Protein Expression Profiling and Molecular Classification of Gastric Cancer by the Tissue Array Method

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Abstract

Purpose: Gastric cancer is heterogeneous clinically and histologically, and prognosis prediction by tumor grade or type is difficult. Although previous studies have suggested that frozen tissue–based molecular classifications effectively predict prognosis, prognostic classification on formalin-fixed tissue is needed, especially in early gastric cancer.

Experimental Design: We immunostained 659 consecutive gastric cancers using 56 tumor-associated antibodies and the tissue array method. Hierarchical cluster analyses were done before and after feature selection. To optimize classifier number and prediction accuracy for prognosis, a supervised analysis using a support vector machine algorithm was used.

Results: Of 56 gene products, 27 survival-associated proteins were selected (feature selection), and hierarchical clustering identified two clusters: cluster 1 and cluster 2. Cluster 1 cancers were more likely to have intestinal type, earlier stage, and better prognosis than cluster 2 ($P < 0.05$). In 187 early gastric cancers (pT1), cluster 2 was associated with the presence of metastatic lymph nodes ($P = 0.026$). Kaplan-Meier survival curves stratified by pathologic tumor-lymph node metastasis revealed that cluster 2 was associated with poor prognosis in stage I or II cancer ($P < 0.05$). Support vector machines and genetic algorithms selected nine classifiers from the whole data set, another nine classifiers for stage I and II, and eight classifiers for stage III and IV. The prediction accuracies for patient outcome were 73.1%, 88.1%, and 76%, respectively.

Conclusions: Protein expression profiling using the tissue array method provided a useful means for the molecular classification of gastric cancer into survival-predictive subgroups. The molecular classification predicted lymph node metastasis and prognosis in early stage gastric cancer.

The incidence and mortality of gastric cancer have declined steadily over the past several decades. Nonetheless, gastric cancer remains a major public health issue as the fourth most common cancer and the second leading cause of cancer death worldwide (1, 2). Gastric cancer is a heterogeneous disease both histologically and genetically, and patient outcome is difficult to predict using classic histologic and molecular classifications. Many histopathologic classifications, including histologic type and grade by WHO (3), Lauren's classification (4), Ming's classification (5), and Goseki classification (6) have been applied for the prediction of patient survival, but their usefulness remains controversial (3, 7).

The incidence of small and early gastric cancer is high in Asia because the increased usage of upper endoscopy has led to the earlier detection of lesions (8). However, despite curative resection of the primary tumor, some early gastric cancer patients succumb to the disease as a result of local or distant tumor recurrence. Adjuvant chemotherapy benefits some patients with early gastric cancer, but it is not necessary in all patients. Therefore, additional markers are required to identify those patients at risk of recurrence or poor prognosis. Recently, large-scale molecular techniques such as DNA microarrays have contributed to our understanding of the molecular complexity of gastric cancer and prognostic classification according to gene expression profile has been achieved using frozen tissue (9, 10). However, in small and early gastric cancer patients, the detection and sampling of proper cancer tissue using gross examination is difficult. In addition, the cost, complexity, and interpretation of DNA microarrays are currently unsuitable for routine use in standard clinical settings. Therefore, prognostic classification on formalin-fixed paraffin-embedded tissue is required, especially in small and early gastric cancer.

Many candidate gene products for the prediction of patient survival have been reported in gastric cancer (11, 12). Genetic alterations including those of p53, MUC1, CEA, E-cadherin, p16, and CD44 have been reported to play important roles in the development and progression of the disease (13–18). Although much has been learned of the genetic factors

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Received 1/21/07; revised 3/23/07; accepted 4/6/07.

Grant support: 21C Frontier Functional Human Genome Project grant FG06-11-03 from the Ministry of Science and Technology of Korea.

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doi:10.1158/1078-0432.CCR-07-0173

Table 1. Antibodies used for immunohistochemical study

Antibody	Retrieval methods	Dilution	Source	Nonneoplastic mucosa	Altered Expression in cancer
a-caspase-3	Microwave	1:100	IMGENEX	Faint stain	Nucleus + cytoplasm
APC	Microwave	1:400	Abcam	Cytoplasm ± nucleus	Loss
β-Catenin	Microwave	1:200	Transduction	Membranous	Nucleus
bcl-2	Microwave	1:100	DAKO	Negative	Nucleus
Caspase-1	Microwave	1:200	Santa Cruz Biotechnology	Cytoplasm	Loss
Caveolin-1	Microwave	1:250	Transduction	Negative	Cytoplasm + membranous
CD10	Autoclave	1:80	Novocastra	Negative	Membranous
CD24	Microwave		Neomarker	Negative	Membranous + cytoplasm
CD44	Microwave	1:40	Novocastra	Negative	Membranous
CEA	Microwave	1:50	DAKO	Negative	Cytoplasm
C-erbB2	Microwave	1:75	DAKO	Negative	Membranous
c-fos	Microwave	1:100	Santa Cruz Biotechnology	Nucleus	Nucleus
c-kit	Microwave	1:250	DAKO	Negative	Cytoplasm
Cytokeratin5	Microwave	1:100	Abcam	Negative	Membranous
Cytokeratin6	Microwave	1:100	Novocastra	Membranous*	Membranous
Cytokeratin7	Microwave	1:50	DAKO	Membranous	Membranous
Cytokeratin8	Microwave	1:100	DAKO	Membranous	Membranous
Cytokeratin14	Microwave	1:100	Novocastra	Negative	Membranous
Cytokeratin16	Microwave	1:20	Novocastra	Negative	Membranous
Cytokeratin17	Microwave	1:40	Novocastra	Negative	Membranous
Cytokeratin18	Microwave	1:100	DAKO	Membranous	Membranous
Cytokeratin19	Microwave	1:100	DAKO	Membranous	Membranous
Cytokeratin20	Microwave	1:50	DAKO	Membranous*	Membranous
DNA-PKcs	Microwave	1:100	Santa Cruz Biotechnology	Nucleus	Loss
E-cadherin	Microwave	1:200	Transduction	Membranous	Loss
FHIT	Microwave	1:250	Zymed	Nucleus	Loss
Gst-p	Microwave	1:5,000		Cytoplasm	Loss
HDAC1	Microwave	1:150	Santa Cruz Biotechnology	Nucleus	Loss
Hexokinase II	Microwave	1:100	Santa Cruz Biotechnology	Negative	Cytoplasm
Id4	Microwave	1:200	Santa Cruz Biotechnology	Faint stain	Cytoplasm
IRS-1	Microwave	1:200	Santa Cruz Biotechnology		
KAI1	Microwave	1:200	Santa Cruz Biotechnology	Cytoplasm	Loss
MAGE-A	Microwave	1:50	Zymed	Negative	Nucleus + cytoplasm
MGMT	Microwave	1:50	Chemicon	Nucleus	Loss
MUC1	Microwave	1:100	Novocastra	Negative	Cytoplasm
MUC2	Microwave	1:100	Novocastra	Cytoplasm*	Cytoplasm
MUC5AC	Microwave	1:100	Novocastra	Cytoplasm †	Cytoplasm
MUC6	Microwave	1:100	Novocastra	Cytoplasm †	Cytoplasm
Osteonectin	Microwave	1:100	DSHB	Cytoplasm	Loss
P16	Autoclave	1:50	PharMingen	Nucleus	Loss
P53	Microwave	1:100	DAKO	Negative	Nucleus
P63	Microwave	1:100	Santa Cruz Biotechnology	Negative	Nucleus
PML	Microwave	1:200	Medical and Biological Laboratories	Nucleus	Loss
PTEN	Microwave	1:50	A.G. Scientific	Cytoplasm	Loss
rad9	Microwave	1:100	IMGENEX	Nucleus	Loss
Rb	Microwave	1:50	PharMingen	Nucleus	Loss
S100A2	Microwave	1:100	DAKO	Negative	Nucleus + cytoplasm
S100A4	Microwave	1:500	DAKO	Negative	Nucleus + cytoplasm
S100A6	Microwave	1:750	DAKO	Faint stain	Nucleus + cytoplasm
SAP97	Autoclave	1:50	StressGen Biotechnologies Corp.	Membranous	Loss
smad4	Microwave	1:50	Santa Cruz Biotechnology	Nucleus	Loss
smad7	Microwave	1:100	Santa Cruz Biotechnology	Negative	Cytoplasm
Sp1	Microwave	1:200	Santa Cruz Biotechnology	Nucleus	Loss
TCF4	Autoclave	1:50	Upstate	Nucleus	Nucleus
VEGF	Microwave	1:250	Santa Cruz Biotechnology	Cytoplasm	Cytoplasm
XIAP	Microwave	1:50	BD Bioscience	Negative	Cytoplasm

Abbreviations: APC, adenomatous polyposis coli; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; FHIT, fragile histidine triad; HDAC1, histone deacetylase 1; IRS-1, insulin receptor substrate-1; KAI1, kangai 1; MAGE-A, melanoma antigen A; MGMT, O^6-methylguanine DNA-methyltransferase; PML, promyelocytic leukemia; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SAP97, synapse-associated protein 97; TCF4, T-cell factor 4; XIAP, X-linked inhibitor of apoptosis protein.

*Positive staining in intestinal metaplasia and negative staining in gastric glands.

†MUC5AC was positive in superficial glands and MUC6 was positive in deep glands of gastric mucosa.

predicting survival, only a few genetic alterations have been used for the diagnosis and management of patients with gastric cancer. Recently, large-scale molecular studies on formalin-fixed tissue have become possible by tissue array method using immunohistochemical approach. Such large-scale studies involve a relatively large number of markers in addition to a large number of cases. Moreover, combined or cluster analysis using multiple markers has been reported to be significantly correlated with patient survival (19, 20). Jacquemier et al. identified a set of 21 proteins using supervised analysis in breast cancer, the expressions of which were significantly correlated with metastasis-free survival (20).

In this study, we immunostained 659 consecutive gastric cancers using 56 tumor-associated antibodies and the tissue array method. Gastric cancer was subclassified using classic hierarchical clustering before and after feature selection. To obtain the optimal number of classifiers and prediction accuracy for patient outcome, we did supervised analysis using a support vector machine (SVM) algorithm.

Materials and Methods

Specimens. A total of 659 consecutive, surgically resected cases of primary gastric cancer treated over a period of 1 year were identified in

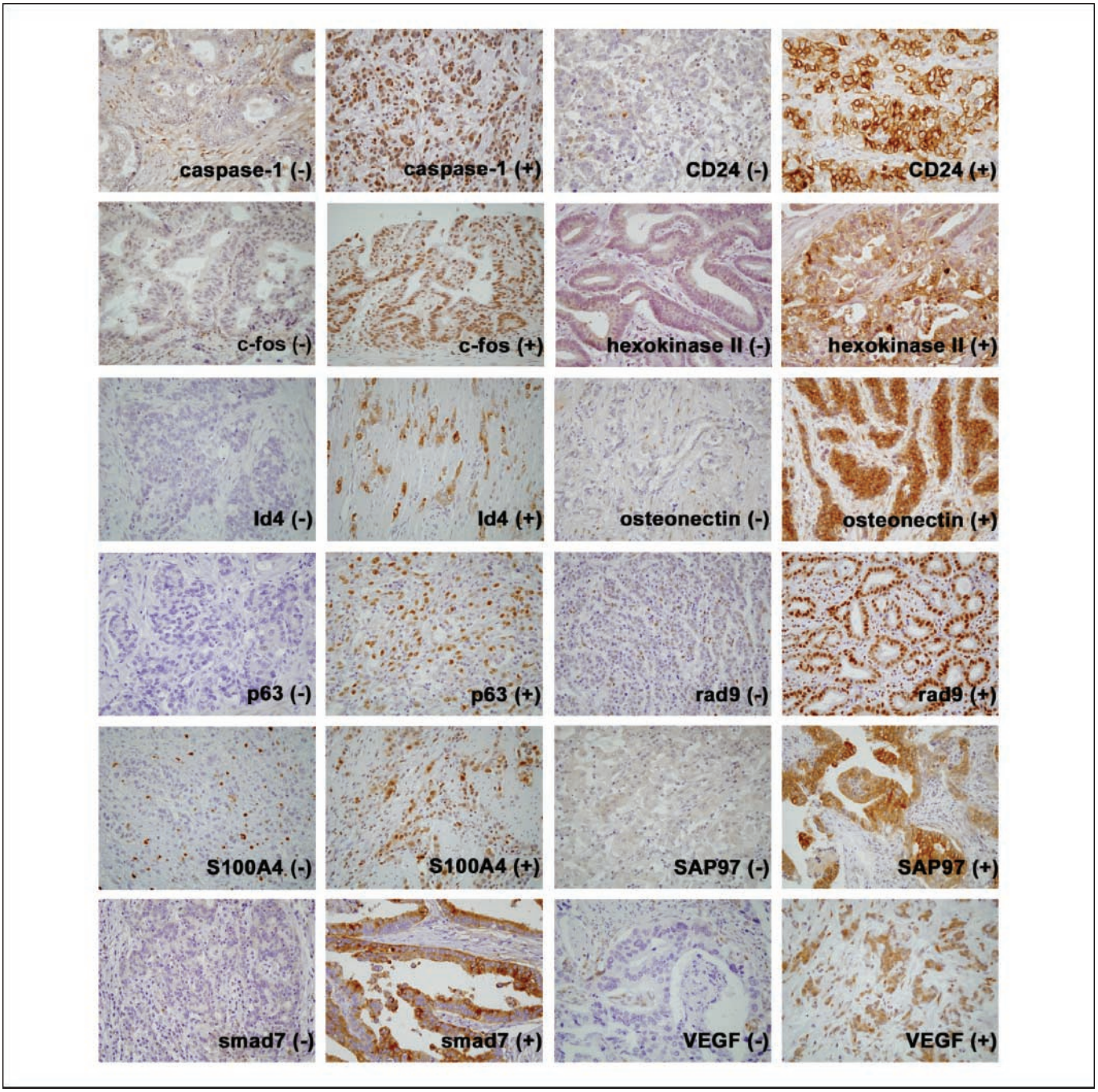

Fig. 1. Representative expressions of proteins studied by immunohistochemistry (original magnification, ×400).

Table 2. Expression rates of the 56 proteins in gastric cancer and 5-y survival rates

Antibody	Expression rate (%)		Univariate survival analysis using Kaplan-Meier curve 5-y survival rate according to expression status		*P*
	Negative	Positive	Negative	Positive	
a-caspase-3	203 (33.4)	405 (66.6)	51.91 ± 3.56	70.71 ± 2.29	<0.0001
APC	159 (25.1)	475 (74.9)	59.09 ± 3.93	67.05 ± 2.19	0.0325
β-Catenin	493 (81.0)	116 (19.0)	66.43 ± 2.15	58.13 ± 4.71	Not significant
bcl-2	564 (90.5)	59 (9.5)	64.11 ± 2.05	72.28 ± 5.90	Not significant
Caspase-1	116 (18.6)	507 (81.4)	43.01 ± 4.68	70.05 ± 2.06	<0.0001
Caveolin-1	630 (98.6)	9 (1.4)	44.44 ± 16.56	65.61 ± 1.92	0.0475
CD10	568 (91.9)	50 (8.1)	63.75 ± 2.04	72.87 ± 6.44	Not significant
CD24	429 (68.2)	200 (31.8)	69.66 ± 2.25	56.17 ± 3.57	0.0002
CD44	499 (82.2)	108 (17.8)	64.38 ± 2.18	62.75 ± 4.73	Not significant
CEA	359 (56.5)	276 (43.5)	69.00 ± 2.47	60.30 ± 3.00	0.0344
C-erbB2	595 (95.0)	31 (5.0)	65.44 ± 1.97	63.40 ± 8.79	Not significant
c-fos	393 (61.7)	244 (38.3)	58.81 ± 2.52	75.35 ± 2.79	<0.0001
c-kit	612 (98.7)	8 (1.3)	65.15 ± 1.95	62.50 ± 17.12	Not significant
Cytokeratin5	609 (97.8)	14 (2.2)	65.00 ± 1.96	66.08 ± 13.94	Not significant
Cytokeratin6	378 (66.5)	190 (33.5)	56.99 ± 2.59	77.60 ± 3.05	<0.0001
Cytokeratin7	175 (29.4)	421 (70.6)	60.25 ± 3.78	66.50 ± 2.33	Not significant
Cytokeratin8	25 (4.3)	555 (95.7)	60.00 ± 9.80	64.60 ± 2.06	Not significant
Cytokeratin14	629 (99.5)	3 (0.5)	65.88 ± 1.92	33.33 ± 27.22	Not significant
Cytokeratin16	587 (97.5)	15 (2.5)	64.54 ± 2.00	61.90 ± 13.44	Not significant
Cytokeratin17	627 (99.7)	2 (0.3)	65.61 ± 1.92	50.00 ± 35.36	Not significant
Cytokeratin18	27 (4.3)	595 (95.7)	58.20 ± 9.65	65.13 ± 1.98	Not significant
Cytokeratin19	68 (11.1)	546 (88.9)	65.67 ± 5.80	64.38 ± 2.08	Not significant
Cytokeratin20	455 (73.4)	165 (26.6)	65.38 ± 2.27	62.91 ± 3.80	Not significant
DNA-PKcs	114 (20.2)	450 (79.8)	54.69 ± 4.70	65.29 ± 2.29	0.0135
E-cadherin	265 (42.3)	361 (57.7)	55.33 ± 3.09	72.59 ± 2.39	<0.0001
FHIT	286 (47.5)	316 (52.5)	61.76 ± 2.91	67.76 ± 2.67	Not significant
Gst-p	18 (2.9)	605 (97.1)	71.43 ± 10.84	64.06 ± 1.98	Not significant
HDAC1	12 (2.0)	599 (98.0)	57.14 ± 14.62	65.05 ± 1.98	Not significant
Hexokinase II	523 (82.9)	108 (17.1)	68.11 ± 2.06	48.70 ± 4.92	<0.0001
Id4	308 (53.3)	270 (46.7)	60.01 ± 2.83	67.62 ± 2.89	Not significant
IRS-1	105 (17.5)	495 (82.5)	54.31 ± 4.97	66.43 ± 2.14	0.0303
KAI1	108 (17.1)	524 (82.9)	47.33 ± 4.85	68.97 ± 2.05	<0.0001
MAGE-A	561 (87.7)	79 (12.3)	67.07 ± 2.01	55.86 ± 5.66	Not significant
MGMT	89 (13.9)	549 (86.1)	56.34 ± 5.33	66.89 ± 2.04	0.0487
MUC1	494 (77.2)	146 (22.8)	70.22 ± 2.09	49.02 ± 4.18	<0.0001
MUC2	438 (71.8)	172 (28.2)	63.05 ± 2.34	67.01 ± 3.64	Not significant
MUC5AC	347 (54.6)	289 (45.4)	62.03 ± 2.65	69.55 ± 2.74	Not significant
MUC6	513 (81.8)	114 (18.2)	64.38 ± 2.15	67.58 ± 4.44	Not significant
Osteonectin	342 (55.9)	270 (44.1)	55.24 ± 2.73	76.46 ± 2.62	<0.0001
P16	179 (29.3)	432 (70.7)	60.63 ± 3.70	65.90 ± 2.32	Not significant
P53	429 (66.9)	212 (33.1)	69.84 ± 2.25	56.01 ± 3.46	0.0012
P63	616 (97.9)	13 (2.1)	65.79 ± 1.94	26.92 ± 12.98	0.0002
PML	74 (11.6)	562 (88.4)	54.19 ± 5.87	66.57 ± 2.02	0.0066
PTEN	157 (24.8)	477 (75.2)	45.46 ± 4.07	71.74 ± 2.09	<0.0001
rad9	55 (8.8)	573 (91.2)	41.54 ± 6.68	67.01 ± 1.99	<0.0001
Rb	19 (3.1)	596 (96.9)	63.16 ± 11.07	64.45 ± 1.99	Not significant
S100A2	616 (97.2)	18 (2.8)	65.24 ± 1.94	51.34 ± 12.43	Not significant
S100A4	545 (88.0)	74 (12.0)	67.31 ± 2.04	42.65 ± 5.79	<0.0001
S100A6	86 (14.7)	500 (85.3)	64.22 ± 5.24	63.23 ± 2.19	Not significant
SAP97	409 (64.6)	224 (35.4)	58.44 ± 2.48	77.21 ± 2.83	<0.0001
smad4	87 (13.7)	550 (86.3)	52.16 ± 5.40	67.09 ± 2.03	0.0019
smad7	465 (73.0)	172 (27.0)	69.14 ± 2.17	55.92 ± 3.83	0.0005
Sp1	21 (3.4)	595 (96.6)	52.38 ± 10.90	65.21 ± 1.98	Not significant
TCF4	390 (61.6)	243 (38.4)	56.41 ± 2.55	79.83 ± 2.60	<0.0001
VEGF	248 (39.6)	378 (60.4)	52.41 ± 3.23	72.97 ± 2.31	<0.0001
XIAP	467 (77.8)	133 (22.2)	65.03 ± 2.24	58.84 ± 4.34	Not significant

Abbreviations: APC, adenomatous polyposis coli; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; FHIT, fragile histidine triad; HDAC1, histone deacetylase 1; IRS-1, insulin receptor substrate-1; KAI1, kangai 1; MAGE-A, melanoma antigen A; MGMT, O^6-methylguanine DNA-methyltransferase; PML, promyelocytic leukemia; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SAP97, synapse-associated protein 97; TCF4, T-cell factor 4; XIAP, X-linked inhibitor of apoptosis protein.

the files of the Department of Pathology, Seoul National University College of Medicine (Seoul, Korea). Age, sex, tumor location, lymphatic invasion, vascular invasion, and pathologic tumor-lymph node metastasis (pTNM) stage (21) were evaluated by reviewing medical charts and pathologic records. The mean age of the 659 patients was 54.7 years, and 93% underwent curative resection (R0 according to the American Joint Committee on Cancer guideline). The study included 438 men and 221 women, and included 436 advanced and 223 early

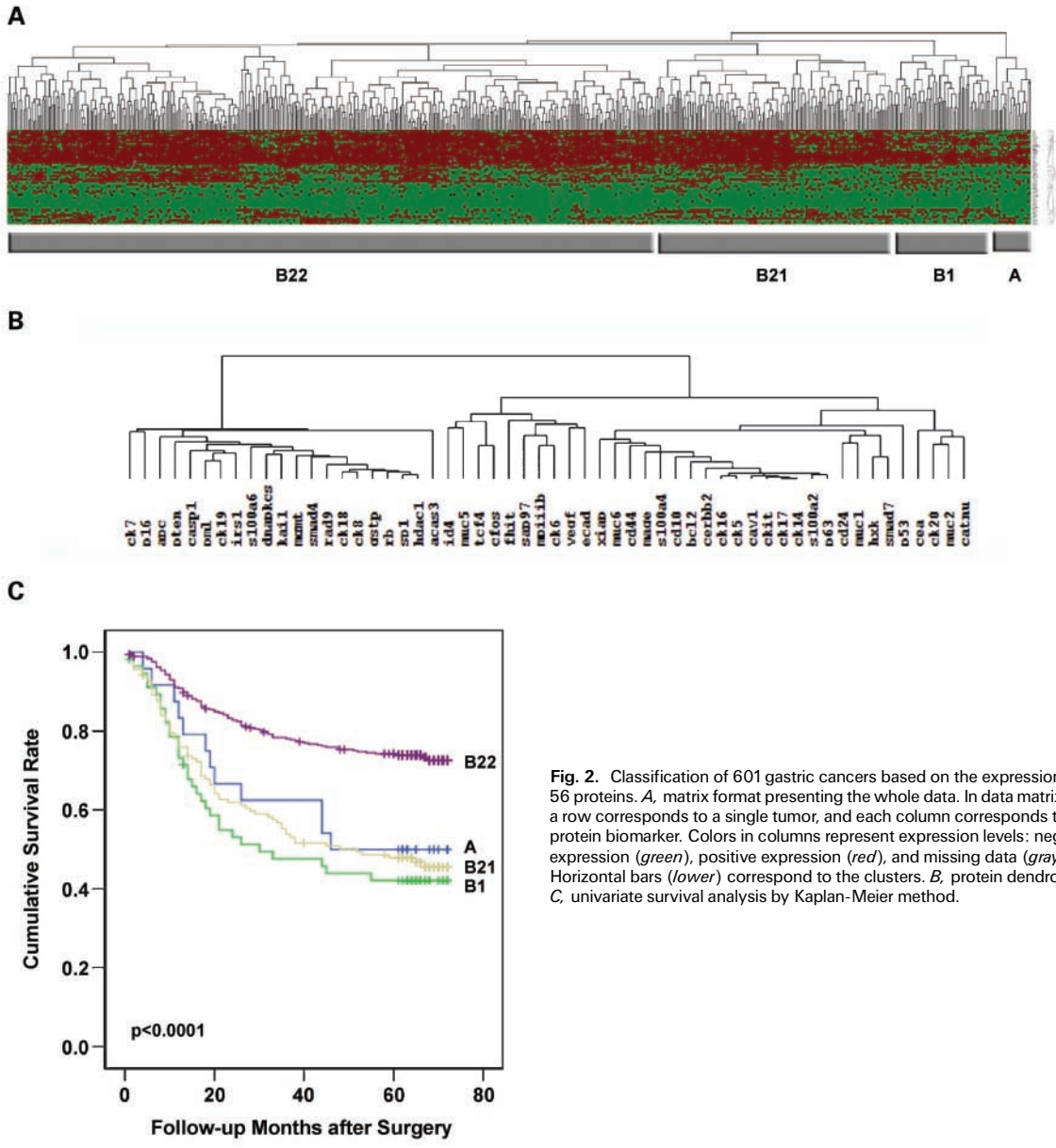

Fig. 2. Classification of 601 gastric cancers based on the expression of the 56 proteins. *A,* matrix format presenting the whole data. In data matrix (*upper*), a row corresponds to a single tumor, and each column corresponds to a single protein biomarker. Colors in columns represent expression levels: negative expression (*green*), positive expression (*red*), and missing data (*gray*). Horizontal bars (*lower*) correspond to the clusters. *B,* protein dendrogram. *C,* univariate survival analysis by Kaplan-Meier method.

gastric cancers. No patient had received preoperative chemotherapy or radiotherapy. Tissue slides were reviewed for histologic classifications (according to WHO and Lauren's classifications; refs. 3, 4). Patient clinical outcome was followed-up from the date of surgery up to a period of 1 to 72 months (mean, 52 months). The cases lost to follow-up and deaths from any other cause other than gastric cancer were regarded as censored data for the analysis of survival rates. This study was approved by the Institutional Review Board for Human Subject Research at Seoul National University Hospital.

Tissue array methods. Twelve array blocks containing a total of 659 cases were prepared as described previously (Superbiochips Laboratories; refs. 19, 22). Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumors (donor blocks) and arranged in recipient paraffin blocks (tissue array blocks) using a trephine. As it has previously been proven that staining results obtained from different intratumoral areas in various tumors agree well (22–25), a core was sampled in each case. An adequate case was defined as a tumor occupying >10% of the core area. Each block contained three internal controls consisting of nonneoplastic gastric mucosa from the body, antrum, and intestinal metaplasia. Four-micrometer-thick sections were cut from each tissue array block, deparaffinized and dehydrated.

Immunohistochemistry. Immunohistochemical staining against tumor-associated gene products was done using a streptavidin peroxidase procedure following an antigen retrieval process using microwaves or autoclaves. Commercially available antibodies were tested using a human control slide and a stomach cancer control slide for immunohistochemistry (Superbiochips Laboratories). After the test procedure, 56 antibodies, which were properly stained in each positive and negative control, were selected for this study. Table 1 and Fig. 1 list the antibodies used. The expression status of nonneoplastic gastric glands and altered expression patterns in gastric cancer are described in Table 1. For statistical analysis of collected data, immunostaining results were considered positive when ≥10% of neoplastic cells were stained

(19, 22). Immunoreactivity was assessed microscopically by two independent pathologists unaware of the clinical details of individual patients. The case-by-case final consensus result was discussed and determined in a common session.

Cluster analyses. Expression data were recorded as follows: -1 was designated negative staining, 1 was designated positive staining, missing data were left blank in the data table. In this study, hierarchical cluster analyses were done using the Cluster program (complete linkage clustering) and results were displayed using TreeView (26). All cases with missing values in >20% of the columns were excluded from the cluster analyses. We could perform an unsupervised analysis on 56 gene expressions in 601 gastric cancer samples (91.2%) of 659 consecutive samples. Of these 56 genes, 27 genes which showed significant correlations with patient outcome using the Kaplan-Meier survival method were selected (feature selection). After feature selection, we did a cluster analysis using these 27 gene expression profiles in 614 samples (93.2%) of 659 consecutive samples.

Supervised analysis using SVM and genetic algorithm. SVM and genetic algorithm were used to identify protein classifier sets associated with survival status (27). SVM is well known for its competency among off-the-shelf classification algorithms and its performance comes from mapping of the original feature space (data space) to the higher dimensional space. The hyperplane obtained in the higher dimensional space generally shows excellent classification performance. In addition to the classification power of the SVM algorithm, selection of the input variables was important to the performance of the classification algorithm. However, it is too computationally intensive to regard all the possible combinations of 56 genes. Therefore, the genetic algorithm was applied to select an optimal subset of tumor-associated genes that yielded the best result for classification of the patient's survival status. The genetic algorithm finds an optimal solution by simulating the natural genetic selection procedure. At each step of the gene selection, prediction accuracy with 10-fold cross-validation was calculated. Cross-validation is a widely used method for assessing the performance of a classification algorithm. It separates the whole data set into a training and test data set. For example, a 10-fold cross-validation assigns 9/10 of the whole data into the training set, and 1/10 of the whole data into the test set. When the training and test sets are assigned, the classification model is extracted from the training set and the performance of the model is examined in the test set. This process is repeated until all instances of the data set are assigned to the test set at once. By combining the SVM and genetic algorithm, the classifier

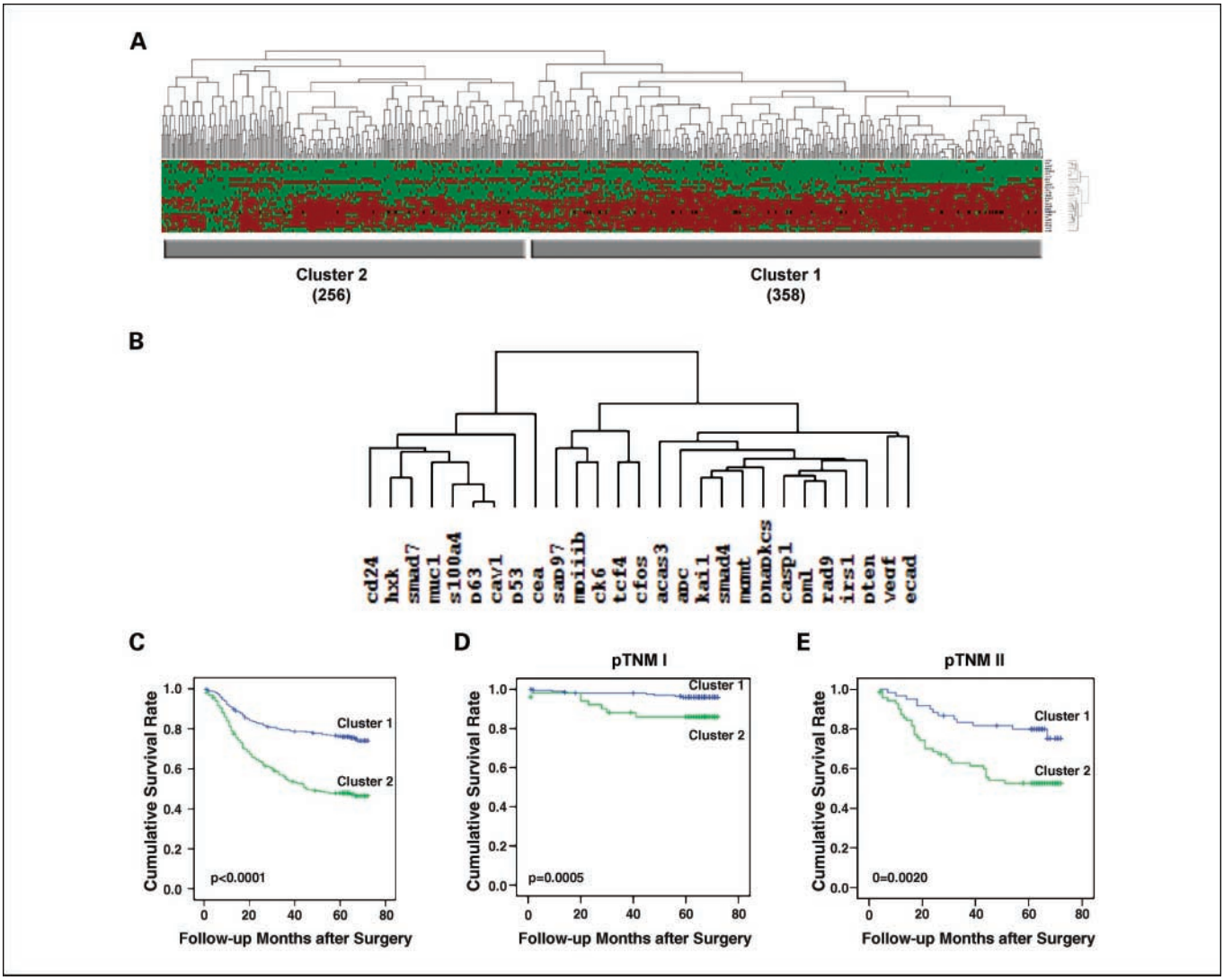

Fig. 3. Classification of 614 gastric cancers based on the expression of the 27 survival-associated proteins after feature selection. *A,* matrix format presenting the data. Gastric cancer was divided into two subgroups, clusters 1 and 2. *B,* protein dendrogram. *C,* univariate survival analysis by Kaplan-Meier method. *D,* Kaplan-Meier survival curve in stage I cancers. *E,* Kaplan-Meier survival curve in stage II cancers.

consisting of a subset of 56 tumor-associated genes yielding the best classification performance with a 10-fold cross-validation was extracted. All computations were done using the R program package (e1071 package for SVM and genalg package for genetic algorithm; ref. 28).

Statistical analyses. Either the χ^2 test or Fisher's exact test (two-sided) was done to determine the correlation between gene expression status and clinicopathologic variables. Survival curves were estimated using the Kaplan-Meier product-limit method, and the significance of the differences between survival curves were determined using a log-rank test. Multivariate survival analyses were done using the Cox proportional hazards model. The association between clustering and regional lymph node metastasis was evaluated by multivariate logistic regression. Results were considered to be statistically significant for $P < 0.05$. All statistical analyses were conducted using SPSS 12.0 statistical software program (SPSS).

Results

Expression profiling of 56 proteins and hierarchical cluster analysis. The staining results of the 56 antibodies are summarized in Table 2. By Kaplan-Meier analyses, the expression status of 27 proteins were found to be significantly associated with patient survival ($P < 0.05$). The overall expression patterns for 601 samples of gastric cancer were analyzed by hierarchical clustering after excluding those with values missing in >20% of the columns. The combined protein expression patterns defined two clusters: cluster A (24 cases) and cluster B (577 cases). Cluster B was subdivided into three clusters, cluster B1 (57 cases), cluster B21 (140 cases), and cluster B22 (380 cases; Fig. 2A and B). Cluster B22 cases tended to have better survival than clusters A, B1, or B21 ($P < 0.0001$; Fig. 2C). Multivariate analysis, including pTNM stage (II-IV versus I) and the molecular classification (cluster B22 versus clusters A + B21 + B1), showed that the molecular classification was an independent prognostic indicator of survival (hazard ratio, 0.654; 95% confidence interval, 0.498-0.858; $P = 0.002$).

Hierarchical cluster analysis of gastric cancer after feature selection. Twenty-seven survival-associated proteins were selected after univariate survival analyses of the 56-protein

Table 3. Clinicopathologic characteristics of the two clusters by hierarchical cluster analysis with 27 survival-associated proteins

Characteristics	Clusters by hierarchical clustering (%)		Total	*P*
	Cluster 1	Cluster 2		
Age (y, mean ± SD)	54.34 ± 12.48	55.62 ± 12.84	614	0.800
Gender				0.376
Male	243 (59.6)	165 (40.4)	408	
Female	115 (55.8)	91 (44.2)	206	
Location				0.002*
Low	175 (61.4)	110 (38.6)	285	
Middle	159 (59.6)	108 (40.4)	267	
Upper	8 (50.0)	8 (50.0)	16	
Whole	16 (34.8)	30 (65.2)	46	
Tumor size (cm)	4.65 ± 2.79	6.23 ± 3.04	614	0.091
WHO classification				0.001*
WD	42 (80.8)	10 (19.2)	52	
MD	121 (67.6)	58 (32.4)	179	
PD	130 (49.1)	135 (50.9)	265	
Mucinous	18 (47.4)	20 (52.6)	38	
SRC	47 (58.8)	33 (41.2)	80	
Lauren classification				0.002
Intestinal	162 (70.1)	69 (29.9)	231	
Diffuse	165 (48.8)	173 (51.2)	338	
Mixed	31 (68.9)	14 (31.1)	45	
Depth of invasion				<0.001*
Advanced	200 (46.8)	227 (53.2)	427	
Early	158 (84.5)	29 (15.5)	187	
Lymph node metastasis				<0.001*
Absent	185 (79.7)	47 (20.3)	232	
Present	173 (45.3)	209 (54.7)	382	
Distant metastasis				0.010*
Absent	337 (59.8)	227 (40.2)	564	
Present	17 (39.5)	26 (60.5)	43	
Stage				<0.001*
I	201 (79.4)	52 (20.6)	253	
II	61 (46.2)	71 (53.8)	132	
III	56 (41.5)	79 (58.5)	135	
IV	40 (42.6)	54 (57.4)	94	
Lymphatic invasion				<0.001*
Absent	277 (64.1)	155 (35.9)	432	
Present	81 (44.5)	101 (55.5)	182	
Total	358	256	614	

*$P < 0.05$.

Table 4. Prediction of lymph node metastasis in 187 early gastric cancers

	Lymph node metastasis (no. of patients)				Logistic regression analysis	
	Absent	Present	Total	*P* value	Odds ratio (95% confidence interval)	*P*
Clustering				0.026		0.027
Cluster 1*	137	21	158		1.00	
Cluster 2	20	9	29		3.098 (1.135-8.456)	
Depth				<0.001		0.002
Mucosa*	85	5	90		1.00	
Submucosa	72	25	97		5.329 (1.870-15.186)	
Lymphatic invasion				0.006		0.070
Absent*	148	23	171		1.00	
Present	9	7	16		2.928 (0.915-9.372)	
Lauren classification				0.725	—	—
Intestinal	84	15	99			
Diffuse	73	15	88			

*Patients in this category served as the reference group.

expression. A total of 614 gastric cancer cases were analyzed by hierarchical clustering analysis after excluding those with values missing in >20% of the columns. Tumors were separated into two main branches (Fig. 3A). The right branch, i.e., cluster 1, consisted of 356 cases and was characterized by intestinal type (according to Lauren's classification; $P = 0.002$), lower lymph node metastasis ($P < 0.001$), and left pTNM stage ($P < 0.001$) compared with the lower branch, i.e., cluster 2, which consisted of 256 cases (Table 3). Of the 614 gastric cancers, 187 cancers were limited to the mucosal or submucosal levels (pT1). In the 187 early gastric cancers, cluster 2 cancers were more likely to have metastatic lymph nodes ($P = 0.026$). By multivariate logistic regression analysis, cluster 2 was significantly correlated with lymph node metastasis independently of lymphatic invasion and depth of invasion ($P = 0.027$; Table 4).

Using a protein dendrogram, 27 survival-associated proteins were classified into three groups (Fig. 3B). Group 1 consisted of proteins overexpressed in gastric cancer, e.g., CD24, hexokinase II, smad7, MUC1, S100A4, and CEA, whereas group 3 consisted of suppressor proteins, e.g., APC, smad4, KAI-1, MGMT, PML, PTEN, and E-cadherin.

To determine whether these two clusters might represent clinically distinct subgroups of patients, univariate survival analysis was done. Cluster 2 was associated with poorer prognosis compared with cluster 1 ($P < 0.0001$; Fig. 3C), and multivariate analysis, including pTNM stage and the above cluster-based molecular classification, showed that the classification was an independent prognostic indicator of survival ($P = 0.013$; Table 5). Kaplan-Meier survival curves stratified by pTNM stage (stages I-IV) revealed that cluster 2 was associated with a probability of lower survival for patients with stage I or II cancer ($P = 0.0005$ and 0.0020, respectively; Fig. 3D and E).

Supervised analysis using the SVM algorithm. After 1,000 iterations, the genetic algorithm selected nine protein classifiers (CEA, c-fos, caspase-1, c-kit, cytokeratin6, cytokeratin19, S100A4, HDAC1, and DNA-PKcs) which showed the best classification performance compared with other combinations of tumor-associated genes in the whole data set. The prediction accuracy of these classifiers for patient survival was 73.1% (sensitivity, 36.7%; specificity, 94.7%). When 421 samples of stage I and II tumors were analyzed, the prediction accuracy was 88.1% for nine classifiers (MUC2, MUC6, smad4, PTEN, MGMT, TCF4, rad9, cytokeratin8, and S100A6). The sensitivity of these classifiers in stage I and II tumors decreased (30%), whereas the specificity increased (99.3%). The molecular classifiers for the data set of stage III and IV tumors ($n = 220$) were p53, p16, KAI1, TCF4, IRS-1, cytokeratin6, GST-p, and osteonectin. The prediction accuracy for patient survival was reduced to 76% compared with that of the classifiers in stage I and II tumors. Sensitivity and specificity for prediction of patient survival showed different features. Sensitivity increased sharply (95.5%) and specificity decreased (33.8%). Results are listed in Table 6.

Discussion

Gastric cancer is a heterogeneous disease histologically and genetically. Histologically, it is subdivided into intestinal and diffuse types by Lauren classification (4), but some gastric cancers cannot be easily classified in this way because these two types are frequently admixed within single tumors. Moreover, it is controversial as to whether Lauren's classification is an independent prognostic factor. Genetically, many earlier studies have reported genetic alterations during gastric carcinogenesis and progression, e.g., *p53* mutations (29), microsatellite instability (30), EBV infection (31), CpG island methylation (32), and chromosomal instability (33). Thus, gastric cancer is associated with various genetic alterations and no single genetic marker can predict gastric cancer biology or prognosis. The potential use of combinations of biomarkers instead of a single marker or histologic feature has been previously commented upon (19, 22, 34). Moreover,

Table 5. Multivariate analysis for factors predictive of survival (Cox proportional hazards model)

Prognostic factor	Hazard ratio (95% confidence interval)	*P*
Molecular classification		
Cluster 2 versus cluster 1	1.418 (1.075-1.869)	0.013
pTNM stage		
II-IV versus I	12.091 (7.079-20.652)	<0.001

high-throughput analysis based on formalin-fixed tissues is possible using the tissue array method. Recent studies have applied tissue array methods to the molecular classifications of various cancers such as breast and brain tumors (20, 35), and protein expression profiling has been found to be clinically useful for the prognostic classification of neoplasms. The present study shows that a molecular classification of gastric cancer can be accomplished based on hierarchical cluster analysis of the immunohistochemical profiles of tumor-associated biomarkers using tissue array sections.

The tissue array method enabled us to immunostain 659 formalin-fixed gastric cancer specimens with 56 tumor-associated antibodies. Moreover, using the large amount of data generated, various hierarchical cluster analyses could be done. The overall protein expression patterns defined four survival-associated clusters, i.e., cluster A (24 cases), cluster B1 (57 cases), cluster B21 (140 cases), and cluster B22 (380 cases). Patients with cluster B22 cancers had the best prognosis among the four clusters ($P < 0.0001$). However, 56 markers are too many for practical use. To more accurately predict patient survival, and to reduce the number of markers, we did a feature selection using Kaplan-Meier survival analyses. Twenty-seven survival-associated proteins were selected as described in Materials and Methods. Tumors were separated into two main branches by hierarchical clustering, i.e., cluster 1 (356 cases) and cluster 2 (256 cases), and these two clusters were found to have distinct clinicopathologic features and patient outcomes.

Recently, the incidence, and thus the importance, of small and early gastric cancers have increased because of the increased use of upper gastrointestinal endoscopy (8). Minimal resection and surgery such as endoscopic mucosal resection and laparoscopic surgery have been increasingly used to treat early gastric cancer (36). Moreover, the prediction of lymph node metastasis in endoscopic mucosal resection specimens is clinically important and the prediction of patient survival in early gastric cancer specimens would allow accurate decision-making concerning postoperative management. In the present study, Kaplan-Meier survival curves that were stratified according to pTNM revealed that cluster 2 was significantly associated with poor survival for stage I and II cancers ($P = 0.0005$ and 0.0020, respectively). Our study contained 187 cancers that were limited to the mucosa or submucosa (pT1). Cluster 2 cancers were also more associated with the presence of metastatic lymph nodes in 187 early gastric cancers (pT1; $P = 0.026$), independently of lymphatic invasion ($P = 0.035$), indicating that the molecular classification may be applied to the prediction of lymph node metastasis or prognosis in early gastric cancer.

Using the protein dendrogram, 27 survival-associated proteins were classified into three groups. Whereas group 1 consisted of proteins overexpressed in gastric cancer, group 3 consisted of proteins lost in gastric cancer. In a previous study of breast cancer (20), a protein dendrogram revealed four major protein clusters, i.e., estrogen receptor–associated proteins, a differentiation cluster, a mitosis cluster, and a proliferation cluster. However, in the present study, the protein groups were not well defined, which is probably due to the more heterogeneous nature of gastric cancer.

In this study, the classifications based on the 27 selected proteins were significantly associated with clinicopathologic features and prognosis. However, 27 proteins are too many for routine use. To obtain an optimal number of protein classifiers for the accurate prediction of patient outcome, 1,000 iterations were done using the genetic algorithm, and as a result, nine classifiers were chosen in the whole data set with a prediction accuracy for patient outcome of 73.1%, sensitivity of 36.7%, and specificity of 94.7%. When 421 stage I and II samples were analyzed, another nine classifiers were chosen and prediction accuracy was 88.1%, sensitivity was 30%, and specificity was 99.3%. The identified classifiers for the stage III and IV samples ($n = 220$) were p53, p16, KAI1, TCF4, IRS-1, cytokeratin6, GST-p, and osteonectin, and these had a prediction accuracy of 76%, sensitivity of 95.5%, and a specificity of 33.8%. These classifier numbers were found to be optimal for practical use in pathology laboratories. There was a large gap between the sensitivity and specificity of the classification analysis. This result was due to our analysis scheme. In this analysis, we used the genetic algorithm to select an optimal subset of tumor-associated genes that yielded the highest prediction accuracy with a 10-fold cross-validation. During selection, only the prediction accuracy was considered. The selected classifier genes therefore generated the optimal prediction accuracy with the sacrifice of sensitivity or specificity. Even if the sensitivity was low, the classifier genes from stage I and II data could be applied as indicators for good prognosis because the specificity is high. In cases with classifiers from stage III and IV, they could be used as the screening test for unfavorable outcome because the sensitivity is high. Further validation is needed concerning the use of the above classifiers for routine pathologic diagnoses.

Table 6. Prediction for patient outcome of molecular classifications by supervised analysis using the SVM algorithm

	Prediction accuracy (%)	Sensitivity (%)	Specificity (%)
Whole data set	73.1	36.7	94.7
Stage I and II data set	88.1	30	99.3
Stage III and IV data set	76	95.5	33.8

In summary, we immunostained 659 consecutive gastric cancers with 56 tumor-associated antibodies using the tissue array method. With 27 survival-associated gene products, the hierarchical cluster analysis identified two clusters with different clinicopathologic features and prognoses. Moreover, the molecular classification predicted lymph node metastasis and prognosis in early stage gastric cancer. To optimize classifier numbers and accurately predict patient outcome, we did the supervised analysis using the SVM algorithm. The genetic algorithm selected nine classifiers in the whole data set, another nine classifiers in stages I and II, and eight classifiers in stages III and IV. Prediction accuracies for the patient outcomes of these classifiers were 73.1%, 88.1%, and 76%, respectively.

Acknowledgments

We thank S.P. Kim, S.H. Chung, and Superbiochip Laboratories (Seoul, Korea) for their technical assistance.

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