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A 70-Gene Signature for Predicting Treatment Outcome in Advanced-Stage Cervical Cancer

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Cervical cancer is the fourth most common cancer in women worldwide. The current approaches still have limitations in predicting the therapy outcome of each individual because of cancer heterogeneity. The goal of this study was to establish a gene expression signature that could help when choosing the right therapeutic method for the treatment of advanced-stage cervical cancer. The 666 patients were collected from four independent datasets. The 70-gene expression signature was established using univariate Cox proportional hazard regression analysis. The 70-gene signature was significantly different between low- and high-risk groups in the training dataset (p = 4.24e−6) and in the combined three validation datasets (p = 4.37e−3). Treatment of advanced-stage cancer patients in the high-risk group with molecular-targeted therapy combined with chemoradiotherapy yielded a better survival rate than with only chemoradiotherapy (p = 0.0746). However, treatment of the patients in the low-risk group with the combined therapy resulted in significantly lower survival (p = 0.00283). Functional classification of 70 genes revealed involvement of the angiogenesis pathway, specifically phosphatidylinositol 3-kinase signaling (p = 0.040), extracellular matrix organization (p = 0.0452), and cell adhesion (p = 0.011). The 70-gene signature could predict the prognosis and indicate an optimal therapeutic modality in molecular-targeted therapy or chemotherapy for advanced-stage cervical cancer.

INTRODUCTION

Cervical cancer is the fourth most frequent cancer in women, and about 570,000 new cases were diagnosed in 2018, representing 6.6% of all female cancers.[1] Early-stage cervical cancer rate increased because of the development of diagnosis technique and screening test. However, many patients are still diagnosed in the advanced stage with poor prognosis. Therefore, it is very important for cervical cancer patients to get a timely, accurate diagnosis and appropriate treatment to increase the survival rate. The National Comprehensive Cancer Network (NCCN) and the International Federation of Gynecology and Obstetrics (FIGO) suggested standard treatment guidelines, such as surgery, radiotherapy, and chemotherapy, for treatment of the patients since the time of diagnosis and according to the disease stage. Even though patients are treated based on these guidelines, each patient has a different prognosis because of the tumor heterogeneity. Therefore, proper stratification of patients, depending on their clinical conditions, is required. In this way, a more effective treatment could be provided to the patient.

Many clinical and pathological studies have descried a number of prognostic factors for cervical cancer, such as clinical stage, tumor histology, depth of invasion, tumor grade, size of primary tumor, lymph node involvement, parametrium involvement, and lymph-vascular space invasion.[2–7] A more successful result was obtained when the therapeutic methods, such as surgery, radiotherapy, and chemotherapy, were applied to patients with certain characteristics. NCCN recommends either surgery or radiotherapy for the treatment of patients with FIGO stage IA1–IIA1 called early stage. In contrast, the treatment of choice for advanced cancer with stages IIB–IVA consists of a combined therapy including external beam radiation therapy (EBRT), cisplatin-containing chemotherapy, and brachytherapy.[8–10] In case of metastasis, targeted therapy and immunotherapy have been applied.[11,12] Despite the development of many treatment options, the survival rate in advanced-stage cancer remains poor.

Recently, the gene expression profiles obtained from microarray, next generation sequencing (NGS), and subsequent oncogenic signaling pathway analysis have become useful for predicting prognosis of the disease and for discovering therapeutic targets in various cancers.[13–15] In estrogen receptor-positive breast cancer, a 21-gene signature has been widely used to improve disease-free survival and to predict chemo-resistance to anthracyclines and a beneficial outcome with tamoxifen administration.[16,17] In this regard, microarray data have been studied for diagnosis, prognosis, or prediction of therapeutic response in cervical cancer as well.[18–21] However, no favorable

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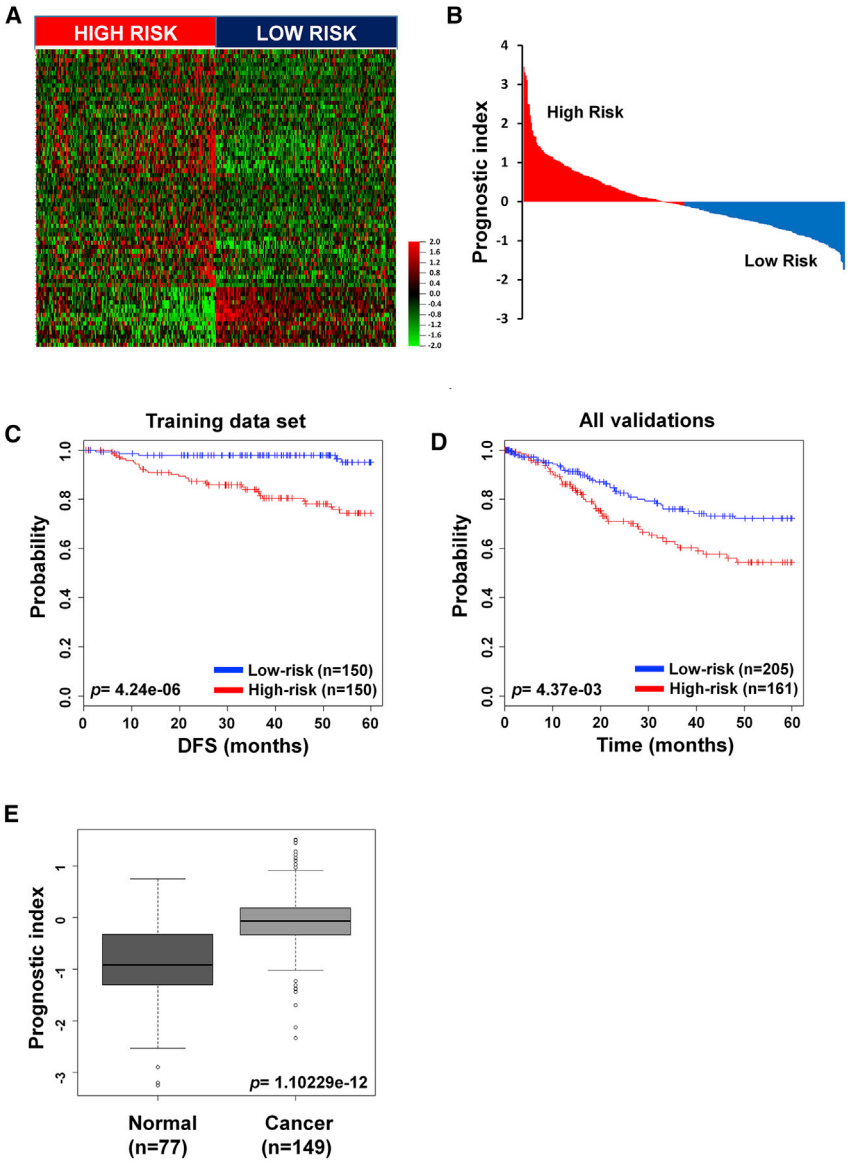

Figure 1. Hierarchical Clustering and Classification by 70 Genes

(A) Heatmap of the median centered 70 genes' expression profiles between high- and low-risk groups in the training dataset (red, relative high expression; green, relative low expression). (B) The relative prognostic index based on 70 genes' expression of each patient. The weight of each gene was calculated by the Cox proportional hazard regression model. (C and D) The 70-gene signature of patients with disease-free survival (DFS) in the training dataset (C) and overall survival (OS) in combined validation datasets (D). Each group was classified by the 70-gene signature into low and high risk, and evaluated by Kaplan-Meier analysis. (E) Prognostic index value of the 70-gene signature in cervical cancer and healthy tissue. The p values were computed by the log rank test.

results have been obtained yet. Therefore, more reliable gene signatures are warranted to further achieve predictive accuracy of prognosis and effective treatments.

In this study, we established a novel prognostic gene signature to distinguish low- and high-risk patients. In addition, we assessed the association between the gene signatures and clinicopathological factors. Finally, we provided a patient profile that could receive the most benefit from molecular-targeted therapy.

RESULTS

Stratification by Hierarchical Clustering

In order to identify a prognostic gene signature that distinguished low- and high-risk patients, we analyzed gene expression profiles based on survival data. Gene Expression Omnibus (GEO): GSE44001 was assigned as the training dataset; meanwhile, The Cancer Genome Atlas (TCGA) RNA sequencing (RNA-seq) and microarray data (GEO: GSE39001 and GSE52904) corresponded to validation datasets. Patients were classified into low- (n = 150) and high-risk (n = 150) groups by the expression pattern of 70 genes and their prognostic indexes (PIs) (Figures 1A and 1B). There was a significant survival difference between the low- and high-risk groups (p = 4.24e−6; Figure 1C). The 70-gene signature was applied to other datasets for validation, and a statistically significant difference of survival between the two groups was observed (p = 0.00437; Figure 1D). Healthy controls had lower PI than cervical cancer patients (p = 1.102e−12; Figure 1E). In both RNA-seq and microarray datasets, low-risk patients had a better survival rate than high-risk patients (p = 0.0311 and p = 0.0466, respectively; Figures S1A and S1B). The expression pattern of 70 genes was divided by low- and high-risk patients in both microarray and TCGA datasets (Figures S1C and S1D).

Validation of Predicted Gene Signature in Specific Tumor Stages

Next, cervical cancer patients were divided based on NCCN guidelines and FIGO staging system into two groups: early stage (stage I–IIA) and advanced stage (stage IIB–IV). Patients in the early-stage group can be cured by surgery and/or radiotherapy, whereas those with advanced-stage cancer require combined therapy, including radiation and chemotherapy.

As previously reported, the survival rate in our patient data was also different between the early and advanced stages (p = 3.35e−13; Figure 2A). Our 70-gene signature stratified patients' survival in all stages

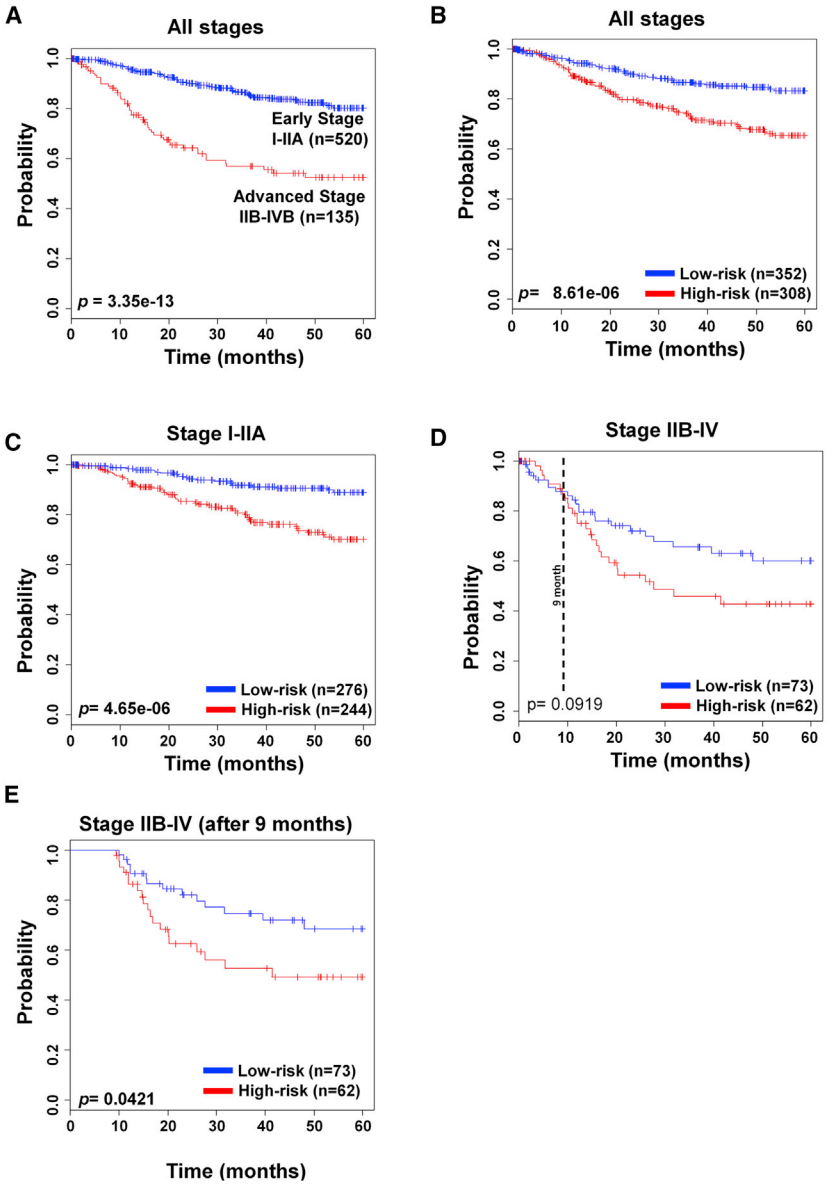

Figure 2. Validation of the 70-Gene Signature in Tumor Stage

(A) Patients were separated according to early- and advanced-stage cancer. (B–E) The 70-gene signature was applied to cancer patients of all stages (B), early stage (C), advanced stage (D), and advanced stage after 9 months (E). Each group was classified by the 70-gene signature into low and high risk, and evaluated by Kaplan-Meier analysis. The p values were computed by the log rank test.

(p = 8.61e−6; Figure 2B). Importantly, the low-risk group had a significantly higher survival rate than the high-risk group in the early stage (p = 4.65e−6; Figure 2C). However, advanced-stage patients did not show the statistical significance (p = 0.0919; Figure 2D), but patients showed significant survival difference between the two groups after 9 months (p = 0.0421; Figure 2E). To evaluate the prognostic accuracy of the 70-gene signature in relation to the cancer stage, we performed univariate and multivariate Cox proportional hazards regression analyses using all of the datasets. As shown in Table S1, stage was significantly associated with overall survival (OS) in both analyses (univariate: hazard ratio [HR], 3.547, 95% confidence interval [CI]: 2.466–5.103, p = 8.8e−12; multivariate: HR, 3.691, 95% CI: 2.564–5.313, p = 2.2e−12). The 70-gene signature was also significantly associated with OS according to univariate and multivariate analyses (univariate: HR, 2.265, 95% CI: 1.566–3.275, p = 1.4e−5; multivariate: HR, 2.369, 95% CI: 1.637–3.428, p = 4.8e−6).

Association of the 70-Gene Signature with Tumor Size and Age

The tumor size is an important clinical diagnosis factor; the threshold size for our study was 4 cm in diameter. As reported, cervical cancer patients with tumor over 4 cm had a worse prognosis than under 4 cm (p = 2.91e−5; Figure 3A). The patients in the low-risk group had lower PI values than those of the high-risk group, even though they carried tumors of over 4 cm in size (Figure 3B). Regardless of tumor size, the high-risk group had poor survival rate in both groups: under and over 4 cm (p = 0.0049 and p = 0.00735, respectively; Figures 3C and 3D). Cox proportional hazard regression test was performed when analyzing the association with tumor size (univariate: HR, 3.703, 95% CI: 1.917–7.152, p = 9.7e−5; multivariate: HR, 2.724, 95% CI: 1.397–5.311, p = 0.00326) and 70-gene signature (univariate: HR, 6.769, 95% CI: 2.632–17.409, p = 7.3e−5; multivariate: HR, 5.491, 95% CI: 2.108–14.304, p = 0.00049) (Table S2).

Patients younger than 65 years of age at the time of diagnosis of cervical cancers have higher chances of longer survival.[22] The 70-gene signature further stratified patients according to age under 65 years into low- and high-risk groups (p = 0.00153; Figure S2A). In this age group, patients were stratified regardless of early or advanced stage (p = 0.0126 and p = 0.0653, respectively; Figures S2B and S2C). The Cox proportional hazard regression test was performed in advanced stage with under 65 years old (univariate: HR, 2.144, 95% CI: 1.346–3.416, p = 0.0013; multivariate: HR, 2.048, 95% CI: 1.284–3.265, p = 0.0033) and 70-gene signature (univariate: HR: 2.134, 95% CI: 1.328–3.432, p = 0.0017; multivariate: HR, 2.095, 95% CI: 1.303–3.368, p = 0.0023) (Table S3).

The Prediction of Therapeutic Effect by the 70-Gene Signature

Next, we analyzed the treatment method, such as hysterectomy, radiotherapy, chemotherapy, and molecular-targeted therapy, that was applied to early-stage or late-stage cancer patients. Following the

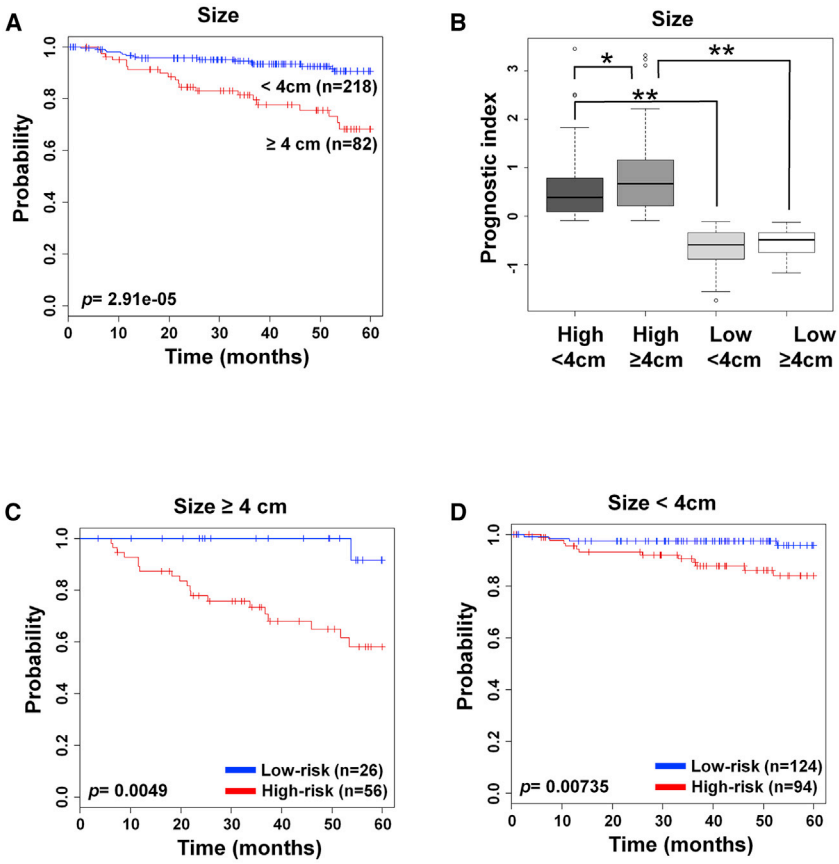

Figure 3. Survival Analysis by the 70-Gene Signature along with Tumor Size

(A) Patients were separated according to the tumor size: under 4 cm and over 4 cm in diameter. (B) The prognostic index of 70-gene signature in the cross-combination of risk group and tumor size. (C and D) The 70-gene signature was applied to patients with tumor sizes over 4 cm (C) and under 4 cm (D). Each group was classified by 70-gene signature into low and high risk, and evaluated by Kaplan-Meier analysis. The p values were computed by the log rank test. *p < 0.05, **p < 0.0001.

NCCN guidelines, the first-line treatment is hysterectomy and/or radiotherapy in early-stage patients. In early-stage patients, there was no difference in patient survival depending on the applied conventional therapy, such as hysterectomy and/or radiotherapy (Figure S3A). Chemotherapy did not affect the survival even in combination with conventional therapies (Figure S3B). However, chemoradiation therapy is the first-line therapy in advanced-stage patients. The chemoradiation therapy showed a significant difference between the low- and high-risk groups (p = 0.0341; Figure S4). Recently, molecular-targeted therapy was provided to increase survival of patients. Survival differences were observed among the various combination therapies in both low- and high-risk patients with advanced-stage cancer (p = 7.04e−5 and p = 0.0208, respectively; Figures 4A and 4B). However, molecular-targeted therapy resulted in the opposite effect between low- and high-risk patients (p = 0.00283 and p = 0.0746, respectively; Figures 4C and 4D). The combined molecular-targeted therapy, hysterectomy, and chemoradiation therapy affected the survival of patients in only the high-risk group (p = 0.705, and p = 0.0374, respectively; Figures 4E and 4F). In this study, the term hysterectomy included radical hysterectomy, simple hysterectomy, and radical trachelectomy.

Classification Analysis Results of 70 Genes in Cervical Cancer

Gene Ontology enrichment analysis in DAVID (Database for Annotation, Visualization, and Integrated Discovery, National Institutes of Health, Bethesda, MD, USA) was used to identify the biological functions of the genes in the 70-gene signature (Table S4) and identified 31 significant terms (biological processes) (Table 1). These terms consist of 29% of cellular process, 16.8% metabolic process, 10.3% biological regulation, and 10.3% response to stimulus (Figure 5A). The 70 genes were analyzed to see the relationship with functional pathway using the PANTHER system. They were involved in various pathways (Figure 5B). The functions of 70 genes were related to binding (44.0%), catalytic activity (34.0%), and other processes (Figure 5C).

DISCUSSION

The previous studies showed that the gene signatures can predict prognosis in cervical cancer patients. In locally advanced cervical cancer, it was suggested that ANXA2-NDRG1-STAT1 gene signature was a candidate for concurrent chemoradiation treatment.[20] Fernandez-Retana et al.[23] established a molecular signature comprising eight degradome-related genes that predicted which patients were at risk for developing distal metastasis among the locally advanced cervical cancer patients. The epigenetic gene regulation has also been extensively analyzed. The methylation pattern observed in promoters of nine genes constituted a potential biomarker for early detection and screening of cervical cancer.[24] In the other studies, gene signatures of microRNAs showed correlation with early diagnosis, prognosis, treatment, occurrence, and cancer development.[25–28] The long non-coding RNAs also correlated with diagnosis, prognosis, recurrence, metastasis, and effective targeted therapy.[29–32]

In clinic, FIGO stage system classifies cancer depending on tumor size, spread to a lymph node, and metastasis. Stages are further subdivided based on the lesion's maximum diameter: stage IB3, diameter of ≥4 cm; and stage IIA2, diameter of ≥4 cm.[33] Cervical cancer is diagnosed most frequently in middle-aged women between the ages of 35 and 44 years. It rarely develops in women younger than 20 years. The older women, over 65 years old, account for 10% of cervical cancer patients and are more likely to die of the disease because they are at advanced stage when

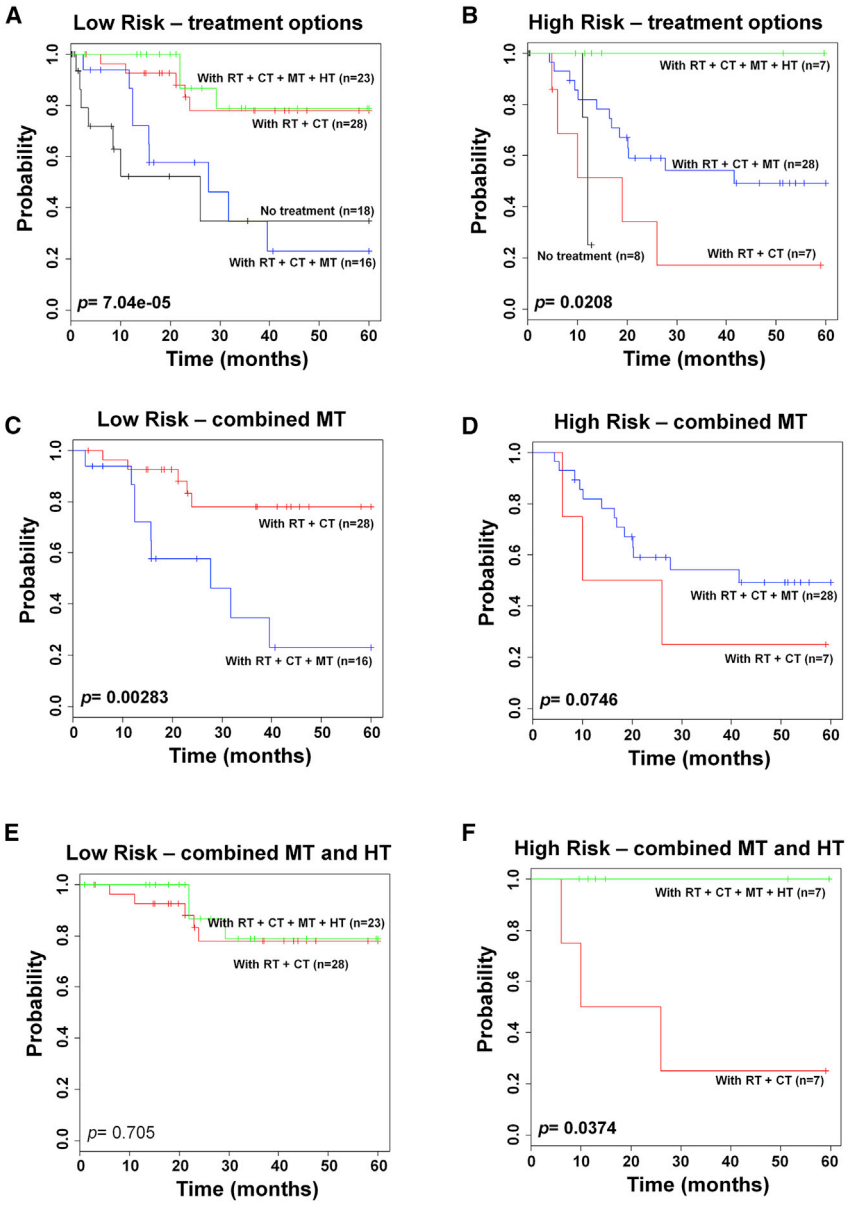

Figure 4. Survival Prediction of Therapeutic Effect by the 70-Gene Signature in Advanced-Stage Cervical Cancer

(A) The therapeutic advantage was evaluated by Kaplan-Meier analysis. Patients were separated according to the treatment options in low-risk. (B) The therapeutic advantage was evaluated by Kaplan-Meier analysis. Patients were separated according to the treatment options in high-risk. (C) Chemoradiation therapy with/without molecular-targeted therapy in low-risk. (D) Chemoradiation therapy with/without molecular-targeted therapy in high-risk. (E) Combined chemoradiation therapy, molecular-targeted therapy, with/without hysterectomy in low-risk. (F) Combined chemoradiation therapy, molecular-targeted therapy, with/without hysterectomy in high-risk. Each group was evaluated by Kaplan-Meier analysis. The p values were obtained from the χ^2 test. CT, chemotherapy; HT, hysterectomy; MT, molecular-targeted therapy; RT, radiotherapy.

diagnosed. Importantly, we showed that the low-risk patients stratified by our 70-gene signature had better survival even in the same tumor stage, size, and age, suggesting that cervical cancer patients were more accurately subclassified if the 70-gene signature was added to the conventional classification system.

Although the prediction of survival and prognosis depends on the FIGO stage, tumor size, and age, it is unknown why prognosis, survival, and drug response are different in the same stage. Radiotherapy and/or hysterectomy is the standard therapy for treatment of early-stage cancer, whereas chemoradiation therapy is generally used for advanced-stage patients. Generally, 80%–90% of patients in early stage are cured using surgery and/or radiotherapy, but it is still difficult to choose the right treatment method for women with advanced-stage cervical cancer. To increase the survival of the patients, chemotherapy and molecular-targeted therapy are currently recommended to early-stage and advanced-stage patients, respectively. In this study, we showed that the 70-gene signature increased accuracy for predicting prognosis of the patients. We observed that chemotherapy had no effect on survival in the early stage of cancer. In contrast, molecular-targeted therapy combined with chemoradiation therapy provided a survival benefit in high-risk patients (p = 0.0746), but had the opposite result in low-risk patients (p = 0.00283). Thus, the 70-gene signature could help to recommend molecular-targeted therapy to high-risk patients suffering from advanced-stage cancer.

Molecular-targeted therapy was approved in recurrent or metastatic cervical cancer.[34] In clinical trials, molecular-targeted therapy increased OS and progression-free survival (PFS), but decreased HR. In a randomized controlled study, vascular endothelial growth factor (VEGF) increased median OS up to 4 months and decreased HR to 0.71.[35] HER2 and epidermal growth factor receptor (EGFR) targeted therapies (clinical trial phase II, randomized controlled study) showed median OS (11 months), time to progression (4.27 months), and stable disease (44%).[36] A single treatment of EGFR targeted agents showed minimal activity or no meaningful benefits.[37–41] programed cell death (PD-1) and programmed cell death ligand 1 (PD-L1) inhibitors increased the response rate in recurrence and metastatic cervical cancer patients.[42] In our study, 31 biological processes were identified from Gene Ontology term

Table 1. Gene Ontology Analysis

Term	Name	Count	p Value	Genes
GO:0031623	receptor internalization	4	0.000698039	GRB2, GHR, DNM2, BTN1A1
GO:0040014	regulation of multicellular organism growth	3	0.004932049	FGFR2, STAT5A, GHR
GO:0001764	neuron migration	4	0.008871214	CXCL12, CDK5R2, BAX, NTRK2
GO:0007155	cell adhesion	7	0.010592445	CXCL12, CX3CL1, NCAN, PCDHB6, APLP1, HAPLN1, COL19A1
GO:0032526	response to retinoic acid	3	0.011967854	RBP4, NCOA1, SCAMP3
GO:0006549	isoleucine metabolic process	2	0.01210023	STAT5A, GHR
GO:0000255	allantoin metabolic process	2	0.01210023	STAT5A, GHR
GO:0007595	lactation	3	0.012533686	NCOA1, STAT5A, GHRHR
GO:0006573	valine metabolic process	2	0.016101492	STAT5A, GHR
GO:0046449	creatinine metabolic process	2	0.016101492	STAT5A, GHR
GO:0001501	skeletal system development	4	0.018107062	ZBTB16, NCAN, HAPLN1, COL19A1
GO:0006417	regulation of translation	3	0.018809178	DDX25, EIF4E, TYMS
GO:0019530	taurine metabolic process	2	0.020086785	STAT5A, GHR
GO:0048562	embryonic organ morphogenesis	2	0.020086785	RBP4, FGFR2
GO:0021987	cerebral cortex development	3	0.021620051	NCOA1, BAX, NTRK2
GO:0060041	retina development in camera-type eye	3	0.023086905	BAX, SERPINF1, NTRK2
GO:0018108	peptidyl-tyrosine phosphorylation	4	0.0241554	BTC, FGFR2, STAT5A, NTRK2
GO:0006101	citrate metabolic process	2	0.028009715	STAT5A, GHR
GO:0051384	response to glucocorticoid	3	0.028530929	TYMS, GHR, GHRHR
GO:0007568	aging	4	0.02932512	GRB2, SERPINF1, TYMS, BTN1A1
GO:0010518	positive regulation of phospholipase activity	2	0.031947477	ARHGAP6, FGFR2
GO:0060670	branching involved in labyrinthine layer morphogenesis	2	0.03586952	GRB2, FGFR2
GO:0060068	vagina development	2	0.03586952	RBP4, BAX
GO:0030324	lung development	3	0.038002374	RBP4, EIF4E, FGFR2
GO:0006105	succinate metabolic process	2	0.039775906	STAT5A, GHR
GO:0014066	regulation of phosphatidylinositol 3-kinase signaling	3	0.039837466	GRB2, BTC, FGFR2
GO:0006600	creatine metabolic process	2	0.043666696	STAT5A, GHR
GO:0030198	extracellular matrix organization	4	0.045163581	MFAP2, NCAN, HAPLN1, COL19A1
GO:0006107	oxaloacetate metabolic process	2	0.047541952	STAT5A, GHR
GO:0002031	G-protein-coupled receptor internalization	2	0.047541952	DNM2, BTN1A1
GO:0045778	positive regulation of ossification	2	0.047541952	ZBTB16, BTN1A1

analysis of 70 genes. Analysis by the PANTHER classification system revealed the angiogenesis pathway as the major target of molecular-targeted therapy. Bevacizumab, an angiogenesis blocker, was associated with regulation of phosphatidylinositol 3-kinase signaling, extracellular matrix organization, and cell adhesion, which were identified in our Gene Ontology analysis (Table 1).

Our study had several strengths. First, we showed that the 70-gene signature was a good biomarker for better prognosis prediction of cervical cancer. Second, it allowed for stratification of the patients into two distinctive risk groups even when the patients were also classified in terms of stage, size, and age. Finally, it provided the guidance on how to select patients (high risk in the advanced stage) who could benefit from optimal molecular-targeted therapy. There are a few limitations in this study. Only a small number of patients were analyzed for outcome determination with each therapeutic modality, and more detailed analysis of each molecular-targeted therapy and chemotherapy drugs was not possible to perform due to limited information. The clinical stages used in this study corresponded to the previous FIGO version because the new staging system was announced in 2019.

In conclusion, the 70-gene signature could constitute a more accurate biomarker for further stratification of patients besides by stage, size, and age. It also provided guidance for identifying patients who could

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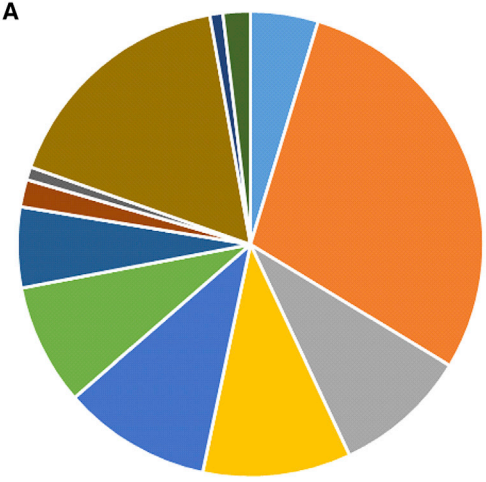

Biological Process	GO No.	%
Cellular component organization or biogenesis	GO:0071840	4.7
Cellular process	GO:0009987	29.0
Localization	GO:0051179	9.3
Biological regulation	GO:0065007	10.3
Response to stimulus	GO:0050896	10.3
Developmental process	GO:0032502	8.4
Multicellular organismal process	GO:0032501	5.6
Locomotion	GO:0040011	1.9
Biological adhesion	GO:0022610	0.9
Metabolic process	GO:0008152	16.8
Growth	GO:0040007	0.9
Immune system process	GO:0002376	1.9

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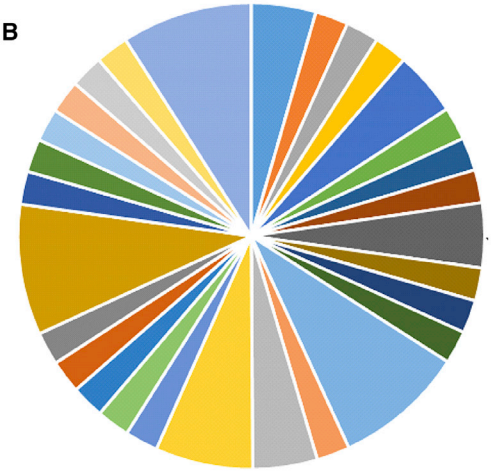

Pathway	P No.	%
Axon guidance mediated by Slit/Robo	P00008	4.5
JAK/STAT signaling pathway	P00038	2.3
Apoptosis signaling pathway	P00006	2.3
P38 MAPK pathway	P05918	2.3
Interleukin signaling pathway	P00036	4.5
Angiogenesis	P00005	2.3
Integrin signaling pathway	P00034	2.3
Insulin/IGF pathway-MAPK kinase/MAP kinase cascade	P00032	2.3
Inflammation mediated by chemokine and cytokine signaling pathway	P00031	4.5
De novo pyrimidine DNA biosynthesis	P02739	2.3
Dopamine receptor mediated signaling pathway	P05912	2.3
Parkinson disease	P00049	2.3
EGF receptor signaling pathway	P00018	9.1
Gonadotropin-releasing hormone receptor pathway	P06664	2.3
PI3K kinase pathway	P00048	4.5
PDGF signaling pathway	P00047	6.8
Cytoskeletal regulation by Rho GTPase	P00016	2.3
Nicotine pharmacodynamics pathway	P06587	2.3
Ras pathway	P04393	2.3
Cadherin signaling pathway	P00012	2.3
B cell activation	P00010	2.3
CCKP signaling map	P06959	9.1
Formyltetrahydroformate biosynthesis	P02743	2.3
Tetrahydrofolate disease	P02742	2.3
Huntington disease	P00029	2.3
P53 pathway	P00059	2.3
Wnt signaling pathway	P00057	2.3
T cell activation	P00053	2.3
FGF signaling pathway	P00021	9.1

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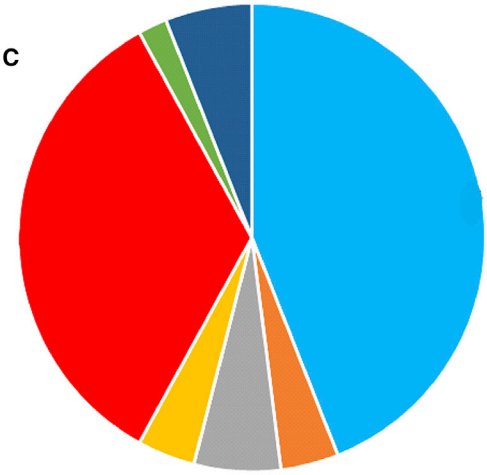

Molecular function	GO No.	%
Binding	GO:00054888	44.0
Receptor activity	GO:0004872	4.0
Structural molecule activity	GO:0005198	6.0
Signal transducer activity	GO:0004871	4.0
Catalytic activity	GO:0003824	34.0
Antioxidant activity	GO:0016209	2.0
Transporter activity	GO:0005215	6.0

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Table 2. Clinical Characteristics of Patients in the Training and Validation Datasets with Cervical Cancer

	Training Set	Validation Set		
Variable	GEO: GSE44001	TCGA	GEO: GSE39001	GEO: GSE52094
Patients used, n	300	290	21	55
Median age (range), years		46 (20–88)	43 (32–67)	50 (24–74)
Tumor size				
<4 cm	218			
≥4 cm	82			
FIGO stage (n)				
I	258	155	14	27
II	42	66	7	8
III	0	42	0	16
IV	0	21	0	4
DFS (range), months	47.5 (0.43–104.13)			
Follow-up (range), months			61 (17–93)	58 (1–86)
OS (range), months		18.4 (0.03–214)		
Hysterectomy				
Yes		155	11	15
No		9	10	40
Radiotherapy				
Yes		175	17	41
No		63	4	14
Chemotherapy				
Yes		145	11	23
No		144	10	32
Molecular-targeted therapy				
Yes		84		
No		44		

DFS, disease-free survival; FIGO, International Federation of Gynecology and Obstetrics; OS, overall survival; TCGA, The Cancer Genome Atlas.

benefit from molecular-targeted therapy in advanced-stage cervical cancer.

MATERIALS AND METHODS

Gene Expression Databases

Three gene expression datasets were obtained from the National Center for Biotechnology Information GEO database (http://www.ncbi.nlm.nih.gov/geo) and one NGS dataset was from TCGA database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). Gene expression data from the GEO: GSE44001 was used as the training dataset. TCGA, GSE39001, and GSE52904 were used as validation datasets (Table S5). Table 2 showed clinicopathological and clinical information of patients in detail, which were provided by TCGA and GEO databases. Healthy controls data were collected from GEO: GSE39001 and GEO: GSE52904.

Development of the Prognostic Gene Expression Signature

The gene expression data in the GEO: GSE44001 dataset were used to develop the gene signature. At first, genes were filtered by more than 1.5-fold absolute value of log2 scale, which represented the same gene expression level. The filtering step leads to 26,156 probes that were selected among various probe sets. In the next step, the disease-free survival (DFS)-associated gene expression signature derived from the training dataset was identified by the univariate Cox proportional hazard regression ($p < 0.001$). Initially, 175 probes were found from the analysis. However, only 70 genes were common among the four datasets (one training and three validation datasets). Then the 70-gene signature was selected as a prognostic signature. To predict prognosis, we applied selected probes from the survival signature to the survival risk prediction analysis. The PI was computed by the formula:

$$\sum_i w_i x_i - 0.250394$$

where w_i and x_i were the weight and logged gene expression for the *i*-th gene, respectively.

The patients were divided into two groups based on a median PI of −0.107633. If the PIs were greater than −0.107633, patients were assigned to the high-risk group. In contrast, patients with PIs equivalent to or less than −0.107633 were assigned to the low-risk group. The cluster analysis was performed with Cluster 3.0. Initially, 175 probes were used for prediction model analysis, and only the 70 genes were common in all of the chip types (Figure S5).

Validation of the Prognostic Gene Expression Signature

The validation of the gene signature was accomplished on independent datasets. Gene expression data from validation datasets were individually adjusted by subtracting the median expression value across the samples. To integrate each validation dataset in order to construct the prediction models, we aligned the 70-gene set in each dataset.

To further refine this model and to sub-stratify the predicted outcomes, we used Compound Covariate Predictor (CCP) as a class prediction algorithm. Gene expression data in the training set were combined to form a classifier according to CCP. The robustness of the classifier was determined by the misclassification rate obtained during

Figure 5. The Functional Classification Analysis of 70 Genes in Cervical Cancer

PANTHER classification system was used to classify the 70 genes according to their functions. (A) Biological process analysis. (B) Functional pathway analysis. (C) Molecular function analysis of the proteins translated by 70 genes.

the leave-one-out cross-validation (LOOCV) in the training set. Kaplan-Meier (KM) survival analyses were performed after the patients were divided into two predicted subgroups, and chi-square (χ^2) and log rank tests were used to evaluate the survival risk between the two predicted subgroups of patients. Univariate and multivariate Cox proportional hazard models were used to evaluate independent prognostic factors associated with survival, gene signature, stage, size, and age, as covariates.

Statistical Methods of Microarray Data

Heatmap was analyzed using BRB-Array Tools Version 4.3 (National Institutes of Health, MD, USA). All other statistical analyses were accomplished in the R language environment (R Foundation for Statistical Computing, Vienna, Austria) and Statistical Package for Social Sciences (SPSS) software (version 20; IBM, Armonk, NY, USA). Cluster analysis was performed with Cluster 3 and Tree View (Stanford University, CA, USA). Statistical significance was defined as $p < 0.05$.

Differential Protein and Gene Categorization and Network Modeling

DAVID was used to classify the genes. DAVID provides a comprehensive set of functional annotation and biological meaning of genes. PANTHER classification system (University of Southern California, CA, USA) was used to classify the proteins, and genes by the function. Protein functions were categorized according to the biological process, protein class, and molecular function through protein's relationship analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.09.001.

AUTHOR CONTRIBUTIONS

N.N.Y.N., Y.H.J., and S.S.K. designed and performed the research, analyzed the data, and wrote the manuscript. T.G.C., J.K., and Y.S. assisted in bioinformatics and statistical analyses. M.H.J. and S.H.K. supervised the study and wrote the manuscript. I.K. and J.H. conceived the project and supervised the study. All authors revised and approved the manuscript. Y.H.J. and S.S.K. shared the corresponding authorship.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Supplemental Information

A 70-Gene Signature for Predicting Treatment Outcome in Advanced-Stage Cervical Cancer

Ngoc Ngo Yen Nguyen, Tae Gyu Choi, Jieun Kim, Min Hyung Jung, Seok Hoon Ko, Yoonhwa Shin, Insug Kang, Joohun Ha, Sung Soo Kim, and Yong Hwa Jo

Figure S1.

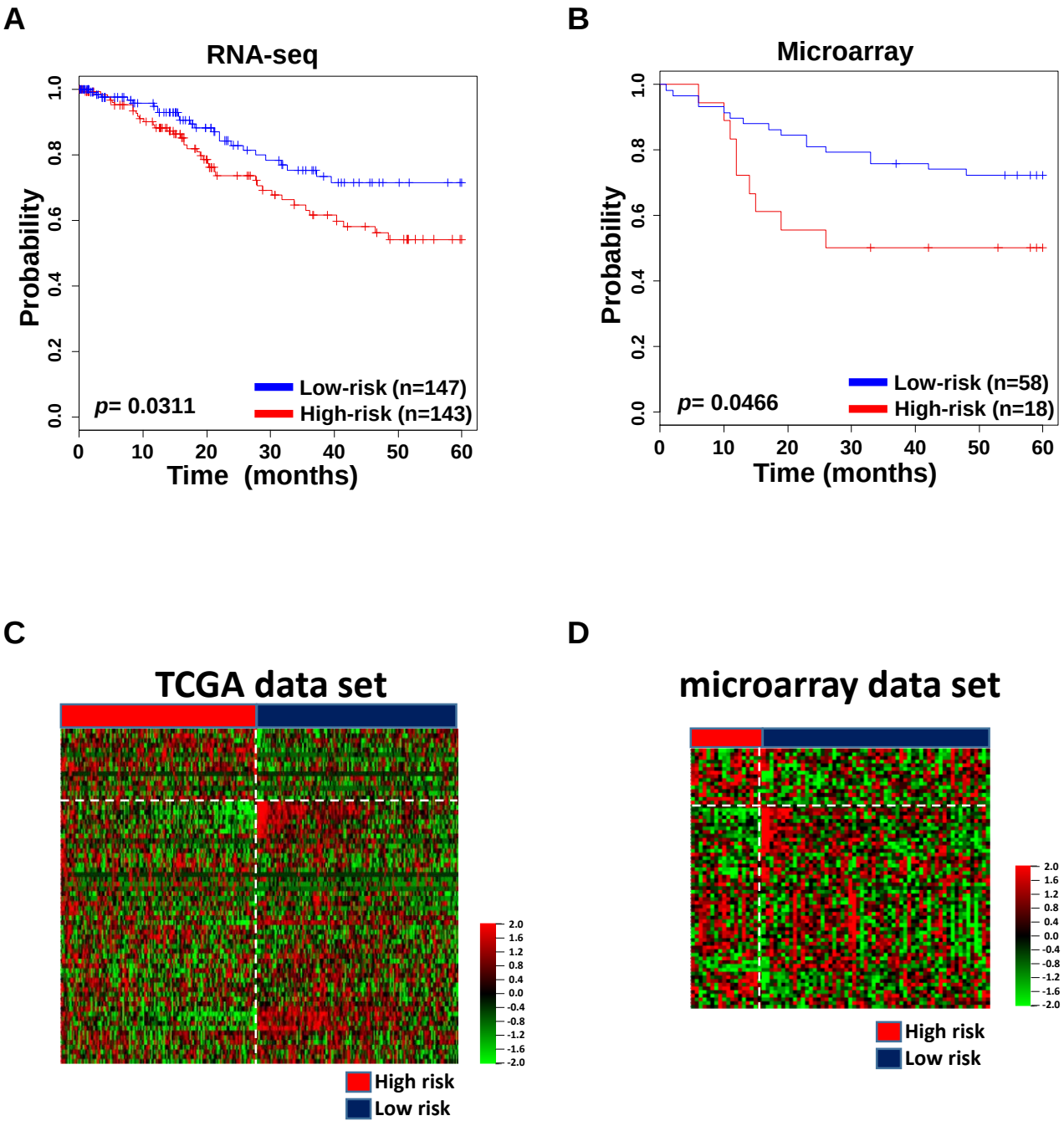
A
RNA-seq
Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Time (months)
p= 0.0311
Low-risk (n=147)
High-risk (n=143)
B
Microarray
Probability
Time (months)
p= 0.0466
Low-risk (n=58)
High-risk (n=18)
C
TCGA data set
2.0
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
High risk
Low risk
D
microarray data set
High risk
Low risk

Figure S1. Validation of the 70-gene signature in independent data sets. (A-B) Incorporation of 70-gene signature into patients with RNA-seq of TCGA (**A)**, microarray of GSE39001 and GSE52904 (**B)**. Each group was classified by 70-gene signature into low- and high-risk, and evaluated by Kaplan-Meier analysis. The *p* values were computed by the log-rank test. **(C)**. Heatmap of median centered 70-gene expression profiles between high- and low-risk groups in TCGA data sets (red, relative high expression; green, relative low expression). **(D)**. Heatmap of median centered 70-gene expression profiles between high- and low-risk groups in TCGA data sets (red, relative high expression; green, relative low expression).

Figure S2.

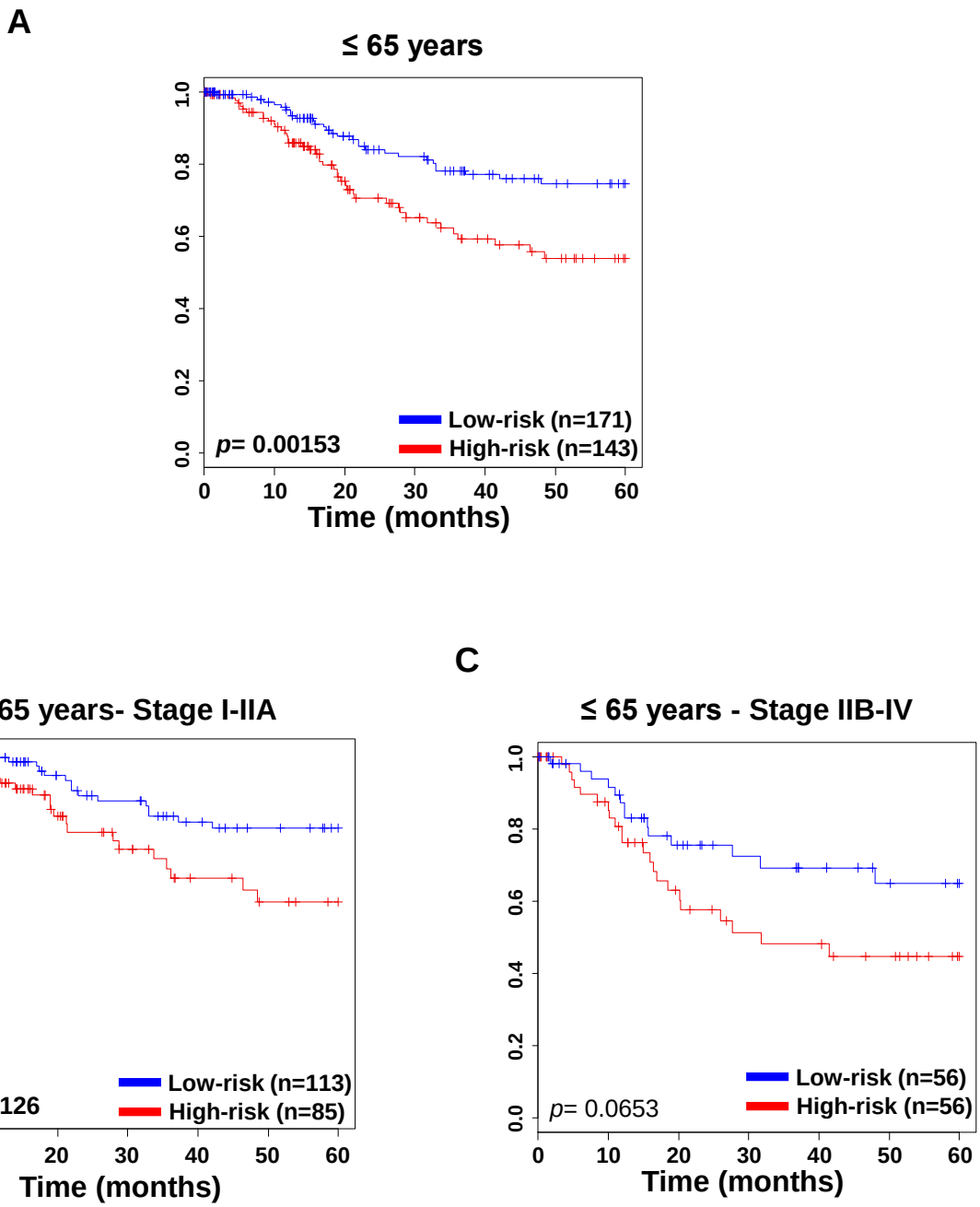
A
≤ 65 years
1.0
0.8
0.6
0.4
0.2
0.0
p= 0.00153
Low-risk (n=171)
High-risk (n=143)
0
10
20
30
40
50
60
Time (months)
B
≤ 65 years- Stage I-IIA
p= 0.0126
Low-risk (n=113)
High-risk (n=85)
Time (months)
C
≤ 65 years - Stage IIB-IV
p= 0.0653
Low-risk (n=56)
High-risk (n=56)
Time (months)

Figure S2. Survival analysis of patients under 65 years old by the 70-gene signature. (A-C) The 70-gene signature was applied to patients under 65 years old **(A)**, early-stage **(B)**, advanced-stage **(C)**. Each group was classified by 70-gene signature into low- and high-risk, and evaluated by Kaplan-Meier analysis. The *p* values were computed by the log-rank test.

Figure S3.

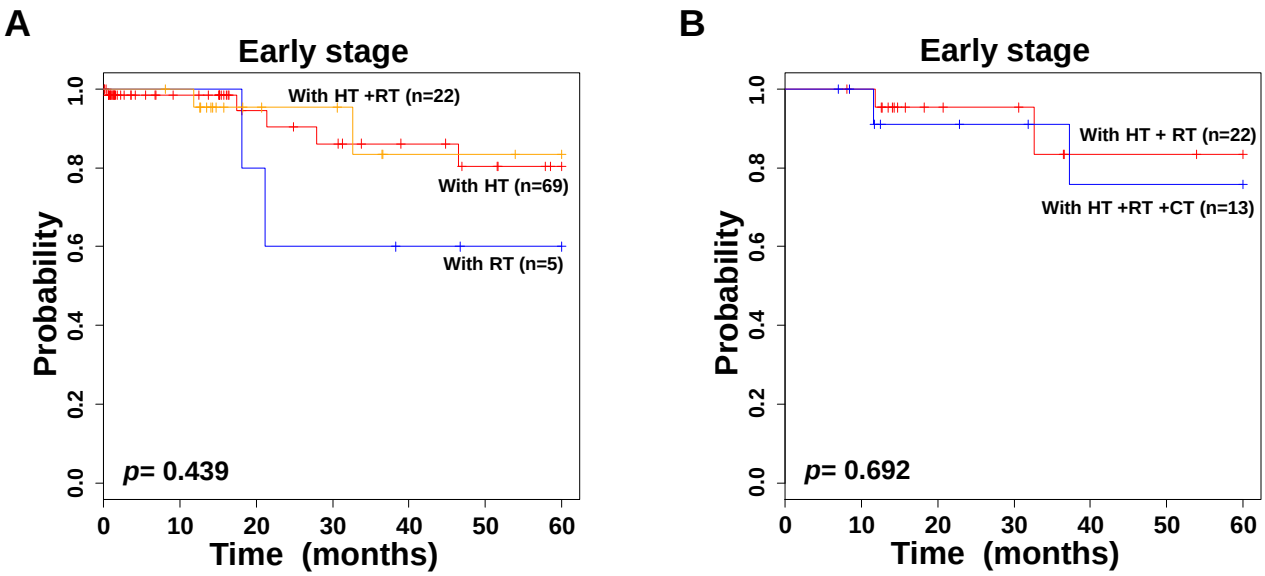
A
Early stage
With HT +RT (n=22)
With HT (n=69)
With RT (n=5)
p= 0.439
Probability
Time (months)
B
Early stage
With HT + RT (n=22)
With HT +RT +CT (n=13)
p= 0.692
Probability
Time (months)

Figure S3. Survival analysis by the treatment methods in patients with early-stage. (A) Patients were separated according to the conventional therapies; hysterectomy and/or radiotherapy. **(B)** Effect of additional chemotherapy with hysterectomy and radiotherapy. The *p* values were computed by the log-rank test. CT, chemotherapy; HT, hysterectomy; RT, radiotherapy

Figure S4.

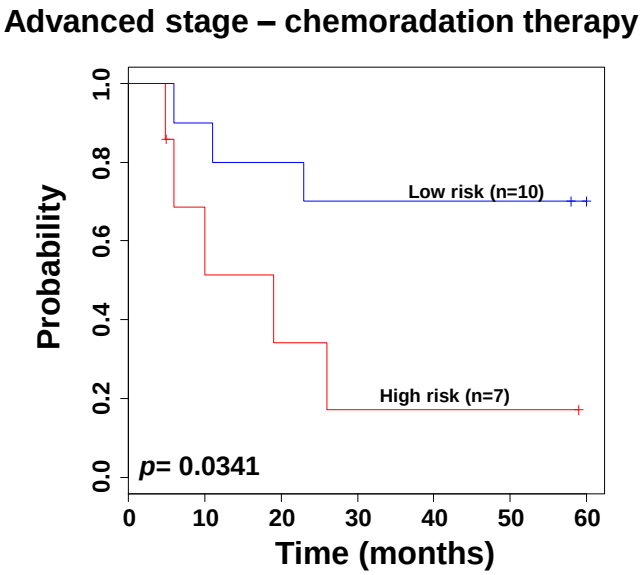
Advanced stage – chemoradation therapy
Probability
0.0
0.2
0.4
0.6
0.8
1.0
Low risk (n=10)
High risk (n=7)
p= 0.0341
0
10
20
30
40
50
60
Time (months)

Figure S4. Survival analysis of advanced-stage patients treated with chemoradiation therapy by the 70-gene signature. Incorporation of 70-gene signature into patients with chemoradiation therapy. Each group was classified by 70-gene signature into low- and high-risk, and evaluated by Kaplan-Meier analysis. The *p* values were computed by the log-rank test.

Figure S5.

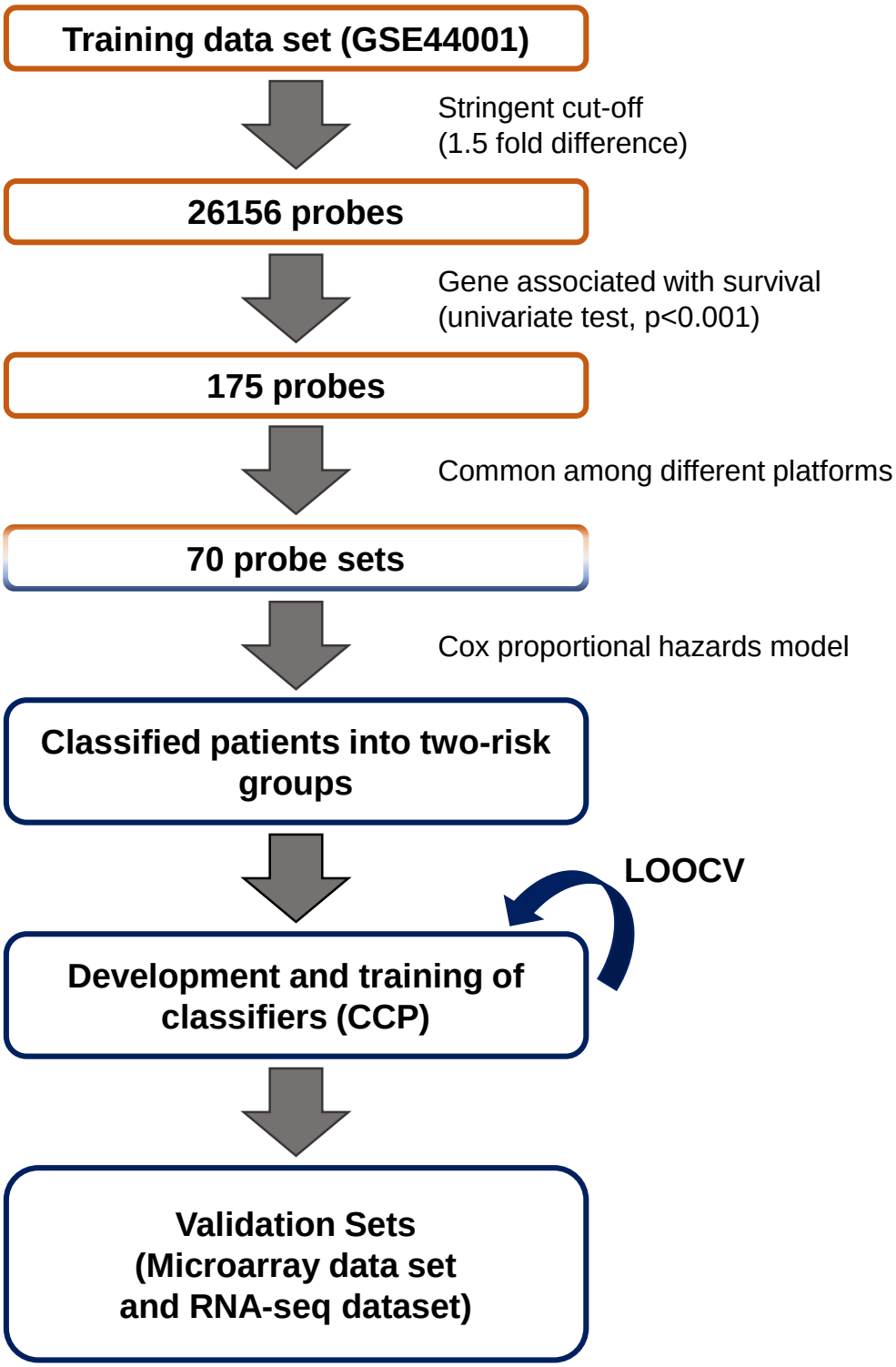
Training data set (GSE44001)
Stringent cut-off
(1.5 fold difference)
26156 probes
Gene associated with survival
(univariate test, p<0.001)
175 probes
Common among different platforms
70 probe sets
Cox proportional hazards model
Classified patients into two-risk groups
LOOCV
Development and training of classifiers (CCP)
Validation Sets
(Microarray data set
and RNA-seq dataset)

Figure S5. Work flowchart of the applied analysis

Table S1. Univariate and multivariate Cox proportional hazard regression of prognosis with stage.

Variables	Univariate			Multivariate		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Stage (Stage IIB-IV)	3.547	2.466 – 5.103	**8.8e-12**	3.691	2.564 – 5.313	**2.2e-12**
Signature (High risk)	2.265	1.566 – 3.275	**1.4e-05**	2.369	1.637 – 3.428	**4.8e-06**

HR, Hazard Ratio; CI, Confidence Interval; the Wald test was used to estimate *p*-values. All statistical tests were two-sided.

Table S2. Univariate and multivariate Cox proportional hazard regression analysis of prognosis with size.

Variables	Univariate			Multivariate		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Size (≥4 cm)	3.703	1.917 - 7.152	**9.7e-05**	2.724	1.397 – 5.311	**0.00326**
Signature (High-risk)	6.769	2.632 -17.409	**7.3e-05**	5.491	2.108 – 14.304	**0.00049**

HR, Hazard Ratio; CI, Confidence Interval; the Wald test was used to estimate *p*-values. All statistical tests were two-sided.

Table S3. Univariate and multivariate Cox proportional hazard regression analysis of prognosis.

Variables	Univariate			Multivariate		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Age under 65year (Advanced Stage)	2.144	1.346-3.416	**0.0013**	2.048	1.284-3.265	**0.0033**
Signature(high)	2.134	1.328 -3.432	**0.0017**	2.095	1.303 – 3.368	**0.0023**

HR, Hazard Ratio; CI, Confidence Interval; the Wald test was used to estimate *p*-values. All statistical tests were two-sided.

Table S5. Cervical cancer RNA expression data sets.

GEO Number	Origin /Year	Chip type	References
TCGA		IlluminaHiSeq	
GSE39001	Mexico 2013	Affymetrix Human HG-Focus Target Array	Espinosa et al.
GSE44001	Korea 2013	Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip	Lee et al.
GSE52904	Mexico 2015	Affymetrix Human Gene 1.0 ST Array [transcript (gene) version]	Medina-Martinez et al.