Development of a survival prediction model for gastric cancer using serine proteases and their inhibitors

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Abstract. Proteolytic enzymes play a key role in the metastatic stage of gastric cancer (GC). In this study, we aimed to identify the serine proteases (SPs) and their inhibitors (serpins) as related to GC. The gene expression profiles of 40 cases of GC were initially detected by cDNA microarray. The results of the differentially expressed SPs and their inhibitor genes from the microarrays were confirmed by real-time PCR. The status of the immunohistochemical staining of the confirmed genes in patients with complete data was used to develop a survival prediction model. Finally, the prediction model was tested in different groups of GC patients. As a result, seven genes, *SERPINB5*, *KLK10*, *KLK11*, *HPN*, *SPINK1*, *SERPINA5* and *PRSS8*, were considered as GC progression-related genes. A survival prediction model including the immunohistochemical scores of three genes and the tumor node metastasis (TNM) score was developed: Survival time (months) = 88.8607 + 2.6395 SERPINB5 - 12.0772 KLK10 + 13.7562 KLK11 - 7.0318 TNM. In conclusion, *SERPINB5*, *KLK10*, *KLK11*, *HPN*, *SPINK1*, *SERPINA5* and *PRSS8* were GC progression-related SPs or serpin genes. The model consisting of the expression profiles of three genes extracted from the microarray study accompanied by the TNM score accurately predicts surgery-related survival of GC patients.

Introduction

There are 300,000 deaths and 400,000 new cases of gastric cancer in China every year (1). Gastric cancer (GC) is the fourth most common type of cancer worldwide. Although there has been a steady decline in the risk of GC incidence and mortality over several decades in most countries, it is the second most common cause of cancer-related death (700,000 deaths annually) (2). The current staging system for GC is inadequate for predicting the outcome of treatment.

The poor prognosis of patients with GC has been attributed mostly to metastases and relapse (3,4). Tumor cells utilize many cellular or biochemical mechanisms to complete metastatic spread, and proteolytic enzymes play a key role in the metastatic stage (5). Mammalian proteolytic enzymes are divided into five classes (aspartic, cysteine, metallo, serine and threonine), and the serine protease (SP) family is the largest (6). It is widely accepted that SPs degrade extracellular matrix and facilitate neoplastic progression. The urokinase plasminogen activator, one of the SPs, promotes tumor cell invasion (7) and the kallikrein prostate-specific antigen predicts prognosis of prostate cancer (8). KLK6 was found to be significantly up-regulated in tissues and sera from patients with colon cancer and was closely associated with a poor prognosis, suggesting that KLK6 may be used as a potential biomarker and a therapeutic target for colon cancer (9). SP inhibitors (serpins) are the largest family of protease inhibitors identified to date. Most serpins act as inhibitors of chymotrypsin-like SPs, others as cross-class serine protease inhibitors of papain-like cysteine proteases and caspases (10-13). Certain serpins have been identified to be involved in the progression of malignant tumor; these include SERPINB3 and SERPINB4 in squamous cell carcinoma (14,15), SERPINB5 in human breast cancer (16), SERPINB13 in head and neck squamous cell carcinoma (17), SERPINH1 in head and neck carcinomas (18), SERPINI2 in breast cancer (19), SERPINB2 and SERPINE1 in breast cancer and other cancers (20), and SERPINB2, SERPINB3, SERPINB4, SERPINB7, SERPINB11, SERPINB12 and SERPINB13 in oral squamous cell carcinoma (21).

Yet, which SPs and their inhibitors are associated with the progression of GC globally? Minimal research has been carried out concerning this issue. In the present study, we focused on SPs and serpins, the largest protease and protease inhibitor families, since they carry out diverse functions, including clotting, fibrinolytic cascades, complement activation, fertilization, pro-hormone conversion, apoptosis, extracellular matrix maintenance and remodeling, angiogenesis and tumor cell invasion (22,23). We aimed to compare

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the difference in gene expression profiles between cancerous and non-cancerous gastric tissues by cDNA microarrays, and to identify the SPs and serpins associated with the progression of GC. The results from the microarrays were confirmed by real-time PCR and immunohistochemistry (IHC). A survival prediction model was further developed using the IHC data.

Materials and methods

Patients and clinical features. Human GC tissues and their paired normal gastric tissues were obtained with informed consent from 140 patients who underwent radical resection for GC in 2000 and 2003 at the Department of Surgery, Ruijin Hospital, Shanghai, China. The corresponding non-cancerous gastric tissue was obtained at least 6 cm from the tumor. The diagnoses were confirmed by histopathology. Stage and grade were established using the tumor node metastases (TNM) and World Health Organization classification systems. For stage IIIB and stage IV by clinical staging, pre-operative chemotherapy was offered. All of the patients, except stage IA of surgical pathologic staging, received fluoropyrimidine-based chemoradiotherapy. All patients were followed up systematically. This follow-up included a complete history and physical examination every 4 months for 3 years, then annually thereafter, complete blood count, platelets, multichannel serum chemistry analysis and other investigations (such as endoscopy and other radiologic studies). Of these 140 patients, 100 were enrolled in 2000 and included in the IHC analyses; 40 were enrolled in 2003 and initially included in the microarray analyses. In total, 10 of the 40 patients in 2003 were later included in the real-time PCR analyses. In addition to the 10 patients, half of the 40 patients were involved in the IHC analyses.

The specimens were snap-frozen in liquid nitrogen immediately after resection and then stored at -80˚C until use or fixed in 10% formalin and paraffin embedded by conventional techniques for IHC staining. The Ethics Committee of Ruijin Hospital approved the use of these tissues for research purposes.

RNA preparation. Total RNAs were extracted from each tissue sample using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and further purified with an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality of the total RNA samples was determined by electrophoresis through formaldehyde agarose gels, and the 18S and 28S RNA bands were visualized under ultraviolet light.

Microarray assays and statistical analysis. The cDNA microarray used in the present study consisted of 13,824 genes/ESTs; this microarray was the same as that used by Huang *et al* (24) and constructed by the National Engineering Center for Biochips at Shanghai (Shanghai, China). The microarray and the experimental procedure were confirmed to be feasible by previous studies (24,25). Thus, it was used instead of a new customized microarray containing SP and serpin genes only. The microarray contained 50 common SPs and serpin genes.

Total RNA (100 μg) was used to prepare fluorescent dye-labeled first-strand cDNA, using SuperScript™ II RNase H Reverse Transcriptase according to the relevant protocol (Invitrogen, Carlsbad, CA, USA). Cy3-dCTP and Cy5-dCTP

(Amersham, Piscataway, NJ, USA) were used to label the GC specimens and the corresponding non-cancerous gastric specimen, respectively. The dye-labeled probes were purified by QIAquick Nucleotide Removal kit (Qiagen) according to the specified protocol. A total of 30 pmol of each probe was used in the two-color array hybridization. The hybridization and scanning procedures were identical as those described previously (24).

To avoid systematic error for each microarray sample, a space and intensity-dependent normalization based on a LOWESS program was employed (26) to normalize the logarithm transformed background corrected signal intensities for the dual-channel data. Thereafter, normalized data were withdrawn for each SP and serpin gene for each specimen. The difference in C (GC specimen) and N (corresponding non-cancerous gastric specimen) was estimated by the ln C-ln N and T test performed using SAS to compare the difference between the GC specimen and its corresponding non-cancerous gastric specimen transcript. All ratios were filtered using a P-value of ≤0.05.

Real-time PCR. Real-time PCR reactions were performed according to a previously reported protocol (27). The primer sequences were as follows: sense 5'-GTTCCAGACATTCT CGCTTC-3' and anti-sense 5'-ATAGTAGCCTGAGCAT GTGC-3' for *SERPINB5* (107 bp); sense 5'-CCTGCTCCA GCATCACTATC-3' and anti-sense 5'-GGTCCAGTCCAGC ACATATC-3' for *KLK10* (93 bp); sense 5'-AGCTCTCCAGC CTCATCATC-3' and anti-sense 5'-CAACAGCCTTCTTCTG CATC-3' for *SERPINH1* (120 bp); sense 5'-CAGAAGTGTGA GAACGCCTAC-3' and anti-sense 5'-CCTTGAAGAGA CTGGTTACAG-3' for *KLK11* (131 bp); sense 5'-GTCATCTC CGTGTGTGATTG-3' and anti-sense 5'-TCATAGCGAA GGCTGACTTG-3' for *HPN* (149 bp); sense 5'-AACGCCAG ACTTCTATCCTC-3' and anti-sense 5'-CAACAATAAGGC CAGTCAGG-3' for *SPINK1* (102 bp); sense 5'-GATGAAGAA GAGAGTCGAGG-3' and anti-sense 5'-GAAGAAGATGTT CTGGCTGG-3' for *SERPINA5* (124 bp); sense 5'-CAAGGAAG CCTATGAGGTCAAG-3' and anti-sense 5'-TGAGTTGGA GGAGTGCAAT-3' for *PRSS8* (146 bp); sense 5'-AAGCAC TGTGCATCACCTTG-3' and anti-sense 5'-CAGAGTTGG AGCACTTGCTG-3' for *TMPRSS2* (102 bp); sense 5'-GGA CCTGACCTGCCGTCTAG-3' and anti-sense 5'-GTAGCC CAGGATGCCCTTGA-3' for *GAPDH* (100 bp).

The results of the real-time PCR are presented as Ct values. The relative changes in gene expression were calculated by the ΔΔCt method (28).

Immunohistochemistry. IHC was performed according to our previously reported protocol (29). The primary antibodies used in the present study were Hepsin polyclonal antibody (Abcam, Cambridge, MA, USA), anti-human Kallikrein 11 antibody (R&D Systems, Minneapolis, MN, USA), goat polyclonal antibody against KLK10 (Santa Cruz, Santa Cruz, CA USA) and mouse monoclonal antibody to SERPINB5 (Novocastra, Newcastle-upon-Tyne, UK). Negative control slides were treated without the primary antibody under equivalent conditions. For the secondary developing reagents, EnVision™ System labeled Polymer-HRP/M/R (DakoCytomation, Glostrup, Denmark) and UltraSensitive™ S-P (Goat) kit

Table I. Differentially expressed genes as determined by the microarray.

Gene name	GeneBank no.	Fold change[a]
Up-regulated		
KLK10	NM_002776	7.181442890
SERPINB5	NM_002639	5.827452523
SERPINH1	NM_001235	4.313578371
Down-regulated		
TMPRSS2	NM_005656	0.823943603
PRSS8	NM_002773	0.659704511
HPN	NM_002151	0.527439252
SERPINA5	NM_000624	0.392424461
SPINK1	NM_003122	0.381989951
KLK11	NM_006853	0.193303929

[a]GC specimen/the corresponding non-cancerous gastric specimen.

(Maixin Bio, Fuzhou, China) were used. Slides were developed with diaminobenzidine (DAB; DakoCytomation) and counterstained with hematoxylin.

Pathologists without knowledge of the patient outcomes scored the immunostained slides independently as previously described (30). In brief, a proportion (proportion of positive-staining tumor cells) score (0, none; 1, <1/100; 2, 1/100-1/10; 3, 1/10-1/3; 4, 1/3-2/3; and 5, >2/3), and an intensity score (0, none; 1, weak; 2, intermediate; and 3, strong) were assigned. These two scores were then added to obtain a total score for each slide.

Statistical analysis. Results of the real-time PCR were evaluated by the Wilcoxon signed rank test. Associations between gene expression profiles as assessed by real-time PCR and IHC were analyzed using non-parametric Spearman rank correlation coefficients. Survival curves were computed with the Kaplan-Meier method and were compared using the log-rank test. A two-sided Fisher's exact test was used in univariate analysis of potential prognostic factors regarding overall survival. Stepwise regression analysis and the best subset regression were used to develop a prediction model of survival. A P-value <0.05 was taken as the level of significance. Statistical analyses were performed using the SAS 6.12 software (SAS Inc., Cary, NC, USA) or GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Selection and confirmation of the serine protease-related genes for the prediction model. Nine serpins or SP genes were determined to be differentially expressed by the microarray experiments. The gene list and fold changes are shown in Table I; it includes three up-regulated and six down-regulated genes. The up-regulated genes included two serpins (*SERPINB5* and *SERPINH1*) and one SP (*KLK10*); the six down-regulated genes included two Serpins (*SPINK1* and *SERPINA5*) and four SPs (*KLK11*, *HPN*, *PRSS8* and *TMPRSS2*).

Real-time PCR was used to verify the differential expression of the genes selected by the microarray. A total of 10 pairs
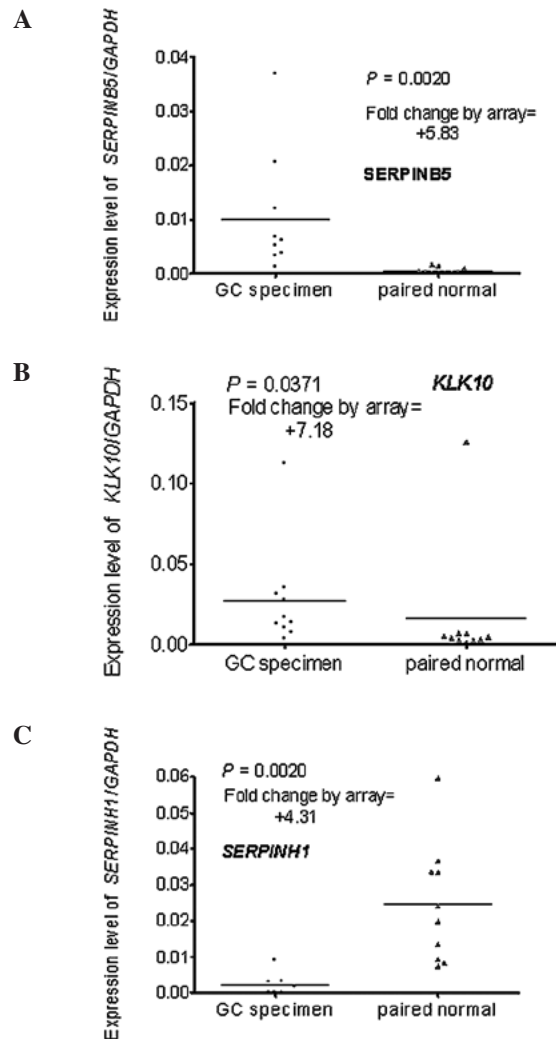

Figure 1. Expression profiles of the three up-regulated genes in the microarray assay as determined by real-time PCR. (A) *SERPINB5* was up-regulated with a 5.83-fold change in the GC specimens by microarray and demonstrated the same expression profile upon real-time PCR. (B) *KLK10* was up-regulated with a 7.18-fold change in the GC specimens by microarray and demonstrated the same expression profile upon real-time PCR. (C) *SERPINH1* showed a contradictory expression profile between the microarray and real-time PCR.

of RNA stock used in the real-time PCR were the same as those in the microarray, the remaining 30 had no remaining RNA or the RNA was of inadequate quality. The results of the real-time PCR were compared to those of the microarray. One of the three up-regulated genes (*SERPINH1*) was excluded as it was down-regulated in the real-time PCR assay (Fig. 1A-C). One of the six down-regulated genes (*TMPRSS2*) was excluded as the difference did not reach statistical significance (P=0.6250) (Fig. 2A-F).

Therefore, seven genes were further analyzed. Four genes (*SERPINB5*, *KLK10*, *KLK11* and *HPN*), which exhibited the highest differential fold change and for which a primary antibody for IHC was commercially available, were included in the IHC assay.

Correlation of the RNA and protein expression profiles of SERPINB5, KLK10, KLK11 and HPN. The expression profiles of the aforementioned four genes were assayed by real-time PCR and IHC. The RNA expression profiles were positively
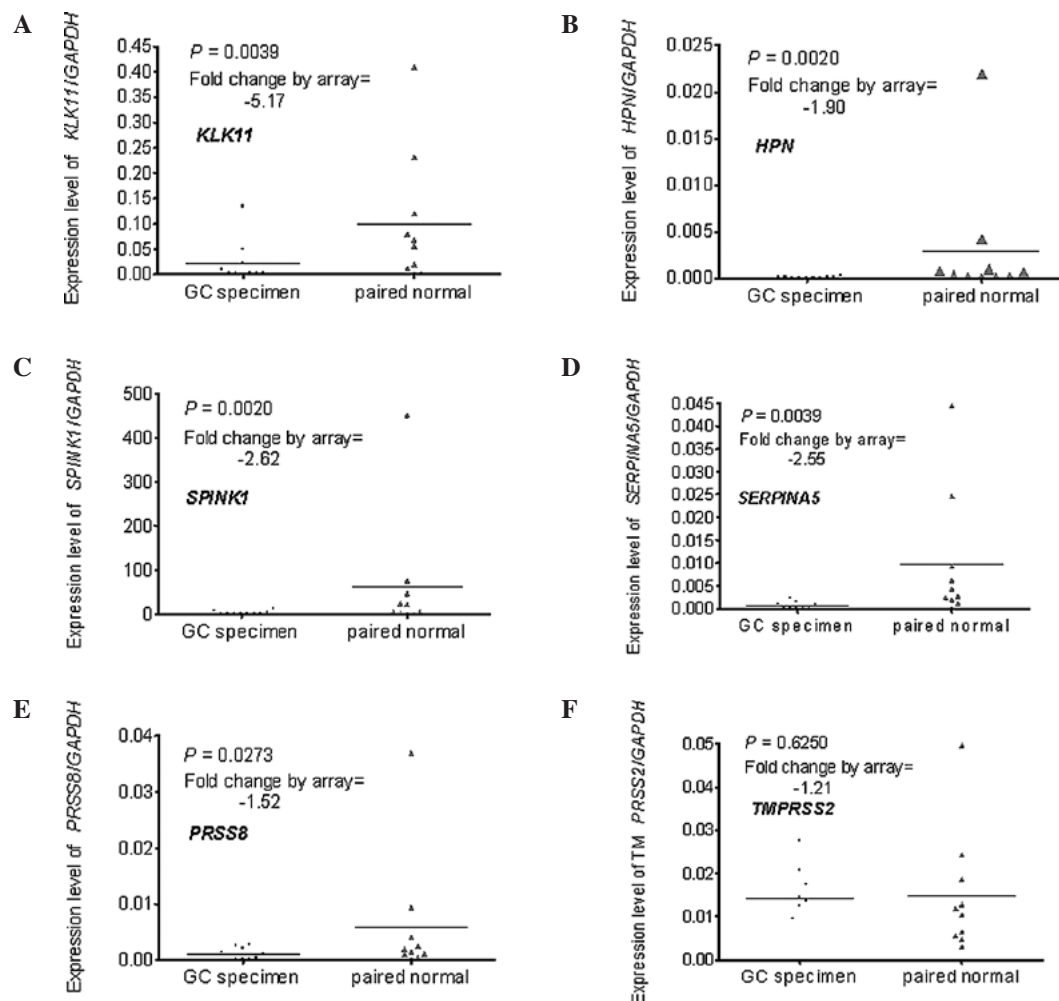
Figure 2. Expression profiles of the down-regulated genes in the microarray assay as determined by real-time PCR. (A) *KLK11*, (B) *HPN*, (C) *SPINK1*, (D) *SERPINA5* and (E) *PRSS8* were all down-regulated in the GC specimens by real-time PCR and exhibited the same expression profiles as the microarray. (F) *TMPRSS2* was excluded as the difference did not reach statistical significance (P=0.6250) by real-time PCR.

correlated with the protein expression profiles (r=0.1172 for SERPINB5, r=0.3433 for KLK10, r=0.5145 for KLK11, r=0.5092 for HPN), but the correlations did not reach statistical significance (P=0.7472 for SERPINB5, P=0.3315 for KLK10, P=0.1281 for KLK11, P=0.1328 for HPN) (Fig. 3).

Prediction of survival using the IHC score and clinical and pathological characteristics. IHC was performed in 120 patients, including 100 patients who underwent GC surgery in 2000 and 20 patients in 2003 who were enrolled in the microarray. An IHC score <3 was recognized as negative, 4-6 as weakly positive and 7-8 as positive. SERPINB5 and KLK10 were positively expressed in all of the patients (IHC score >4), while the patients with weak positive expression of SERPINB5 and KLK10 had a more favorable prognosis (Fig. 4A and B). KLK11 and HPN were negatively expressed in most of the patients. The number of patients with negative expression of KLK11 and HPN was 66 and 83, respectively, and negative expression of these genes indicated a worse survival (Fig. 4C and D).

To elucidate the factors contributing to improved survival, an analysis was carried out to identify the prognostic factors for overall survival using univariate analyses. A total of

10 factors were considered in the analyses, including patient age and gender, tumor size, WHO classification and differentiation of the tumor, TNM score (e.g., score of T3N3M0 = 3+3+0 = 6), and IHC scores for SERPINB5, KLK10, KLK11 and HPN. Tumor size, TNM score, IHC scores for SERPINB5, KLK10, KLK11 and HPN were shown to be indicative of patient survival (Table II); these six factors were used in further analyses.

Translation of expression of SERPINB5, KLK10, KLK11 and HPN together with tumor size and TNM score in the prediction model of survival. Complete data from 42 patients who underwent GC surgery in 2000 were used to develop a prediction model of survival by stepwise regression analysis and the best subset regression. It identified 63 models totally using the data of expression of SERPINB5, KLK10, KLK11 and HPN together with tumor size and TNM score. The following was identified as the most accurate model: Survival time (months) = 88.8607 + 2.6395 SERPINB5 - 12.0772 KLK10 + 13.7562 KLK11 - 7.0318 TNM, where SERPINB5, KLK10 and KLK11 are the IHC scores of these genes and TNM indicates the TNM score. This model had the highest adjusted R^2 value (0.9556) and the least CP value (4.4748).
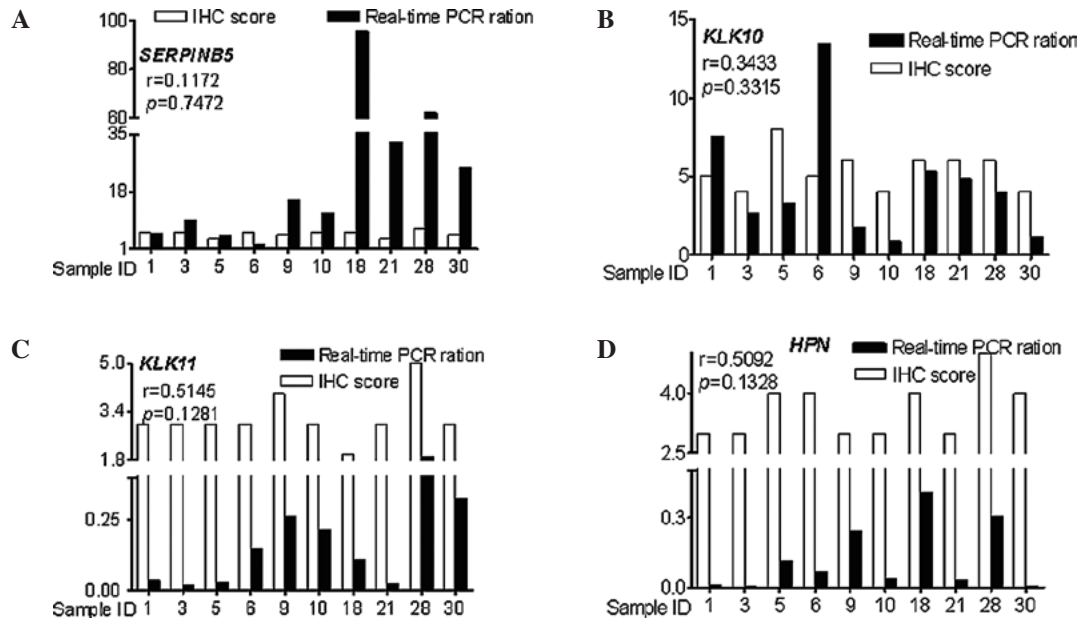
Figure 3. Correlation of the RNA and protein expression profiles of SERPINB5, KLK10, KLK11 and HPN. The RNA expression profiles were positively correlated with the protein expression profiles, yet the correlations did not reach statistical significance: (A) r=0.1172, P=0.7472 for SERPINB5; (B) r=0.3433, P=0.3315 for KLK10; (C) r=0.5145, P=0.1281 for KLK11; (D) r=0.5092, P=0.1328 for HPN.
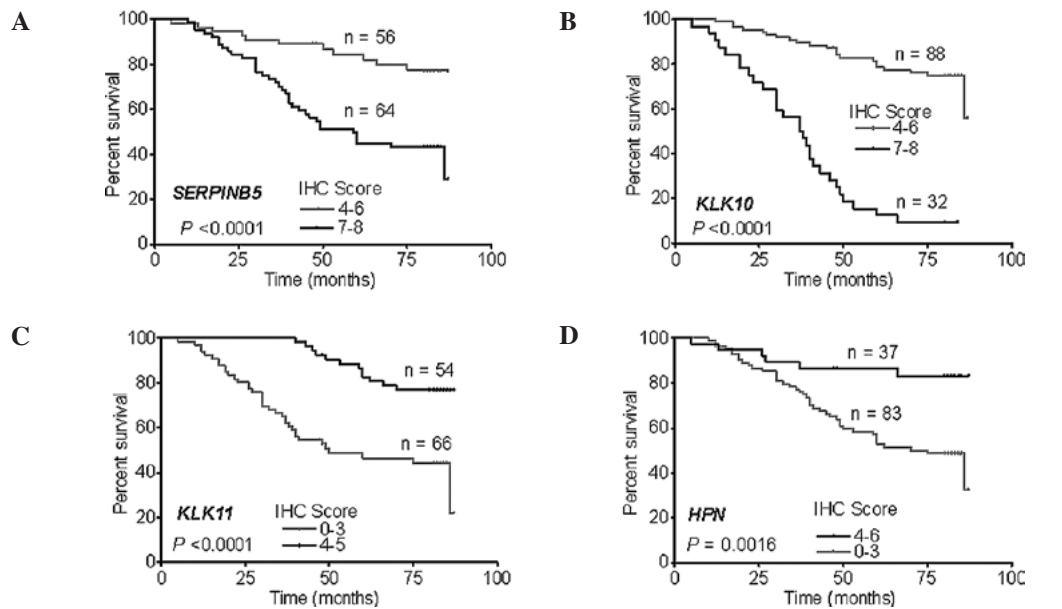

Figure 4. The survival curves of the patients were assessed according to the IHC score. The survival rate of the entire test group of patients was divided according to the IHC scores of (A) SERPINB5 and (B) KLK10. Results showed that SERPINB5 and KLK10 were positively expressed in all patients and the patients with positive expression (IHC scores 7 and 8) exhibited a worse prognosis. The survival rate of the entire test group of patients was divided according to the IHC scores of (C) KLK11 and (D) HPN. KLK11 and HPN were negatively expressed in most of the patients, while the patients with negative expression of KLK11 and HPN demonstrated worse survival rates.

Survival prediction for the different groups of GC patients. The prediction model consisting of SERPINB5, KLK10, KLK11 and TNM was applied to the different test groups of GC patients. This was considered to be accurately predictive, for complete data, when the prediction value was in the range of actual survival time ± 5 months; for censored data, the prediction value was higher than the actual survival time. Survival of 47 patients out of 58 patients of 2000 was correctly predicted, and yielded a sensitivity of 0.8103. In particular, this prediction model correctly predicted 5 death events and 14 survivals for 20 patients of 2003 (Table III). A highly predictive power was achieved for this prediction model for the GC patients in the independent test group.

Discussion

Microarray technology has rapidly evolved during the past decade. The main objectives of microarray studies are i) to

Table II. Univariate analysis of significant prognostic factors for overall survival in the GC patients.

Variable	Overall survival		
	n	Events	P-value
Age at diagnosis (years)			
≤65	87	31	0.311
>65	33	17	
Gender			
Male	77	31	0.959
Female	43	17	
Tumor size (cm)			
≤5	86	26	0.03
>5	34	22	
WHO classification			
1	104	38	0.469
2	7	4	
3	9	6	
TNM score			
1	14	2	0.006
2	31	4	
3	22	5	
4	32	19	
5	16	13	
6	5	5	
Differentiation			
1	13	3	0.612
2	63	25	
3	44	20	
SERPINB5			
Weakly positive	56	11	0.005
Positive	64	37	
KLK10			
Weakly positive	88	19	0.001
Positive	32	29	
KLK11			
Negative	66	36	0.016
Positive	56	12	
HPN			
Negative	84	42	0.014
Positive	36	6	

Table III. Frequency distribution of accuracy in all of the patients of 2003 (n=20).

Predicted status	Clinical status		Total
	Death	Survival	
Death	5	0	5
Survival	1	14	15
Total	6	14	20

Sensitivity, 83.33%; specificity, 100%; negative predictive value, 93.33%; positive predictive value, 100%.

variance in the hybridization-based systems. Studies have demonstrated that the combination of microarray and RT-PCR technologies is a highly efficient and reliable approach for the identification of clinically important diagnostic and prognostic biomarkers, as well as for the identification of novel therapeutic target candidates in pancreatic cancer (34). However, protein is mainly the effective molecule in cells. We found that the correlations between the RNA and protein expression profiles did not reach statistical significance. In our study, the cDNA microarray was used to identify differentially expressed genes, and a survival prediction model of clinical applicability was developed using the IHC data of these genes.

Nine SPs and serpin genes were found to be differentially expressed between the GC and non-cancerous gastric tissues after the microarray assay, but two genes were excluded after confirmation by real-time PCR. Therefore, seven genes were further analyzed: *SERPINB5*, *KLK10*, *KLK11*, *HPN*, *SPINK1*, *SERPINA5* and *PRSS8*. The first four genes were included in the IHC assay. Finally, three, *SERPINB5*, *KLK10* and *KLK11*, were identified to be involved in the most effective prediction model.

SERPINB5 (maspin, mammary serine protease inhibitor) was identified in 1994 by subtractive hybridization analysis of normal mammary tissue and breast cancer cell lines (16). SERPINB5 regulates the invasive activity of tumor cells (35), inhibits angiogenesis (36), primary tumor growth as well as invasion and metastasis (37). However, high expression of maspin is associated with early tumor relapse in breast cancer (38), and SERPINB5 may contribute to gastric carcinogenesis and have a potential role in tumor metastasis in GC (39,40). Our microarray data, real-time PCR and IHC assay demonstrated that the expression of SERPINB5 was up-regulated at the RNA and protein levels in the GC tissues. These may indicate that SerpineB5 is a 'bad' gene for survival. However, the final model indicated expression of SerpineB5 to be a good predictor for survival. The predictive effectiveness of SerpineB5 may be poor; further studies are required to explain this discrepancy.

KLK10 and KLK11 belong to the kallikreins which are enzymes which historically release vasoactive peptides from high molecular weight precursors. In humans, there are two categories of kallikreins: plasma and tissue kallikreins. Human tissue kallikreins have attracted increased attention due to their role as biomarkers for the screening, diagnosis, prognosis and monitoring of various types of cancers, including those of the prostate, ovarian, breast, testicular and lung (41). KLK10

identify homogeneous subtypes of a disease on the basis of gene expression, ii) to find genes that are differentially expressed in tumors with different characteristics, or iii) to develop a rule on the basis of gene expression allowing the prediction of patient prognosis or of the response to a particular treatment (31). Using cDNA microarray, a classifier containing 153 genes with weights was generated (32). Microarrays can also be used to identify leukemia subtypes (33). However, these significant results have seldom been put into clinical application as they rely on massive data output gathered from a relatively large number of genes, sophisticated algorithms for analyzing the data and costly experiment platforms, let alone the technical

is significantly up-regulated in pancreatic, colon, ovarian and gastric cancer (42,43); this is consistent with our findings concerning KLK10. KLK11 is down-regulated in renal cell carcinoma and prostate cancer (44,45), similar to our findings in GC. It appears that these genes have only limited predictive power in isolation, but we get a more efficient survival prediction model combining the expression profiles of these genes with the TNM status of the tumor.

The prognostic model presented here may predict the survival of GC patients after radical resection; this may influence the clinical treatment of GC patients by aiding in the selection of patients for adjuvant therapy and developing meaningful clinical trials for new regimens.

Although the genes in our model were selected by microarray, the model is independent of the microarray platform. It requires only IHC, which is a common assay and can be performed easily in any laboratory or pathological department of any hospital. Certainly, our model is developed from the data of a relatively small population of patients and from one digestive surgery center, therefore it may require various modifications in future practice.

Acknowledgements

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