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Gene expression signature–based prognostic risk score in gastric cancer

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Abstract

Purpose—Despite continual efforts to develop a prognostic model of gastric cancer by using clinical and pathological parameters, a clinical test that can discriminate patients with good outcomes from those with poor outcomes after gastric cancer surgery has not been established. We aim to develop practical biomarker-based risk score that can predict relapse of gastric cancer after surgical treatment.

Experimental Design—Using microarray technologies, we generated and analyzed gene expression profiling data from 65 gastric cancer patients to identify biomarker genes associated

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No potential conflicts of interest were disclosed.

Additional Sources: Primary microarray data is available in NCBI's Gene Expression Omnibus public database (<http://www.ncbi.nlm.nih.gov/geo/>) (accession number GSE13861).

with relapse. The association of expression patterns of identified genes with relapse and overall survival was validated in independent gastric cancer patients.

Results—We uncovered two subgroups of gastric cancer that were strongly associated with the prognosis. For the easy translation of our findings into practice, we developed a scoring system based on the expression of six genes that predicted the likelihood of relapse after curative resection. In multivariate analysis, the risk score was an independent predictor of relapse in a cohort of 96 patients. We were able to validate the robustness of the 6-gene signature in an additional independent cohort.

Conclusions—The risk score derived from the 6-gene set successfully prognosticated the relapse of gastric cancer patients after gastrectomy.

INTRODUCTION

Gastric cancer is the second-leading cause of cancer-related death in the world(1). Surgery remains the gold standard in the treatment of gastric cancer(2;3). In the United States, however, only a small fraction of patients with gastric cancer who undergo curative resection have early-stage disease, and the prognosis for patients with more advanced stage (II or III) remains poor because of the high rate of relapse after gastrectomy(4;5). Preoperative staging techniques, including laparoscopy and non-invasive imaging systems (i.e., endoscopic ultrasonography and positron emission tomography), have a relatively low sensitivity for discriminating patients with favorable clinical biology vs. poor clinical biology(6). Moreover, the outcomes of patients considered to have a similar clinical or pathologic stage remain unpredictable, especially when patients are treated similarly. For example, not all patients with stage III tumor survive 5 years even after successful curative resection(7;8), and the outcomes remain uneven whether or not preoperative or postoperative therapy is administered. This inherent clinical heterogeneity is most likely due to the diverse molecular profile of gastric cancer. Thus, identifying the diversity in the molecular profile of gastric cancer that governs the clinical behavior of tumors could lead to new and more effective clinical strategies. Microarray technologies have been successfully used to predict clinical outcomes and survival as well as classify different types of cancer(9–13). Recent studies in gastric cancer have identified genes that differ according to histological factors and age, as well as those for gastric cancer prognosis prediction(14–16). However, these studies have failed to create molecular prognostic tests that could be practical for gastric cancer patients.

In the present study, we characterized tumor transcriptome at the systems level to identify potential markers that could be used to divide patients into distinct subclasses that haven't been recognized by current staging system.

Materials and Methods

Patients and samples

Tumor specimens and clinical data from 213 gastric cancer patients undergoing gastrectomy as primary treatment were obtained from Yonsei University Severance hospital, Seoul, South Korea. Sixty-five surgically removed frozen gastric adenocarcinoma tissues, with 19 normal surrounding tissue samples, from gastric cancer patients were used for microarray experiments (Yonsei gastric cancer [YGC] cohort). In addition, six frozen tissue samples from gastrointestinal stromal tumor (GIST) patients were included in the microarray experiments as reference for distinct tumors resided in gastric tissues. To validate gene expression patterns found by microarray analysis, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments were performed with RNA from 96 formalin-fixed, paraffin-embedded (FFPE) tissues from a separate gastric adenocarcinoma

patient group from Yonsei Gangnam Severance hospital (GSH1 cohort). For validation of risk score, FFPE tissues of independent patient group from Yonsei Gangnam Severance hospital (GSH2 cohort, n=52) were used for qRT-PCR. Tissue specimens used in microarray and qRT-PCR were obtained from the surgical specimens. All samples were collected after obtaining written informed consent from patients, and the study was approved by the Institutional Review Board of The University of Texas M. D. Anderson Cancer Center (Houston, USA), the Yonsei University Severance Hospital (Seoul, Korea), and Yonsei Gangnam Severance Hospital (Seoul, Korea). Clinical data also were obtained retrospectively. All of the experiments and analyses were done in the Department of Systems Biology at M. D. Anderson Cancer Center.

Experimental procedures for microarray

Total RNA was extracted from the fresh frozen tissues by using a mirVana™ RNA Isolation labeling kit (Ambion, Inc.). Five-hundred nanograms of total RNA were used for labeling and hybridization, according to the manufacturer's protocols (Illumina). The microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package in the R language environment(17). The expression level of each gene was transformed into a log 2 base before further analysis. Primary microarray data is available in NCBI's GEO database (microarray platform, GPL6884; microarray data, GSE13861).

Statistical analysis of microarray data

BRB-ArrayTools were primarily used for all statistical analysis (18;19). Gene expression differences were considered statistically significant if the *P* value was less than 0.001. Cluster analysis was performed with Cluster and Treeview(20). Kaplan–Meier plots and the log-rank test were used to estimate patient prognosis. Multivariate Cox proportional hazards regression analysis was used to evaluate independent prognostic factors associated with survival, and gene signature, tumor stage, and pathologic characteristics were used as covariates. A *P* value of less than 0.05 was considered to indicate statistical significance, and all tests were two-tailed.

Selection of genes for quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay and experimental procedures

To select the candidate genes during the course of validation experiments, we used receiver operating characteristic (ROC) curves from censored relapse-free survival (RFS) data using the nearest neighbor estimation method, with a cut-off of 36 months and under the curve (AUC) were calculated with 95% confidence intervals (21). First ROC models were constructed by using gene expression data from microarray experiments. From 2755 gene features identified from microarray experiments, top 27 genes with highest AUC values (14 genes) or lowest AUC values (13 genes) were selected for validation with qRT-PCR experiments in GSH1 cohort. Using qRT-PCR based gene expression data from GSH1 cohort, we constructed second ROC curves to further select genes with AUCs of more than 0.55 as risk genes and less than 0.45 as protective genes. Out of 27 candidate genes, only six genes were within the range of selection cut-off.

Total RNA was extracted from the FFPE sections according to the manufacturer's instruction manual (RecoverAll™ Total Nucleic Acid Isolation; Ambion, Inc.). Real-time RT-PCR amplification was performed using the 7900HT Fast Real-Time PCR System with a 384-well block module (Applied Biosystems). Cycling conditions were 45°C for 10 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Relative amounts of mRNA were calculated from the threshold cycle (C_T)

number using expression of cyclophilin A (PPIA) as an endogenous control. All PCR experiments were duplicated and the values averaged.

Development of six-gene risk scoring system

To generate a risk score with six genes, we adopted a previously established strategy using the Cox regression coefficient of each gene (22;23). The risk score of each patient was derived from sum of multiplication of reference-normalized expression level of the gene by its corresponding coefficient. Risk score = $(0.097 \times \text{CTNNB1 value}) + (0.141 \times \text{EXOCS3 value}) + (0.148 \times \text{TOP2A value}) + (-0.0898 \times \text{LBA1 value}) + (-0.0985 \times \text{CCL5 value}) + (-0.0618 \times \text{LZTR1 value})$. GSH1 patients were dichotomized into a high-risk and low-risk group using the 50th percentile (median) cut-off of the risk score as the threshold value. The coefficient and the threshold value derived from the GSH1 cohort were directly applied to the gene expression data from the exploration data set (YGC cohort) and an independent test sets (GSH2 patient cohorts).

RESULTS

Two major subclasses revealed by hierarchical clustering of gene expression patterns are highly associated with prognosis

We applied hierarchical clustering analysis to gene expression data from 65 human primary tumor tissue samples (YGC cohort in Table 1), and six gastrointestinal stromal tumor (GIST) tissue samples. Unsupervised clustering revealed three distinctive subtypes with clear differences in overall gene expression patterns (Fig. 1A). Most of the gastric cancer tissues were subdivided into one of two subgroups (C1 and C2). Intriguingly, a few gastric cancer tumors (C3) were co-clustered with the GIST tissues, indicating that a small percentage of gastric cancers may acquire sarcomatoid features during progression. When clinical relevance was examined, relapse-free survival (systemic) was found to differ significantly between the two major clusters (C1 and C2). Kaplan-Meier plots and log-rank tests indicated C2 patients had a significantly better relapse-free survival (RFS) than C1 or C3 patients ($P = 0.001$ by the log-rank test; Fig. 1B). When only patients with stage III tumors were considered for analysis, the differences in prognosis between C1 and C2 patients were still significant ($P = 0.005$ by the log-rank test; Fig. 1C), indicating that the molecular features of these tumors reflected in gene expression patterns might be strong independent predictors of clinical outcomes. Because the number of gastric cancer patients in group C3 was too small ($n=5$), these patients were removed from further analysis.

Prognostic Gene Expression Signature in Gastric Cancer

Because the C1 subgroup was strongly associated with poor prognosis, we next sought to identify genes whose expression is unique to the C1 subgroup by cross-comparing gene lists from different statistical tests. We first generated two different gene lists by applying two-sample t-tests ($P < 0.001$). Gene list A represents the genes that were differentially expressed between C1 and C2. Gene list B represents the genes that were differentially expressed between C2 and normal gastric tissues (Supplementary Fig. S1). When gene expression patterns of all tissues were compared together, three different patterns were observed: A not B (2755 genes), A and B (241 genes), and B not A (1437 genes). Genes in the A not B category displayed a poor prognostic C1-specific gene expression pattern and are potential markers for predicting relapse-free survival (Fig. 2). Because the use of a complex algorithm with a long gene list from microarray data may not be practical in the clinic, we tried to identify a small number of genes whose expression patterns can still reliably predict relapse-free survival. Out of 2755 genes in A not B category, we further selected candidate genes based on receiver operating characteristic (ROC) models analysis. Top 27 genes with highest AUC values (14 genes) or lowest AUC values (13 genes) were

selected for validation with qRT-PCR experiments. We next tested, using qRT-PCR, whether expression of these genes or their subsets could predict the relapse-free survival in an independent cohort (GSH1).

Six-gene Signature and Risk Score

Using gene expression data from qRT-PCR experiments with tissues from GSH1 cohort, we again constructed another ROC models to assess the prognostic relevance of gene expression in 96 GSH1 patients. Considering AUC over 0.55 and under 0.45 to be significant, we found that the expression of six genes (*CTNNB1*, *EXOSC3*, *TOP2A*, *LBA1*, *LZTR1*, and *CCL5*) had a non-trivial correlation with relapse-free survival. We next tested whether we could use expression of the six genes as a prognostic signature in the GSH1 cohort. When hierarchical clustering analysis was applied to the six-gene expression data, the 96 patients were divided into two subgroups with significantly different relapse-free survival ($P = 0.017$ by the log-rank test; Supplementary Fig. S2).

Because use of hierarchical clustering analysis methods in clinical practice has proven to be difficult (24), we developed risk score methods using the Cox regression coefficient of each gene (Supplementary Table S1) (22;23). Patients in the GSH1 cohort were dichotomized according to their risk score, and the relapse-free survival rate was significantly lower in the patient group with the high risk score ($P = 0.048$ by the log-rank test; Fig. 3). Gene expression data from the YGC cohort were re-analyzed with the six-gene-based risk score. With direct application of the Cox regression coefficient from the GSH1 cohort and the 50th percentile cut-off threshold, relapse-free survival in two patient groups differed significantly ($P = 0.04$ by the log-rank test; Supplementary Fig. S3).

In the GSH1 cohort, the prognostic association between our newly identified six-gene signature and other known clinical and pathological risk factors for gastric cancer progression was assessed by univariate and multivariate analyses. As expected, in addition to stage and lymph node status, which are already well-known risk factors, the six-gene signature was a significant risk factor for shorter relapse-free survival in univariate analysis (Table 2). Multivariate analysis that included all relevant pathological variables revealed that the gene signature remained an independent prognostic risk factor for relapse-free survival.

Validation of Risk Score in Independent Cohorts

We next sought to validate risk score in another independent cohort, especially in stage III patients (GSH2 cohort, $n=52$, Supplementary Table S2). Expression data of 6 genes from FFPE tissues were obtained by applying qRT-PCR and used to generate risk score. When patients in the GSH2 cohort were dichotomized according to their risk score, both of relapse-free survival and overall survival rate were significantly lower in the patient group with the high risk score ($P = 0.028$ and $P = 0.032$ respectively by the log-rank test; Fig. 4A and 4D).

To test whether the 6-gene based risk score is independent of American Joint Committee on Cancer (AJCC) stage, patients in GSH2 cohort were re-stratified according to sub-stage and risk score. As expected, prognosis of stage IIIB is significantly worse than that of stage IIIA (Supplementary Fig. S4A and S4C). When the risk score was applied to stage IIIA and IIIB separately, the risk score successfully identified a population of high risk patients in both subgroups (Fig. 4B, 4C, 4E, and 4F). In fact, when all of stratifications were combined together, the risk score identified gastric cancer patients in IIIA subgroup whose risk of relapse was similar to or worse than that of IIIB (Supplementary Fig. S4B and S4D). Since nodal stage is best known predictor of relapse, we assessed the utility of the risk score in T3

patients who differed only with N stage (T3N1 and T3N2). Nodal stage is well associated with relapse-free survival (Supplementary Fig. 5A). Within these groups, the risk score clearly identified high risk patients (Supplementary Fig. 5B, 5C, and 5D). Taken together, this data indicate that the risk score provides information on the risk of relapse independent of current staging systems and demonstrate that the risk score captures biological differences among gastric cancers that are not encompassed in the current staging criteria.

DISCUSSION

Using a series of independent experiments and complementary data analyses (Supplementary Fig. S6), we have identified and validated robust prognostic markers in gastric cancer and developed a prognostic risk score that can be easily translated into the clinic. First, we used microarray technology to uncover potential prognostic subgroups of gastric cancer patients and identify candidate genes for prognostic markers. The association of gene expression patterns with prognosis was significant (Fig. 1), which suggests that our gene expression signature well reflects clinical differences between subgroups of patients with gastric cancer. However, the difficulty of acquiring fresh-frozen tissues from patients and the complexity of data analysis makes it hard to use this approach in the clinic. To overcome this limitation, we switched from microarray-based technology to qRT-PCR technology, simpler and easily accessible technology in clinics, to measure gene expression and identified a small number of genes (*CTNNB1*, *EXOSC3*, *TOP2A*, *LBA1*, *LZTR1*, and *CCL5*) whose expression patterns can reliably predict the prognosis of gastric cancer patients. The robustness of the prognostic gene expression signature was validated in an independent cohort using the reduced gene set. For easy translation of our finding to the clinic, we developed a risk score for relapse after curative resection of tumor (Fig. 3). Finally, the robustness of our risk score was validated in an independent cohort, especially in stage III patients who shows most heterogeneous clinical outcome (Fig. 4 and Supplementary Fig. S5).

The unique molecular characteristics of each subgroup of gastric cancer may lead to new therapeutic strategies. *CTNNB1* is a key mediator of the *WNT* signaling pathway that regulates cell-fate decisions and cell proliferation during gut development (25). Activated mutation of *CTNNB1* was reported in gastric cancer, and abnormal expression of *CTNNB1* in gastric cancer was significantly associated with poorer survival (26;27), supporting the notion that our gene expression data may well recapitulate the molecular abnormality of gastric carcinogenesis. *TOP2A* encodes a DNA topoisomerase II, an enzyme that controls the topologic state of DNA during transcription. This gene is currently the target of several anticancer agents, and a variety of its mutations have been associated with the development of drug resistance (28–30).

The development of the six-gene-based risk score has strong clinical implications. Using simple qRT-PCR technology and paraffin-embedded tissues, which are routinely acquired at diagnosis, we could identify gastric cancer patients at higher risk. One of limitations of our study is its retrospective character. Thus, to validate its true clinical relevance of the risk score, it will be necessary that 6-gene based risk score is integrated into prospective randomized trials in form of a biologic stratification criterion. In addition, since this new approach has not been applied to samples from small pre-treatment biopsies, the reliability of the new approach should be extensively tested before its use in clinical trials.

Prognostic characteristics of the risk score may not be sufficient to change current clinical practice since they only provide information on probable course of the disease, not the probable response to treatments. However, biomarker study in breast cancer showed that 21-gene prognostic marker can also be used as predictive marker for standard adjuvant

chemotherapy (31). Thus, in future study, it will be interesting to test whether the risk score can also be good predictive marker for response to adjuvant chemotherapy.

Translational Relevance

Gastric cancer is the second-leading cause of cancer-related death in the world, and prognosis is difficult to predict for individual patients. Most of gastric cancer patients receive similar treatments, typically surgery followed by chemotherapy, since there are no reliable biomarkers to optimize therapy. Our study identified the prognostic gene expression signatures and limited number of prognostic biomarkers. We developed a score based on these six genes which significantly associated with survival and early relapse. This method requires the determination of only six genes by using simple RT-PCR technology and easily accessible paraffin-embedded tissues, which are routinely acquired at diagnosis. This will open up new opportunities to optimize treatment of gastric cancer patients according to molecular subtypes of tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Hierarchical clustering analysis of gene expression data from the YGC cohort
 (A) Hierarchical clustering of gene expression data from 65 gastric cancer and 6 GIST patients in the YGC cohort. Genes with expression levels that were at least 2-fold different in at least 15 tissues, relative to the median value across tissues, were selected for hierarchical clustering analysis (2,077 gene features). The data are presented in matrix format, in which rows represent individual genes and columns represent each tissue. Each cell in the matrix represents the expression level of a gene feature in an individual tissue. The color red or green in cells reflects relative high or low expression levels, respectively, as indicated in the scale bar (log2 transformed scale).

(B) Kaplan-Meier plots of three gastric cancer clusters in the YGC cohort. The six patients with GIST were excluded from the plotting.

(C) Kaplan-Meier plots of stage III patients in 2 clusters (C1 and C2) in the YGC cohort. (No stage III patients were identified in C3.)

Fig. 2. Gene Expression signature unique to Cluster C1

Measured gene expression values were log 2-transformed and median-centered across samples before generating heatmap.

Fig. 3. Risk score based on six-gene signature and RFS of patients in GSH1

(A) The relative risk score based on the six-gene signature of each patient. (Each bar represents the risk score of an individual patient.) The regression coefficients of each gene were calculated by Cox regression analysis (Supplementary Table 1). The risk score was used to dichotomize patients into high- or low-risk groups, with the 50th percentile as the cut-off. To avoid the ambiguity of a risk score near the median value, patients in the upper and lower 5th percentiles from the risk score median were removed from Kaplan-Meier plotting. Blank bars near the median indicate these patients.

(B) Kaplan-Meier plots of two risk score risk groups in the GSH1 cohort. *P*-values were obtained from the log-rank test.

Fig. 4. Kaplan–Meier survival plots of overall survival and relapse-free survival in AJCC stage III gastric cancer patients in GSH2 cohort

Patients were stratified by risk score in all stage III (A and D), risk score in IIIA (B and E), in IIIB (C and F). *P*-values were obtained from the log-rank test.

Table 1

Clinical Characteristics of the Patients

Characteristics	YGC (n=65)	GSH1 (n=96)
Age (yr)		
Median	63	60
Range	32–83	26–77
Sex (%)		
Male	46 (71%)	60 (62%)
Female	19 (29%)	36 (38%)
Subsite of tumor (%)		
Cardia	5 (8%)	11 (12%)
Body	24 (37%)	47 (49%)
Antrum	31 (48%)	28 (29%)
Diffuse	4 (6%)	10 (10%)
Unknown	1	0
Histologic type of tumor (%)		
Intestinal	23 (35%)	23 (24%)
Diffuse	32 (49%)	71 (74%)
Mixed	10 (16%)	2 (2%)
Cancer stage, TNM class (%)		
I	12 (18.5%)	0 (0%)
II	2 (3%)	36 (38%)
III	34 (52%)	59 (61%)
IV	12 (18.5%)	1 (1%)
Relapse and Survival		
Relapse	27 (41 %)	48 (50%)
Death	20 (30%)	33 (34%)
Adjuvant chemotherapy		
Not received	16 (25%)	10 (10%)
Received	49 (75%)	86 (90%)

Table 2

Univariate and Multivariate Cox Proportional Hazard Regression Analyses of Relapse-Free Survival

	Univariate		Multivariate	
	Hazard Ratio (95% CI)	P-value	Hazard Ratio (95% CI)	P-value
Six Gene Risk Score (High or Low)	1.81 (0.998 – 3.3)	0.0476	2.587 (1.351 – 4.953)	0.004
T (T2 or T3)	4.42 (2.21 – 8.81)	<0.001	3.969 (1.906 – 8.265)	<0.001
N (N1 or N2)	2.94 (1.65 – 5.22)	<0.001	3.389 (1.686 – 6.815)	<0.001
Age (>60 or not)	0.652 (0.368 – 1.16)	0.140	0.701 (0.354 – 1.390)	0.31
Gender (M or F)	0.56 (0.317 – 0.991)	0.043	0.567 (0.306 – 1.059)	0.075
Adjuvant chemotherapy (Yes or No)	0.423 (0.189 – 0.947)	0.031	0.358 (0.142 – 0.904)	0.03
Lauren classification (Intestinal or Diffuse)	0.935 (0.475 – 1.84)	0.844	1.384 (0.612 – 3.131)	0.44