 0.0004 2173 0.00045 2105 0.00045 2105 0.00047 2155 0.00057 2118 0.00057 2155 0.00057	968_1_at Neural cell adhesion molecule I 972_at Forkhead box D4-like I 758_at EST 978_at EST 978_at EST 978_at EST 978_at EST 978_at FLJ12528 978_at FLJ12528 978_at FLO228 protein kinase regulatory suburit 1B 978_at Miogen-activated protein kinase-activated protein kinase as 978_at OVC10-2 979_at FD0 blood group 906_at Hypothetical gene CG018 815_at KIAA06532 255_at FPAPF interacting protein AA 55_at FO/powerase (DNA directed), gamma 885_at EST 93_at EST 93_at EST				0.00111 0.00112 0.00113 0.00115 0.00115 0.00123 0.00123 0.00124	203003_at 207567_at 214536_at 214831_at 207839_s_at 207484_s_at 210223_s_at	MADS box transcription enhancer factor 2, polypeptide D Solute carrier family 13, member 2 ARS component B FLJ21291 NAG-5 protein HLA-8 associated transcript 8 HLA-8 associated transcript 8
0.0006 2170 0.0006 2170 0.0006 2170 0.0007 2018 0.0007 2018 0.0007 2018 0.0007 2018 0.0001 2218 0.0001 2219 0.0001 2219 0.0001 2219 0.00016 2201 0.00016 2201 0.00022 2149 0.00022 2149 0.0003 2170 0.0003 2170 0.00	1072_att Forkhead box D4-like I 1075_att EST 1885_att FLI12528 1895_att FLI12528 1895_att FLI2528 1895_att CDC28 protein kinase regulatory subunit I B 1875_att Milogen-activated protein kinase-activated protein kinase 3 1875_att FLI10534 1875_att FLI0054 1875_att FLI0054 1875_att FLI0054 1875_att FLI0054 1875_att FLI0054 1875_att FLI0054 1855_att				0.00112 0.00113 0.00115 0.00115 0.00123 0.00123 0.00124	207567_at 214536_at 214831_at 207839_s_at 207884_s_at 210223_s_at 21555_at	Solute carrier family 13, member 2 ARS component 8 FLJ21291 NAG-5 protein HLA-8 associated transcript 8 HLA-8 associated transcript 8 HLA-8 associated transcript 8
0.0000 211 0.00007 2118 0.00007 2000 0.00007 2018 0.00007 2018 0.00012 2199 0.00012 2199 0.00016 2013 0.00012 2199 0.00016 2013 0.00022 2148 0.00022 2148 0.00022 2148 0.00022 2148 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00032 2188 0.00031 2175 0.00031 2175 0.00032 2188 0.00031 2175 0.00032 2188 0.00032 2188 0.00035 2197 0.00045 2105 0.00045 2105 0.00	USA_IL ESI USA_IL ESI USA_ILIZS28 Fatty seid binding protein 7, brain USA_ILIZS28 Fatty seid binding protein 7, brain USA_ILIZS28 Fatty seid binding protein 7, brain USA_ILIZS28 Fatty seid binding protein 1, brain USA_ILIZS28 Fatty seid binding protein 1, brain USA_ILIZS28 OVC10-2 USA_ILIZS28 ABO blood group USA_ILIZS24 ABO blood grou				0.00113 0.00115 0.00117 0.00123 0.00123 0.00124	214536_at 214831_at 207839_s_at 207484_s_at 210223_s_at	ARS component B FLJ21291 NAG-5 protein HLA-B associated transcript 8 Malor histocompatibility complex class Like services
0.00007 2018 0.00007 2018 0.00007 2018 0.00007 2018 0.00017 2018 0.00012 2219 0.00016 2013 0.00022 21499 0.00022 21499 0.00022 2149 0.00022 2149 0.00022 2149 0.00022 2149 0.00022 2149 0.00023 2158 0.00031 2158 0.00031 2158 0.00031 2158 0.00032 2218 0.00032 2218 0.00032 2218 0.00032 2218 0.00032 2218 0.00032 2218 0.00042 2088 0.00042 2088 0.00042 2088 0.00042 2179 0.00042 2179 0.00052 2110 0.00047 2158 0.00047 2158 0.00069 2013 2110 0.00067 2159 0.00078 2139	189_stal FLJ12248 189_stal FLJ12248 190_st Fally scid binding protein 7, brain 1965_stal CDC28 protein kinase regulatory subunit 1 B 187_stal Micogen-activated protein kinase-activated protein kinase 3 187_stal FLJ10534 121_st CVC10-2 127_st Phospboglycerate dehydrogenase 129_st_at ABO Blood group 06_st_at Hypothetical gene CG018 15_st KIAA0652 25_st_at PTPR interacting protein; alpha 1 44s_st_at CAoran-binding protein; AA 55_st_at PONPerase (DNA directed), gamma 885_st_at EST 109_st_at EST				0.00115 0.00117 0.00123 0.00123 0.00124	214831_at 207839_s_at 207484_s_at 210223_s_at 215455_at	FLJ21291 NAG-5 protein HLA-8 associated transcript 8 Make biscompatibility complex class Liike services
0.00007 2018 0.00007 2018 0.0001 20279 0.00016 22219 0.00016 22219 0.00016 22019 0.00021 2169 0.00022 2149 0.00025 2184 0.00028 2208 0.00028 2208 0.00028 2208 0.00032 2163 0.00032 2163 0.00032 2163 0.00034 2174 0.00035 2102 0.00037 2110 0.0004 2147 0.0004 2147 0.0005 2110 0.0006 207 0.0006 207 0.0006 207 0.0006 207 0.0006 207 0.0006 2110 0.0006 2110 0.0006 210 0.0007 2125 0.0007 2125 0.0007 2125 0.0007 2125 0.0007 2155 0.0007 2155 0.0	Jose at Party secio snoing protein /, orain Jose at CZ22 protein kinase regulatory subunit 1B 187s.at Milogen-activated protein kinase 3 187s.at JIJ0534 213_at OVC10-2 297_s.at FLJ10534 292_s.zt ABO blood group 96_s.zt Hypothetical gene CG018 815_s.t KIAA06532 255_s.at PTPR interacting protein, alpha 1 448_s.zt Ovoran-binding protein 2A 855_s.at EXP interacting protein, alpha 1 845_s.at EXT 835_s.at EXP interacting protein 2A 835_s.at EXP interacting protein 2A 835_s.at EXP interacting protein 3A				0.00117 0.00123 0.00123 0.00124	207839_s_at 207484_s_at 210223_s_at 215456_at	NAG-5 protein HLA-B associated transcript 8 Malor bistocompatibility complex class Like second
0.0001 / 2013 0.0001 / 2013 0.00012 / 2199 0.00016 / 2013 0.00022 / 2149 0.00022 / 2149 0.00022 / 2149 0.00022 / 2149 0.00022 / 2148 0.00023 / 2158 0.00023 / 2168 0.00031 / 2163 0.00031 / 2163 0.00031 / 2163 0.00032 / 2148 0.00031 / 2163 0.00034 / 2023 0.00035 / 2019 0.00035 / 2198 0.00042 / 2135 0.00042 / 2143 0.00042 / 2143 0.00045 / 2163 0.00045 / 2163 0.00046 / 2173 0.00051 / 279 0.00052 / 2110 0.00052 / 2110 0.00052 / 2110 0.00052 / 2110 0.00056 / 2153 0.00069 / 2153 0.00067 / 2158 0.00077 / 2158	System COL20 protein kinase regulatory subulit 15 SFT_st Millingen-activitated protein kinase-activated protein kinase 3 SFT_st FL10534 SFT_st FL20534 SFT_st Hypothetical gene CG018 ST_st FL2053-st SFT_st Corant-binding protein 2A SFT_st COArant-binding protein 2A SFT_st EST_st SFT_st EST_st ST_st EST_st SFT_st EST_st ST_st EST_st				0.00123 0.00123 0.00124	207484_s_at 210223_s_at 215456_at	HLA-B associated transcript 8 Major histocompatibility complex, class Like services
0.00012 2194 0.00012 2194 0.00016 2021 0.00016 2021 0.00016 2021 0.00016 2013 0.00022 21469 0.00022 21469 0.00022 2148 0.00022 2128 0.00022 2128 0.00022 2128 0.00031 2176 0.00031 2176 0.00031 2176 0.00031 2176 0.00031 2176 0.00031 2176 0.00031 2176 0.00032 2188 0.00031 2176 0.00032 2188 0.00031 2176 0.00032 2188 0.00032 2188 0.00042 2076 0.00045 2167 0.00045 2167 0.00045 2176 0.00045 2176 0.0005 2170 0.0005 2176 0.0005 2170 0.0	16 Joint State				0.00123	210223_s_at	Major histocompatibility complex class Like company
0.00016 2201 0.00016 2201 0.00016 2201 0.00016 2201 0.00012 2169 0.00022 2169 0.00025 2188 0.00025 2188 0.00025 2188 0.00035 2101 0.00032 2168 0.00032 2168 0.00037 2101 0.00037 2101 0.0004 2174 0.0004 2174 0.0005 2110 0.0005 2100 0.0005 2100 0.0005 2100 0.0005 2100 0.0005 2100 0.0005 2100 0.0005 2100 0.0005 2100 0.0005 21000 0.0005 210000 0.0005 2100000	11 at OVC10-2 13 at OVC10-2 197 at Phosphoglycosta dehydrogenase 297 at ABO blood group 906 x_at Hypothetical gene CG018 815 at KIAA0632 25 _ at PTPRF interacting protein, alpha 1 845 x_at Odorant-binding protein 2A 855 _ at Polymerase (DNA directed), gamma 855 _ at EST 14 _ trail_DNA divorvalue				0.00124	215456 at	major matocompany complex, class rake sequence
0.00016 2013 0.00016 2013 0.00022 21469 0.00022 21469 0.00022 21489 0.00022 21489 0.00022 21489 0.00023 2102 0.00024 2102 0.00031 21761 0.00031 21761 0.00031 21761 0.00031 21761 0.00031 21761 0.00031 21761 0.00032 2188 0.00033 2176 0.00032 2188 0.00032 2188 0.00032 2188 0.00032 2188 0.00032 2188 0.00032 2188 0.00032 2188 0.00045 2105 0.00045	113_at OVC/Ore 97_at Phosphoglycerate dehydrogenase 275_x_at ABO blood group 065_x_at Hypothetical gene CG018 815_at KIAA0652 255_x_at PTPR interacting protein, alpha 1 845_x_at Odorant-binding protein 2A 55_x_at PDynerase (DNA directed), gamma 885_x_at EST 103_x_at EST				0 00405	210400_at	EST
0.00071 21692 0.00072 21692 0.00072 21490 0.00072 21490 0.00072 21490 0.00075 2183 0.00078 21072 0.00078 21072 0.00073 21010 0.00031 2174 0.00031 2174 0.00031 2174 0.00031 2174 0.00031 2174 0.00031 2174 0.0004 2177 0.0004 2177 0.00052 2110 0.00052 2150 0.00052 2150 0.00052 2150 0.00052 2150 0.00052 2150 0.00052 2150 0.00052 2150 0.00052 2150 0.00052 2150 0.00052	yy an Protopology version deny drogenase yy at ADO blood group 906 x, zt Hypothetical gene CG018 15 at K1AA0632 235 _ st PTPRF interacting protein alpha 1 445 x, at Odorant-binding protein 2A 85 _ st EST 85 _ st EST 1				0.00125	220702_at	PRO2037
0.00022 2149 0.00022 2149 0.00025 2158 0.00025 2158 0.00025 2158 0.00025 2158 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00031 2175 0.00032 2168 0.00031 2175 0.00032 2168 0.00031 2175 0.00032 2168 0.00032 2168 0.00032 2168 0.00032 2168 0.00032 2168 0.00032 2168 0.00032 2175 0.00052 2170 0.00052 2170 0.000	227_a in Abo Ginos group 027_a in Abo Ginos group 155_at FIAA0652 255_at FIAPR interacting protein alpha 1 445_at Odoran-binding protein 2A 555_at Polymerase (DNA directed), gamma 885_at EST 107_at EST				0.00127	203684_s_at	B-cell CLL/lymphoma 2
0.00022 2138 0.00022 2138 0.00022 2138 0.00022 2102 0.00032 2138 0.00032 2138 0.00032 2138 0.00033 2148 0.00033 2148 0.00035 2110 0.0003 2130 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2158 0.0004 2157 0.0004 2158 0.0004 2157 0.0004 2158 0.0004 2157 0.0004 2158 0.0004 2157 0.0004 2158 0.0004 2157 0.0004 2158 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2158 0.0005 2158 0.00058 2158 0.00058 2158 0.0005	No 2, at hypotactcal gene COULS 85_at KIAA0652 235_at PTPRF interacting protein, alpha 1 848_x.at Odorant-binding protein 2A 55_at Polymerase (DNA directed), gamma 85_at EST 10_at Uracil-DNA alworotase				0.00127	220128_s_at	FLJ13955
0.0002 2138 0.00026 2102 0.00026 2102 0.00003 12163 0.00031 2163 0.00031 2163 0.00031 2163 0.00034 2033 0.00034 2033 0.00035 2019 0.00035 2019 0.00035 2105 0.00042 2068 0.00045 2105 0.00045 2105 0.00052 2106 0.00052 2105 0.00052 2105 0.000	25 at PTR interacting protein, alpha 1 25 at Odorant-binding protein 2A 25 at Polymerase (DNA directed), gamma 85 at EST Lincib.DNA showsthese				0.00129	217723_x_at	Ribosomal protein S12
0.00052 2102 0.00032 2103 0.00032 2105 0.00032 2105 0.00032 2105 0.00032 2105 0.00032 2105 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0005 215 0.0005	485 x at Odorant-binding protein 2A 635 s at Polymerase (DNA directed), gamma 885 s at EST 30 s at UrridDNA elycosylase				0.0013	213368_x_at	PTPRF interacting protein, alpha 3
0,00013 2176 0,00013 2176 0,00013 2176 0,00014 2013 0,00014 2013 0,00014 2013 0,00014 2013 0,00014 2013 0,00014 2013 0,00015 2019 0,00015 2101 0,00045 2105 0,00045 2105 0,00052 2170 0,00052 2150 0,00052 2150 0,00052 2150 0,00052 2150 0,00052 2150 0,00052 2150 0,00052 2150 0,000	535_s_at Doornaneomong protein 2A 635_s_at Polymerase (DNA directed), gamma 885_s_at EST 330_s_at Lizrdi-DNA elwoorvlase				0.00135	211942_x_at	Ribosomal protein L13a
0.00051 21103 0.00052 2168 0.00052 2168 0.00054 21744 0.00053 2105 0.00054 21744 0.00053 2105 0.00042 2105 0.00042 2105 0.00045 2105 0.00045 2105 0.00045 2105 0.00045 2105 0.00045 2105 0.00047 2158 0.00052 2170 0.00052 2150 0.00052 2150 0.	885_s_at EST 3.0 s at Uncil-DNA elwoorvlase				0.00136	220677_s_at	ADAMTS8
0.0002 2105 0.0004 2023 0.0004 2023 0.0003 2019 0.0003 2019 0.0003 2019 0.0003 2110 0.0004 2147 0.0004 2147 0.0005	330 s at Uracil-DNA elycosylase				0.00139	219809_at	FLJ20195
0.00034 2174 0.00034 2174 0.00037 2110 0.00037 2110 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2147 0.0004 2167 0.00045 2167 0.00045 2167 0.00045 2167 0.00045 2167 0.00047 2158 0.00052 2170 0.00052 21					0.00143	209100_at	Interferon-related developmental regulator 2
0.0003 2005 0.0003 2005 0.0003 2010 0.0003 2010 0.0004 2008 0.0004 2008 0.0004 2008 0.0004 2008 0.0004 2007 0.0004 2017 0.0004 2017 0.0004 2017 0.0004 2017 0.0004 2017 0.0005 2110 0.0005 2110 0.0005 2110 0.0005 2017 0.0005 2017 0.0005 2017 0.0005 2110 0.0005 210 0.0005 210005 210005 210005 210005 210005 2100	400 at Manaia VA (hanna anhanatida 12 munaia)				0.00145	219417_s_at	FLJ20014
0.0003 2009 0.0003 2010 0.0003 2213 0.0004 2147 0.0004 2168 0.0005 2140 0.0005 2105 0.0004 2105 0.0004 2105 0.0004 2170 0.0007 2158 0.0005 2110 0.0005 210 0.0005 200 0.0005 2000 0.0005 2000 0.0005 2000 0.0005 2000 0.0005 2000 0.0005 200000000000000000000000000000000	wy at wyoun wy (neavy polypepude 12, myoun)				0.00146	209320_at	Adenylate cyclase 3
0.00039 2213 0.00042 20683 0.00042 20683 0.00042 20683 0.00045 21403 0.00045 21403 0.00045 21403 0.00045 21053 0.00045 21053 0.00046 22074 0.00046 22074 0.00046 22074 0.00052 2170 0.00052 2150 0.00052	990 3 at ES1				0.00147	213590_at	Solute carrier family 16, member 5
0.00037 2213 0.0004 21473 0.0004 21048 0.0004 21048 0.0004 2105 0.00045 21405 0.00045 2105 0.00046 2207 0.00045 2170 0.00045 2170 0.00052 2110 0.00052 2110 0.00057 2155 0.00077 2155 0.000	10_s_at Natural cylotoxicity triggering receptor 5				0.00147	201957_at	Protein phosphatase 1, regulatory (inhibitor) subunit 128
0.0004/2 2005 0.0004/2 2005 0.0004/2 2005 0.0004/2 2005 0.0004/2 2140 0.0005/2 2140 0.0005/2 2157 0.0005/2 2170 0.0005/2 2170 0.		and the second se			0.00151	221361_at	Olfactory marker protein
0.00042 2008 0.00045 21405 0.00045 21405 0.00046 22077 21580 0.00049 21979 0.00055 21105 0.00055 2210 0.00055 22110 0.00055 22110 0.00055 22110 0.00066 20035 0.00066 20135 0.00066 20135 0.00072 2155 0.00072 2158 0.00072 2158 0.00072 2158 0.00075 2157 0.00055 2177 0.00055 2175 0.00055 2155 0.00055 21555 0.00055 21555 0.00055 21555 0.00055 21555 0.00055 21	736_3_at E31				0.00154	179_at	Postmelotic segregation increased 2-like 5
0.00045 21407 0.00045 21407 0.00045 2105 0.00047 21580 0.00047 21580 0.00047 21580 0.00052 22170 0.00052 22170 0.00052 22170 0.00052 20170 0.00052 20170 0.00052 20170 0.00067 2053 0.00078 2153 0.00078 2153 0.00078 2153 0.00078 2153 0.00078 2153 0.00078 2153 0.00077 2156 0.00077 2156 0.00077 2156 0.00077 2157 0.00067 2157 0.00077 2157 0.00077 0.00077 2157 0.00077 0.00077 0.00077 2157 0.00077 0.00077 2157 0	233 e st Choling kinger				0.00157	209502_s_at	BAI1-associated protein 2
0.00045 21055 0.00046 22074 0.00047 21958 0.00051 20799 0.00052 21710 0.00052 21710 0.00052 21710 0.00052 21710 0.00056 2053 0.00078 2155 0.00078 21	DTS at EST				0.00158	35179_at	Beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I)
0.0004 22077 0.00047 21580 0.00049 21978 0.00059 21978 0.00052 22110 0.00052 2210 0.00052 2210 0.00052 2210 0.00056 2053 0.00066 2053 0.00069 2013 0.00078 2155 0.00078 2157 0.00078 2157 0.00056 2157 0.00057 2156 0.00078 2157 0.00078 2157 0	504 x at Muelin protein zero-like 1		-		0.00159	215616_s_at	KIAA0876
0.00047 21580 0.00047 21580 0.00052 21700 0.00052 21700 0.00052 21700 0.00053 2110 0.00066 20635 0.00069 20635 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00077 2156 0.00077 2157 0.00072 2158 0.00077 2157 0.00072 2158 0.00077 2157 0.00072 2158 0.00077 2157 0.00072 2158 0.00072 2159 0.00072 2150 0.00072 210	743 at PRO0149				0.0016	32540_at	EST
0.00049 21977 0.00052 2110 0.00052 2110 0.00052 2110 0.00052 2210 0.00062 2053 0.00069 2013 0.00069 2013 0.00078 2193 0.00078 2193 0.00078 2193 0.00078 2193 0.00077 2156 0.00087 2157 0.00077 2156	807 s at Plevin B1		-		0.0016	219845_at	Bart-like nomeobox 1
0.00051 2079 0.00052 2110 0.00052 22116 0.00053 2110 0.00052 2017 0.00065 20057 0.00066 20057 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00074 2155 0.00075 2157 0.00075 2157 0.00075 0.00075 2157 0.00075 0.00075 2157 0.00075 2157 0.00075 2157	787 s at Epithelial cell transforming sequence 2 oncogene				0.00162	206141_at	Motybdopterin synthase sulfurylase
0.00052 2170 0.00052 21716 0.00052 22116 0.00053 21110 0.0006 20673 0.00062 20673 0.00062 20673 0.00074 21556 0.00077 2156 0.00077 2156 0.00087 21277 0.00087 21277	200 at Chemokine (C-C motif) ligand 17				0.00163	216407_81	Tax1 (numan 1-ceil leukemia virus type I) binding protein 1
0.00052 22116 0.00053 22116 0.0005 20675 0.00065 20135 0.00069 20135 0.00078 21556 0.00078 21556 0.00077 21566 0.00057 21273 0.00057 21275 0.00057 21275 0.00057 2156	040 x at SRY (sex determining region Y)-box 15				0.00168	214027_X_8t	Lesmin
0.00053 21110 0.0006 20678 0.00062 20678 0.00062 20678 0.00079 21359 0.00078 21931 0.00079 21886 0.00077 21566 0.00087 21277 0.00087 21259	162 at HERV-H LTR-associating 1				0.00168	208118_x_at	LATI-31M protein
0.0006 20673 0.00066 20633 0.00074 21555 0.00079 21880 0.00079 21880 0.00087 21596 0.00087 21593 0.00087 21593	104 s at Myosin VIIA (Usher syndrome 1B)				0.00109	200191_8_8t	MGC4293 Prostate enithelium energific Ete transadiction factor
0.00066 20639 0.00069 20139 0.00074 21556 0.00078 21836 0.00079 21886 0.00087 21566 0.00087 21579 0.00087 21579 0.00087 20599	781 at DnaJ (Hsp40) homolog, subfamily C, member 4			STREET, SQUARE,	0.00174	213442_X_8L	Prostate epimenum-specific Ets transcription factor
0.00069 20135 0.00074 21556 0.00078 21931 0.00079 21886 0.00087 21556 0.00087 21556 0.00087 21556 0.00087 21595 0.00087 20599	393 at Troponin I, skeletal, fast				0.00177	215482 8 81	Fukanotic translation initiation factor 28 submit 4 date 675
0.00074 2155 0.00078 2193 0.00079 2188 0.00087 2156 0.00087 2156 0.00087 2157 0.00087 2059	396 s at Small glutamine-rich tetratricopeptide repeat-containing				0.00170	217420 6 21	Pohymersee (PNA) II (DNA directed) pohymentide A 22040
0.00078 2193 0.00079 2188 0.00087 2156 0.00087 2156 0.00087 2157 0.00087 20598	561 s at FLJ23150				0.00184	221628 8 21	Cutokine like nuclear factor p.pac
0.00079 21886 0.00087 21566 0.00087 21273 0.00087 20598	314 s_at Zinc finger protein 219	and the second se			0.00181	217264 9 21	Sodium channel nonvoltane-neted 1 alpha
0.00087 2156 0.00087 21273 0.00087 20598 0.00087 20598	865_at FLJ22390				0.00183	208730 x at	RAR2 member RAS oncorene family
0.00087 21273 0.00087 20598 0.00089 20598	669 at Major histocompatibility complex, class II, DR beta 4				0.00184	207017 at	PAB27B member PAS oncogene family
0.00087 20598	739 s at Non-metastatic cells 4, protein expressed in				0.00103	200695 at	Protein phosphatase 2 regulatory subunit & sinhs insform
0.00080 20823	986 at Apoptosis-associated tyrosine kinase			States and	0.00104	217485 × ~*	Protein prosphatase 2, regulatory suburit A, alpha isolorm
0.00087 20822	222 at Activin A receptor, type IB	-	_	-	0.00194	217405_X_at	Integrin hete 1 hinding pretain (mehrsin) 2
0.0009 21356	563 s at Tubulin, gamma complex associated protein 2	D			0.00169	219629_at	KIAA0930
0.0009 20707	070 at Retinal G protein coupled receptor	D			0.00166	2125/3_at	Arginino, alutamic acid dinantida (PE) reposte
0.00092 20825	255 s at FK506-binding protein 8 (38kD)				0.00136	2121043_8_at	START domain containing 12
0.00094 21899					0.00136	213103_at	Tauthulin kinase
0.00096 22006	991 at FLJ22087				0.00093	213922_8	Noteh hemolog 2 (Dressphile)
0.00097 20349	991_at FLJ22087 065_at Tenomodulin protein		_	a second s	0.0005/	2123/7_8_81	Chemoldine (C.C. motif) ligand 7
0.00098 21159	991_at FLJ22087 D65_at Tenomodulin protein 451_at LIM domain binding 1				0.00041	2080/5_8_8t	Absort is molecome 2
0.001 20312	P91_at FLJ22087 D65_at Tenomodulin protein 451_at L1M domain binding 1 S57 x at Solute carrier family 21, member 9				0.0003	200013_at	Advantation and SOCS hav containing 4
0.001 20719	991 at FLJ2087 065 at Tenomodulin protein 051 at LIM domain binding 1 557 x.at Solute carrier family 21, member 9 125 x.at Solute carrier family 11, member 2			100	0.00021	217228_81	Nuclear aretain double minute 1
0.00101 20631	99] at FLJ22087 065 at Tenomodulin protein 151 at L1M domain binding 1 557 x, at Solute carrier family 21, member 9 125 x, at Solute carrier family 11, member 2 157 x at K1AA0616				0.00021	213/01_at	Nuclear protein double minute 1

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 glycosy*lase*), 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 antiapoptotic 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 y. 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

A Patient

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 MAP-KAPK3 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

Table 3

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

<i>P</i> ^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.

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).

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 singleinstitution 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.

Acknowledgments

We thank Dr. Richard Simon and Dr. Amy Peng for providing us with the BRB-ArrayTools software, and Dr. Michael B. Eisen for use of the Cluster and TreeView software. We also thank Dr. Hiroki Sasaki for critical review of the manuscript, Dr. Hee Jin Chang for technical advices, and Dr. Cheol-Goo Hur for his help with data analysis.

References

- L.R. Kelland, Preclinical perspectives on platinum resistance, Drugs 59 (Suppl. 4) (2000) 1–8.
- [2] B.A. Teicher, T.S. Herman, S.A. Holden, Y.Y. Wang, M.R. Pfeffer, J.W. Crawford, E. Frei 3rd, Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo, Science 247 (4949 Pt. 1) (1990) 1457–1461.

- [3] R.M. Mader, M. Muller, G.G. Steger, Resistance to 5-fluorouracil, Gen. Pharmacol. 31 (5) (1998) 661–666.
- [4] E.J. Yeoh, M.E. Ross, S.A. Shurtleff, W.K. Williams, D. Patel, R. Mahfouz, F.G. Behm, S.C. Raimondi, M.V. Relling, A. Patel, C. Cheng, D. Campana, D. Wilkins, X. Zhou, J. Li, H. Liu, C.H. Pui, W.E. Evans, C. Naeve, L. Wong, J.R. Downing, Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling, Cancer Cell 1 (2) (2002) 133–143.
- [5] N.K. Kim, Y.S. Park, D.S. Heo, C. Suh, S.Y. Kim, K.C. Park, Y.K. Kang, D.B. Shin, H.T. Kim, H.J. Kim, W.K. Kang, C.I. Suh, Y.J. Bang, A phase III randomized study of 5-fluorouracil and cisplatin versus 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil alone in the treatment of advanced gastric cancer, Cancer 71 (12) (1993) 3813–3818.
- [6] U. Vanhoefer, P. Rougier, H. Wilke, M.P. Ducreux, A.J. Lacave, E. Van Cutsem, M. Planker, J.G. Santos, P. Piedbois, B. Paillot, H. Bodenstein, H. Schmoll, H. Bleiberg, B. Nordlinger, M. Couvreuer, B. Baron, J.A. Wils, Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus etoposide, leucovorin, and fluorouracil versus infusional fluorouracil and cisplatin in advanced gastric cancer: a trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group, J. Clin. Oncol. 18 (14) (2000) 2648–2657.
- [7] A. Ohtsu, Y. Shimada, K. Shirao, N. Boku, I. Hyodo, H. Saito, N. Yamamichi, Y. Miyata, N. Ikeda, S. Yamamoto, H. Fukuda, S. Yoshida, Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with unresectable, advanced gastric cancer: The Japan Clinical Oncology Group Study (JCOG9205), J. Clin. Oncol. 21 (1) (2003) 54–59.
- [8] A.B. Miller, B. Hoogstraten, M. Staquet, A. Winkler, Reporting results of cancer treatment, Cancer 47 (1981) 207–214.
- [9] M. Dole, G. Nunez, A.K. Merchant, J. Maybaum, C.K. Rode, C.A. Bloch, V.P. Castle, Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma, Cancer Res. 54 (12) (1994) 3253– 3259.
- [10] H. Miyake, N. Hanada, H. Nakamura, S. Kagawa, T. Fujiwara, I. Hara, H. Eto, K. Gohji, S. Arakawa, S. Kamidono, H. Saya, Overexpression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin and adenoviral-mediated p53 gene transfer, Oncogene 16 (7) (1998) 933–943.
- [11] V. Wacheck, E. Heere-Ress, J. Halaschek-Wiener, T. Lucas, H. Meyer, H.G. Eichler, B. Jansen, Bcl-2 antisense oligonucleotides chemosensitize human gastric cancer in a SCID mouse xenotransplantation model, J. Mol. Med. 79 (10) (2001) 587–593.
- [12] E. Chu, G.M. Lai, S. Zinn, C.J. Allegra, Resistance of a human ovarian cancer line to 5-fluorouracil associated with decreased levels of 5-fluorouracil in DNA, Mol. Pharmacol. 38 (3) (1990) 410–417.
- [13] D.J. Mauro, J.K. De Riel, R.J. Tallarida, M.A. Sirover, Mechanisms of excision of 5-fluorouracil by uracil DNA glycosylase in normal human cells, Mol. Pharmacol. 43 (6) (1993) 854– 857.
- [14] A. Vaisman, S.E. Lim, S.M. Patrick, W.C. Copeland, D.C. Hinkle, J.J. Turchi, S.G. Chaney, Effect of DNA polymerases and high mobility group protein 1 on the carrier ligand specificity for translesion synthesis past platinum–DNA adducts, Biochemistry 38 (34) (1999) 11026–11039.
- [15] A. Fedier, U.B. Ruefenacht, V.A. Schwarz, U. Haller, D. Fink, Increased sensitivity of p53-deficient cells to anticancer agents due to loss of Pms2, Br. J. Cancer 87 (9) (2002) 1027–1033.
- [16] D. Choubey, S. Walter, Y. Geng, H. Xin, Cytoplasmic localization of the interferon-inducible protein that is encoded by the AIM2 (absent in melanoma) gene from the 200-gene family, FEBS Lett. 474 (1) (2002) 38–42.

- [17] T. Waerner, P. Gardellin, K. Pfizenmaier, A. Weith, N. Kraut, Human RERE is localized to nuclear promyelocytic leukemia oncogenic domains and enhances apoptosis, Cell Growth Differ. 12 (4) (2001) 201–210.
- [18] P. Perego, M. Giarola, S.C. Righetti, R. Supino, C. Caserini, D. Delia, M.A. Pierotti, T. Miyashita, J.C. Reed, F. Zunino, Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems, Cancer Res. 56 (3) (1996) 556–562.
- [19] D.E. Fisher, Apoptosis in cancer therapy: crossing the threshold, Cell 78 (1994) 539–542.
- [20] D.S. Alberts, J.K. Noel, Cisplatin-associated neurotoxicity: can it be prevented? Anticancer Drugs 6 (3) (1995) 369–383.
- [21] S. Ramaswamy, T.R. Golub, DNA microarrays in clinical oncology, J. Clin. Oncol. 20 (7) (2002) 1932–1941.
- [22] V. Luzzi, V. Holtschlag, M.A. Watson, Expression profiling of ductal carcinoma in situ by laser capture microdissection and highdensity oligonucleotide arrays, Am. J. Pathol. 158 (6) (2001) 2005– 2010.