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Original Article

Establishment and validation of a prognostic model for cervical squamous cell carcinoma based on mRNA biomarkers

Zhong Huang*, Fei Fei Liang*, Kai Hu, Ren Sheng Wang

*Department of Radiation Oncology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi, P. R. China. *Equal contributors.*

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Abstract: The purpose of this study is to establish and validate a prognostic model for cervical squamous cell carcinoma (CSCC) based on mRNA biomarkers. Using CSCC data set in TCGA, we identified the differential expression of mRNAs between CSCC and matched healthy cervical tissues, and then we used unifoliate and multivariate Cox regression analysis to assess the correlation between differentially expressed mRNAs and overall survival (OS). This analysis was eventually used to establish a prognostic model for CSCC based on mRNA biomarkers. By single factor and multivariate Cox regression analysis, we found that 4-mRNAs biomarkers can be used as prognostic models for CSCC. According to the median PI, patients were split into the high-risk group and low-risk group, by comparing the OS of the two groups ($P<0.05$). This result is coherent in the validation set. With this prognostic model, patients with CSCC can be separated into the high-risk and low-risk groups for personalized management.

Keywords: Cervical squamous cell carcinoma, mRNA, prognostic model, risk

Introduction

Cervical cancer (CESC) is one of the most common cancers, especially in developing countries [1]. The overall survival rate of CESC has been improved due to the wide use of comprehensive treatments such as surgery, radiotherapy and chemotherapy, but some patients still have recurrence or metastasis within 5 years (including the earlier stage of CESC). This indicates that there are still some high-risk groups in CESC patients [2, 3]. The pathological types of CESC mainly include squamous cell carcinoma and adenocarcinoma, among which cervical squamous cell carcinoma (CSCC) accounts for the majority [4]. Therefore, a reliable prognostic model to identify and personalize the management of these high-risk CSCC patients is of great value.

Although CESC is closely related to human papillomavirus (HPV) infection, the disease's progression is influenced by other factors, such as abnormal gene expression and epigenetic changes [5]. Studies have shown that abnormal gene expression may be an intrinsic condition of the occurrence, development and pretreatment of CESC, which plays a key role in the occurrence and development of CESC [6]. Studies have shown that there are differences in biological behavior and prognosis between CSCC and cervical adenocarcinoma [7]. Therefore, screening the key genes related to the pathogenesis and prognosis of CSCC can not only provide new effective targets for the treatment of CSCC, but also provide reliable biological markers for its prognosis, providing a certain molecular basis for clinical individualized management and treatment [8-10]. However, there are few studies on the prognostic model of CSCC based on mRNA biomarkers.

The main work of this study is to establish and validation the CSCC prognosis model based on mRNAs biomarkers, which can be used to

Table 1. Comparison of clinical features between training set and validation set

Clinical features		Training set		Validation set		Chi-square test (*P* value)
		n	%	n	%	
Stage_T	T0~T2	113	0.837037037	50	0.757575758	0.1977
	T3~T4	15	0.111111111	8	0.121212121	
	Tx	7	0.051851852	8	0.121212121	
Stage_N	N0	69	0.507352941	32	0.470588235	0.38289
	N1	33	0.242647059	13	0.191176471	
	Nx	34	0.25	23	0.338235294	
Stage_M	M0	66	0.532258065	28	0.444444444	0.52492
	M1	5	0.040322581	3	0.047619048	
	Mx	53	0.427419355	32	0.507936508	
Stage	I~II	129	0.777108434	52	0.742857143	0.56981
	III~IV	37	0.222891566	18	0.257142857	
Grade	G1-2	79	0.475903614	37	0.506849315	0.90484
	G3-4	73	0.439759036	30	0.410958904	
	Gx	14	0.084337349	6	0.082191781	
Age (year)	<60	134	0.792899408	56	0.767123288	0.65391
	≥60	35	0.207100592	17	0.232876712	

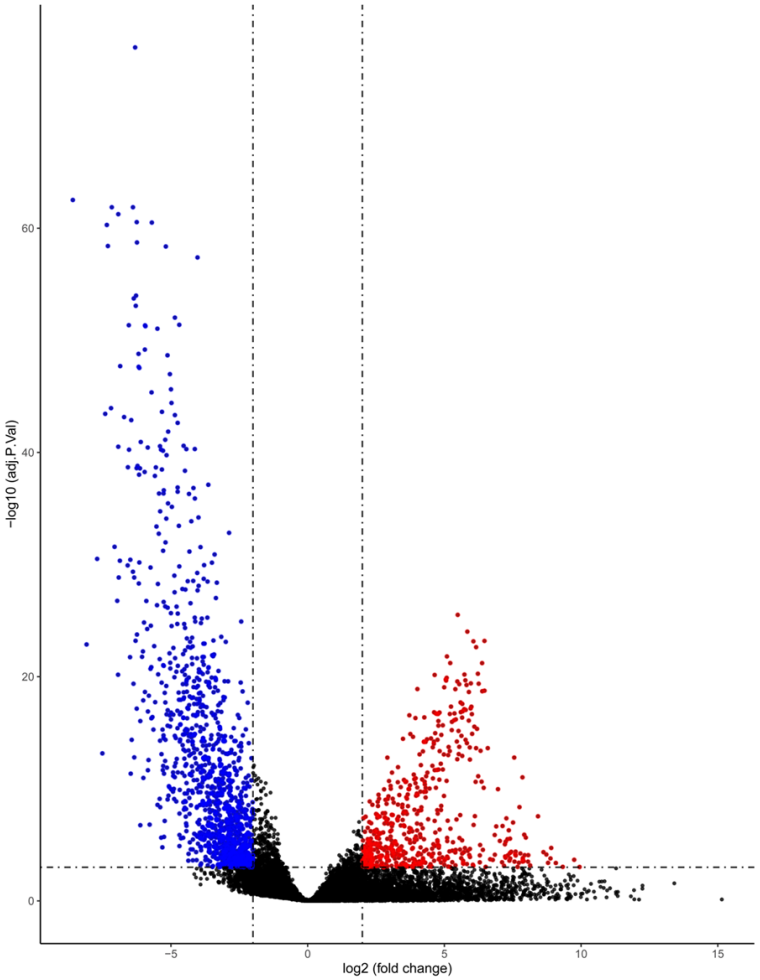


Figure 1. Volcano plot of the differentially expressed genes between CSCC and para cancer tissues. Red indicates up-regulated, blue indicates down-regulated, black indicates no significantly differentially expressed.

evaluate CSCC patients with high-risk and low-risk, to facilitate more personalized diagnosis and treatment in CSCC clinic.

Materials and methods

Data processing and grouping

Download CSCC's RNA sequencing data (displayed as read counts) from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/) (download date: 09-28-2018). The data set includes 255 tissue samples of CSCC and 3 healthy cervical tissue samples, and the corresponding clinical data of CSCC were downloaded (the patients with no follow-up time were deleted). The current study followed the

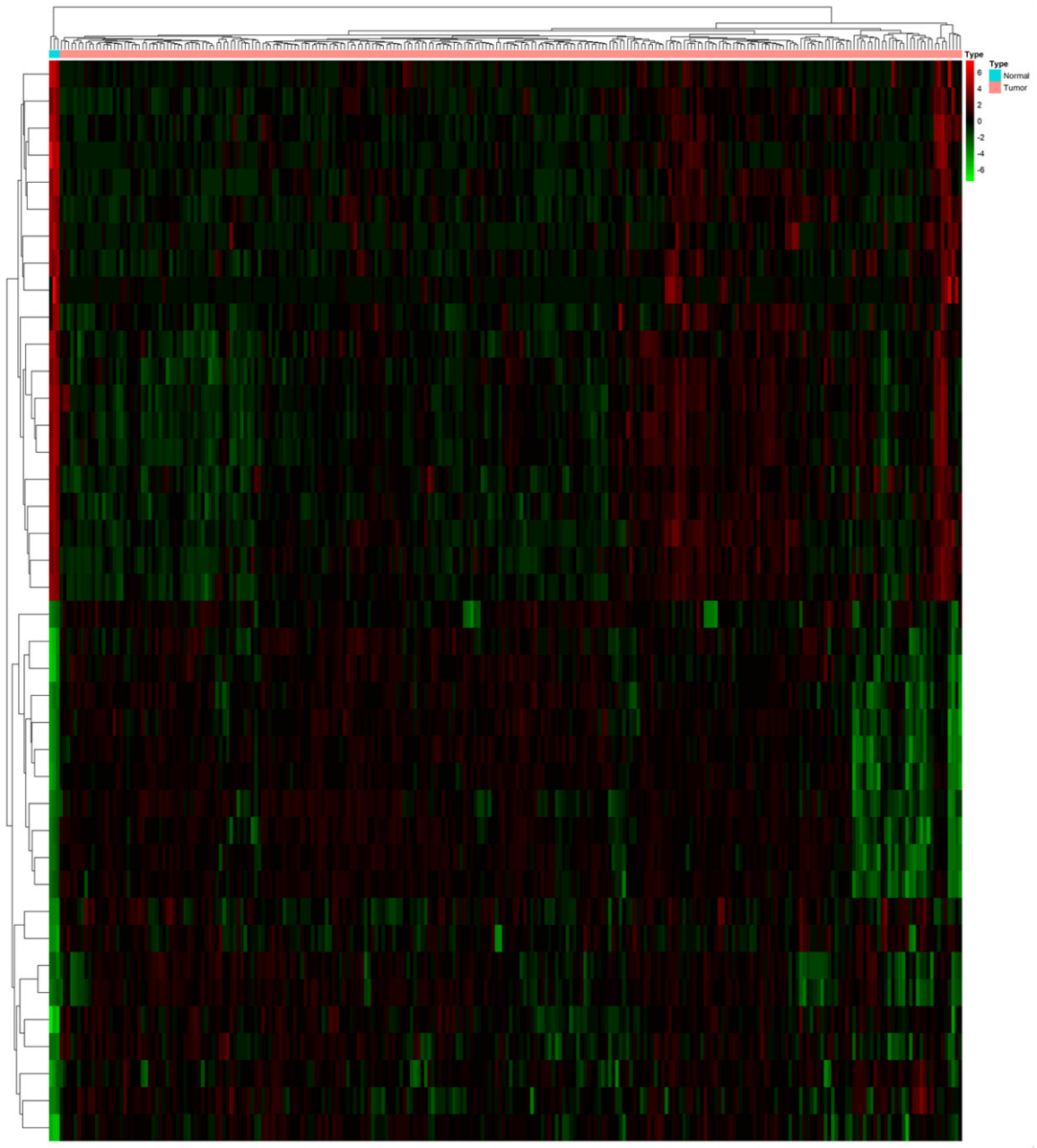


Figure 2. Hierarchical clustering dendrograms of expression patterns of differentially expressed genes that can accurately distinguish between CSCC and para cancer tissues.

TCGA release guidelines and data access policy, and the data downloads did not require approval from the local ethics committee. The download samples were divided strictly according to the random number method, and 70% of the samples (169 cases) were divided as the training set to establish the prognosis model, and 30% of the samples (73 cases) were used as the validation set to validate the model. The clinical characteristics of the two groups were compared by chi-square test (**Table 1**).

Screening of differentially expressed genes (DEGs) and bidirectional hierarchical clustering

The DEGs between CSCC tissue samples and healthy cervical tissue samples were analyzed using the R package of "edge R" [11]. Bidirectional hierarchical clustering (10.1007/s00357-005-0012-9) to these DEGs was performed based on Euclidean distance and displayed the results as a heat map.

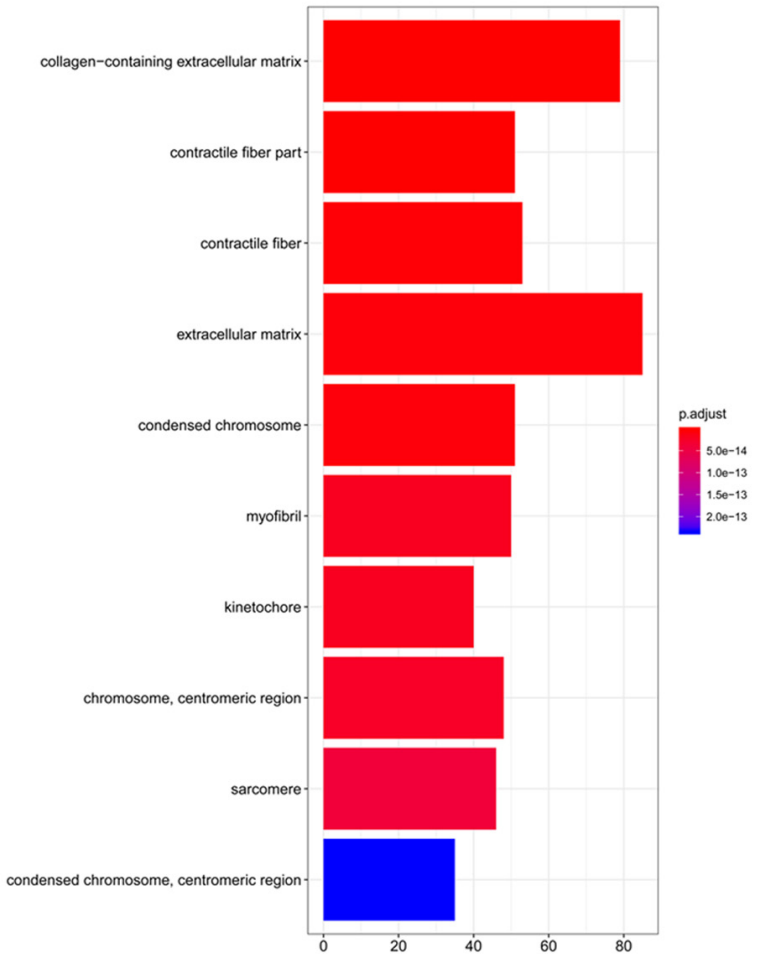


Figure 3. Functional enrichment analysis: cellular component.

Establishment of prognosis model of CSCC in the training set

The mRNAs expression data were converted into log2 (count + 1) before survival analysis. First, univariate Cox regression analysis was used to screen the DEGs affecting the prognosis, then Lasso regression analysis [12] was performed on the variables using the "glmnet" package [13] in R, and multivariate Cox regression analysis was used to establish a prognostic model. Therefore, a prognostic model based on mRNAs biomarker was established.

Finally, an mRNA signature-based prognosis index (PI) score was established based on a linear combination of the expression level multiplied by a regression coefficient derived from the multivariate Cox regression model (β) using the following formula. The "β" value is the estimated regression coefficient of mRNAs and is derived from the multivariate Cox regression analysis, and "M" indicates the expression profiles of the mRNAs.

$$PI = M1 \times \beta 1 + M2 \times \beta 2 + M3 \times \beta 3 + \ldots$$

Evaluation of CSCC prognosis model in training set

We divided the high-risk group and the low-risk group according to the median of PI, and the OS of the two groups was compared by Kaplan-Meier method and log-rank test. The "survival ROC" package [14] in R was used for ROC curve analysis to evaluate the characteristic value of the prognosis model, to investigate the predictive value of 3-year and 5-year survival rate of CSCC, and "survcomp" package [15] was used to calculate the C-index value of the model to evaluate the predictive ability of the model. Besides, we analyzed and demonstrated the correlation between risk score and CSCC prognosis.

Validation of the prognosis model of CSCC in the validation set

Similarly, we calculated the PI of each patient according to the PI formula and calculated the high and low-risk groups according to the median PI. Then, Kaplan-Meier and log-rank test were used to compare the OS of the two groups of patients, and ROC curves were used to assess the prognostic ability of the predictive validation set.

Cox analysis of prognostic models and clinical characteristics

In the training set, the characteristics of clinical data and the PI of each patient were ana-

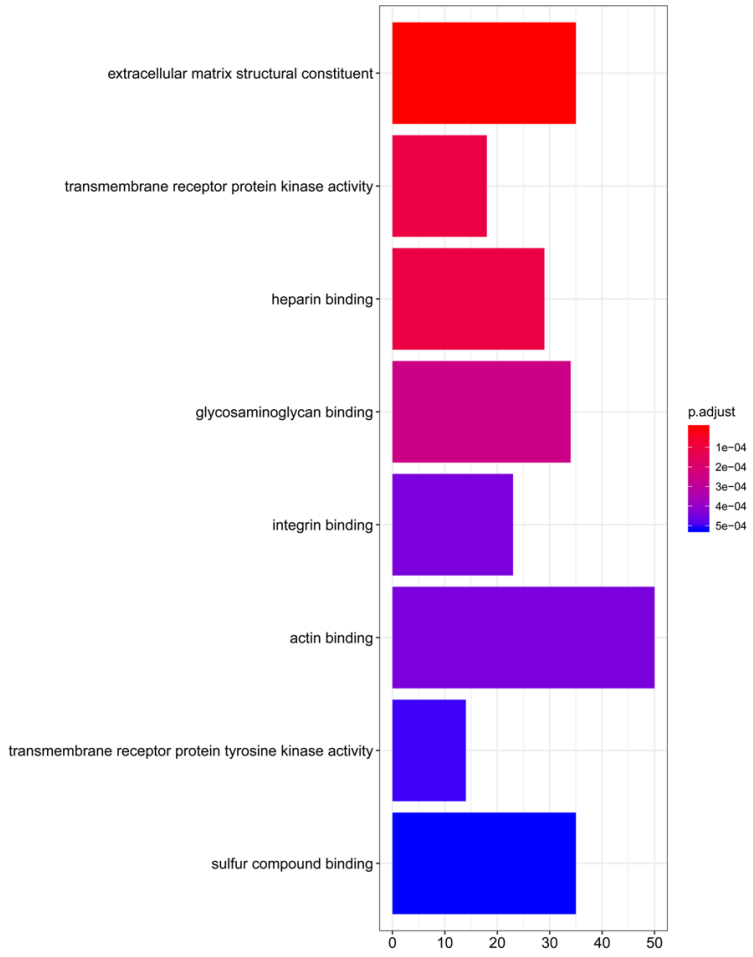


Figure 4. Functional enrichment analysis: molecular function.

lyzed by multi-factor Cox. It is used to assess whether PI is an independent prognostic factor for CSCC.

Statistical analysis

We used R language (version: 3.6.1) as statistical analysis software which is a free software for statistical computing and graphics. Univariate Cox regression analysis was used to screen the DEGs affecting the prognosis, lasso regression analysis was used to shrink variables, multivariate Cox regression analysis was used to establish a prognostic model, Kaplan-Meier and log-rank test were used to compare the OS between high-risk group and low-risk group, and the ROC curve analysis was used to evaluate the characteristic value of the prognosis model.

Result

DEGs and bidirectional hierarchical clustering

According to the cutoff criteria (P<0.0001 and |log2FC| >2), 1441 DEGs were identified between the CSCC and para cancer tissue samples, including 407 genes that were up-regulated and 1034 genes that were down-regulated in CSCC (**Figure 1**). The hierarchical clustering showed that the expression patterns of the 20 most up-regulated and 20 most down-regulated DEGs could accurately distinguish CSCC and para cancer tissue samples (**Figure 2**). In CSCC tissue, MCM5, TSACC were up-regulated and SELP, TMEM55A were down-regulated.

Functional enrichment analysis of DEGs

The R package of "clusterProfiler" [16] was implemented to analyze and visualize functional profiles of gene and gene clusters from Gene Ontology (GO) [17] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [18]. GO and KEGG pathway enrichment analysis for the DEGs was performed using a cluster profile package. P adjusted by false discovery rate <0.05 was considered for a significant in GO function and KEGG pathway. The GO annotation results have three parts: a cellular component (**Figure 3**), a molecular function (**Figure 4**), and a biological process (**Figure 5**). The DEGs were significantly involved in multiple tumor-related and immune-related pathways (**Figure 6**), such as cGMP-PKG signaling pathway, DNA replication, p53 signaling pathway, neuroactive ligand-receptor interaction. This indicated that the occurrence of CSCC was associated with abnormal immune function.

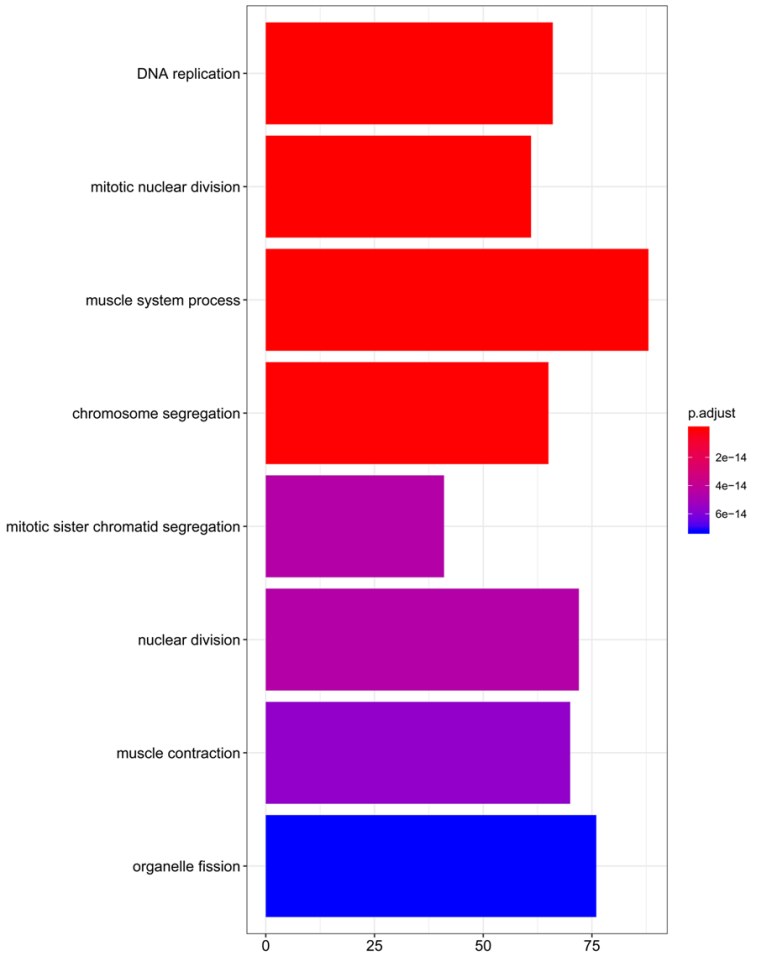


Figure 5. Functional enrichment analysis: biological process.

Establishment of prognosis model of CSCC in the training set

In the training set, univariate Cox proportional risk regression was used to analyze 1441 differentially expressed mRNAs, showing that the *P* values of 18 differentially expressed mRNAs were less than 0.005 (**Table 2**), and then Lasso regression fitting was performed on these 18 mRNAs to obtain 10 mRNAs with prognostic value (**Figure 7**). Then, multivariate Cox proportional risk comparative analysis was applied to determine 4-mRNAs prognosis model (**Figure 8**), with specific regression coefficients shown in the table (**Table 3**). Therefore, we used these 4-mRNAs to establish PI, as following:

PI = SELP* - 0.376918856234018 + TMEM55A*0.310620545470287 + MCM5* - 1.00957285920915 + TSACC*0.42837664381227

Evaluation of CSCC prognosis model in training set

Patients were divided into two groups with high-risk and low-risk according to the median PI. Kaplan-Meier method and log-rank test were used to compare the survival time of patients in the two groups, and the OS in the two groups were significantly different (log-rank, P = 0.00006411) (**Figure 9A**). This indicates that the prognosis model based on the 4-mRNAs biomarker is related to CSCC survival. The time ROC curve and C-index value are used to evaluate the predictive value of the prognostic model tool for CSCC's 3-year and 5-year survival rate. The area under the ROC curve (AUC) were 0.847 (**Figure 9B**), 0.822 (**Figure 9C**) respectively, and the C-index value was 0.77 (**Figure 8**). It can be seen that the prognosis model used to predict CSCC's 3-year or 5-year survival was very promising. At the same time, in the study on the correlation between PI and prognosis, we showed the survival curve (**Figure 10**), survival status map (**Figure 11**) and survival heat map (**Figure 12**). The results showed that with the increase of PI, the risk of affecting the prognosis of CSCC increased, the survival time was relatively shortened, and the risk of death increased. In addition, the prognostic risk of CSCC was negatively correlated with the expression levels of SELP and MCM5, and positively correlated with the expression levels of TMEM55A and TSACC, so as to further verify the reliability of the model.

Validation of the prognosis model of CSCC in the validation set

As in the training set, we ranked the high-risk group and low-risk group according to the me-

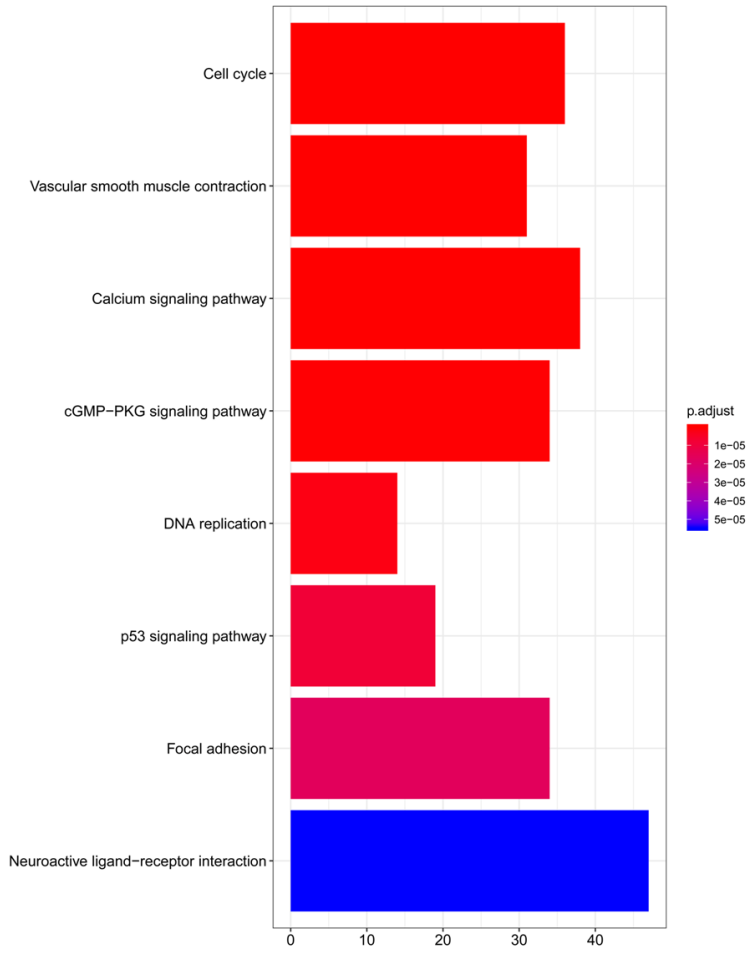


Figure 6. Functional enrichment analysis: Kyoto Encyclopedia of Genes and Genomes pathway.

dian PI. Kaplan-Meier method and log-rank test were used to compare the survival time of patients in the two groups, and the OS in the two groups was significantly different (log-rank, P = 0.004123) (**Figure 9D**). The AUC of the ROC curve in the verification set for 3 years reached 0.751 (**Figure 9E**). Thus, the prognostic model was also validated in the validation set. At the same time, this prognostic model has not been validated in cervical adenocarcinoma (log-rank, P = 0.6424) (**Figure 9F**). This indicates that the CSCC prognostic model is independent.

Multivariate Cox analysis of prognostic models and clinical characteristics

Multivariate Cox analysis was performed on the PI and clinical characteristics of CSCC cases (**Table 4**). We can see that the *P*-value of PI<0.05, and PI can be used as an independent prognostic factor of CSCC. Therefore, we can further believe that the CSCC prognosis model established with these 4-mRNAs biomarkers is reliable.

The expression level and the survival analysis of related mRNA in GEPIA database

In GEPIA database [19] (http://gepia.cancer-pku.cn/) (P<0.05 and |log2FC| >1), we found that SELP (**Figure 13A**), TMEM55A (**Figure 13B**) was down-regulated and MCM5 (**Figure 13C**) was up-regulated in CESC tissue. Although the *P* value of TSACC (**Figure 13D**) was not statistically significant, it still showed a high expression trend. These are generally similar to the results of this study. Meanwhile, in the survival analysis, we found that for the OS of SELP (**Figure 14A**), TMEM55A (**Figure 14B**), and TSACC (**Figure 14C**), P<0.05; but for the OS of MCM5 (**Figure 14D**), P>0.05, which may indicate that MCM5 is specific in CSCC.

The correlation between the expression level of related mRNA and methylation

To explore whether the aberrant expression of the relevant mRNAs is caused by aberrant DNA methylation, differentially methylated CpGs of the relevant mRNAs were screened using the Wanderer tool [20] (http://maplab.imppc.org/wanderer/), P<0.05 was considered significant. We found that the expression levels of SELP (**Figure 15**), TMEM55A (**Figure 16**), MCM5 (**Figure 17**), and TSACC (**Figure 18**) were all related to their methylation levels (P<0.05).

Discussion

Cervical cancer (CESC) is one of the malignant tumors with high incidence in women, and it tends to be younger. Worldwide, 470,000 new

Table 2. Univariate Cox regression analyses of variables (P<0.005)

mRNA	*P* value	HR	(95.0% CI)	
			Lower	Upper
SELP	1.23E-05*	0.682667708	0.5753	0.8101
MCM5	0.000660683*	0.359270098	0.1993	0.6476
ACKR1	0.000919269*	0.8080457	0.7124	0.9166
FAM65B	0.00150319*	0.649194851	0.4972	0.8477
DEF6	0.001547941*	0.442139277	0.2668	0.7328
TMEM55A	0.001699187*	1.580686552	1.188	2.104
HLF	0.001982314*	0.770113366	0.6526	0.9088
CBX7	0.002672453*	0.542412164	0.3639	0.8086
DENND2D	0.002729456*	0.424594396	0.2425	0.7435
FAM107A	0.003355055*	0.727706047	0.5885	0.8999
TSACC	0.003847084*	1.724671801	1.192	2.496
DES	0.003918568*	0.832966567	0.7357	0.9431
ZIC2	0.004093005*	0.838896879	0.7441	0.9458
BCL2	0.004141845*	0.62363151	0.4516	0.8612
CENPM	0.004146257*	0.524782668	0.3377	0.8155
TMEM40	0.004367496*	0.799343322	0.6853	0.9324
OSR2	0.004730993*	0.623952877	0.4498	0.8655
ZIC5	0.004926522*	0.835515454	0.7371	0.947

Abbreviations: HR, hazard ratio; CI, confidence interval; **P* value of <0.05 was considered statistically significant difference.

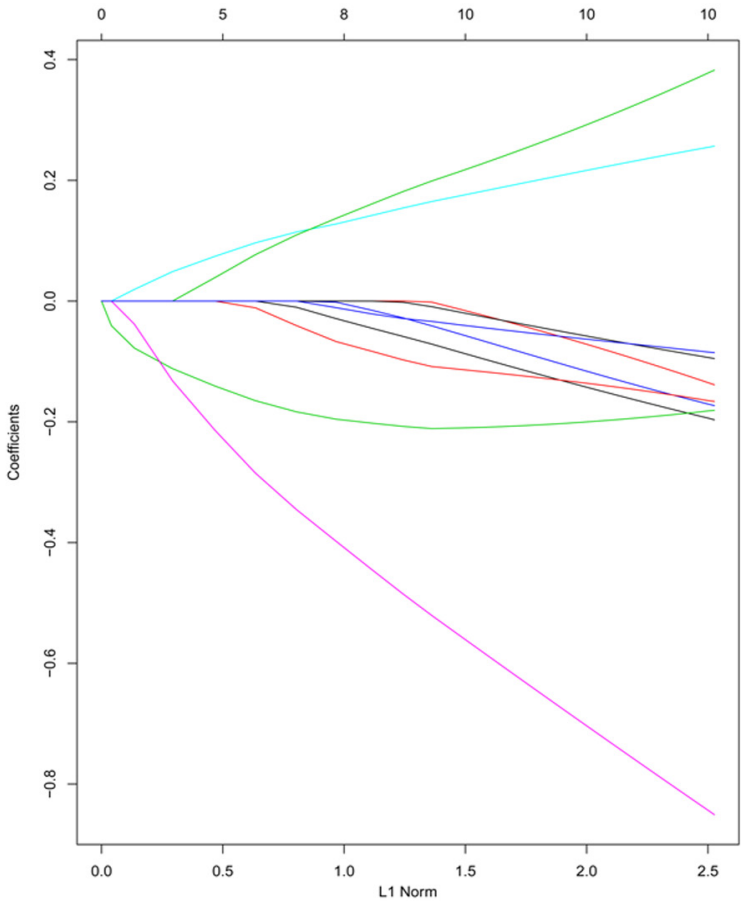


Figure 7. Lasso regression analysis of 13 mRNAs.

cases and 200,000 deaths are reported each year [2, 21, 22]. Human papillomavirus (HPV) infection has been shown to be closely related to the pathogenesis of CESC [23, 24]. The overall treatment effect of CESC is relatively satisfactory, but there are still a considerable number of patients with long-term recurrence and metastasis, and the prognosis of these patients is poor [25]. Therefore, it is necessary to establish the prediction model of CESC prognosis and conduct individualized management of high-risk and low-risk population of CESC, especially for developing countries without screening programs. CSCC is the overwhelming majority of CESC, accounting for about 90% [26]. Studies have shown that

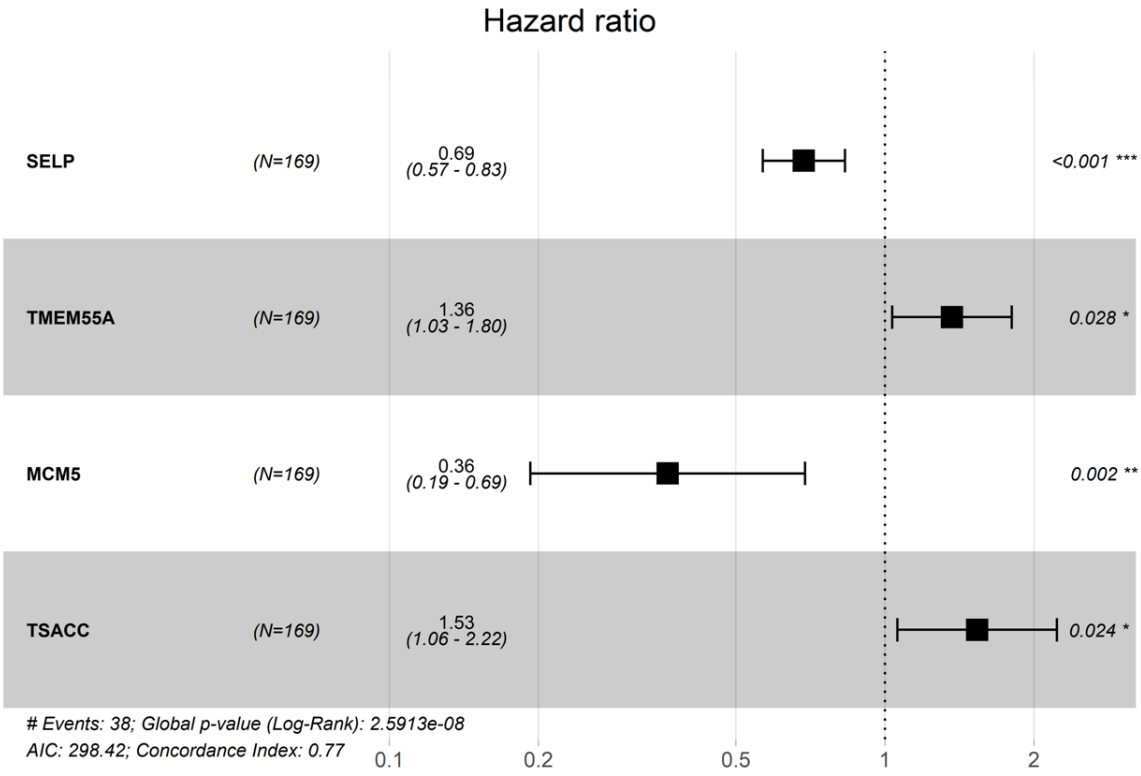


Figure 8. Multifactor regression forest map.

Table 3. Multivariate Cox regression analysis of variables

mRNA	coef	HR	*P* value	95.0% CI	
				Lower	Upper
SELP	-0.376918856	0.685971734	0.000105886*	0.567	0.83
TMEM55A	0.310620545	1.364271444	0.028362999*	1.0335	1.801
MCM5	-1.009572859	0.364374586	0.001932639*	0.1925	0.6898
TSACC	0.428376644	1.534764031	0.023619619*	1.0591	2.2241

Abbreviations: HR, hazard ratio; CI, confidence interval; *p value of <0.05 was considered statistically significant difference.

there are differences in biological behavior and prognosis between CSCC and cervical adenocarcinoma [7]. The clinical management of CSCC is still dependent on the patient's disease stage and there are no personalized biomarkers. mRNA expression levels are highly specific to different types of cells, tissues and organs and can be used as potential biomarkers of clinical relevance [6]. In this study, a CSCC prognostic model based on 4-mRNAs was established and validated as a CSCC prognostic model, which can be used to subdivide CSCC patients into high-risk group and low-risk group for personalized management.

In this study, there were 1441 differentially expressed genes (DEGs) between CSCC and paracancer tissue samples, among which 407 genes were up-regulated and 1034 genes were down-regulated in CSCC. In the GO analysis and KEGG analysis of the DEGs, we found that the DEGs were mainly concentrated in DNA replication, transmembrane receptor protein kinase activity, cGMP-PKG signaling pathway, p53 signaling pathway, etc. Relevant studies have shown that miR-411-mediated direct inhibition of STK17A can induce apoptosis through the p53 signaling pathway and inhibit the proliferation, migration, and invasion of

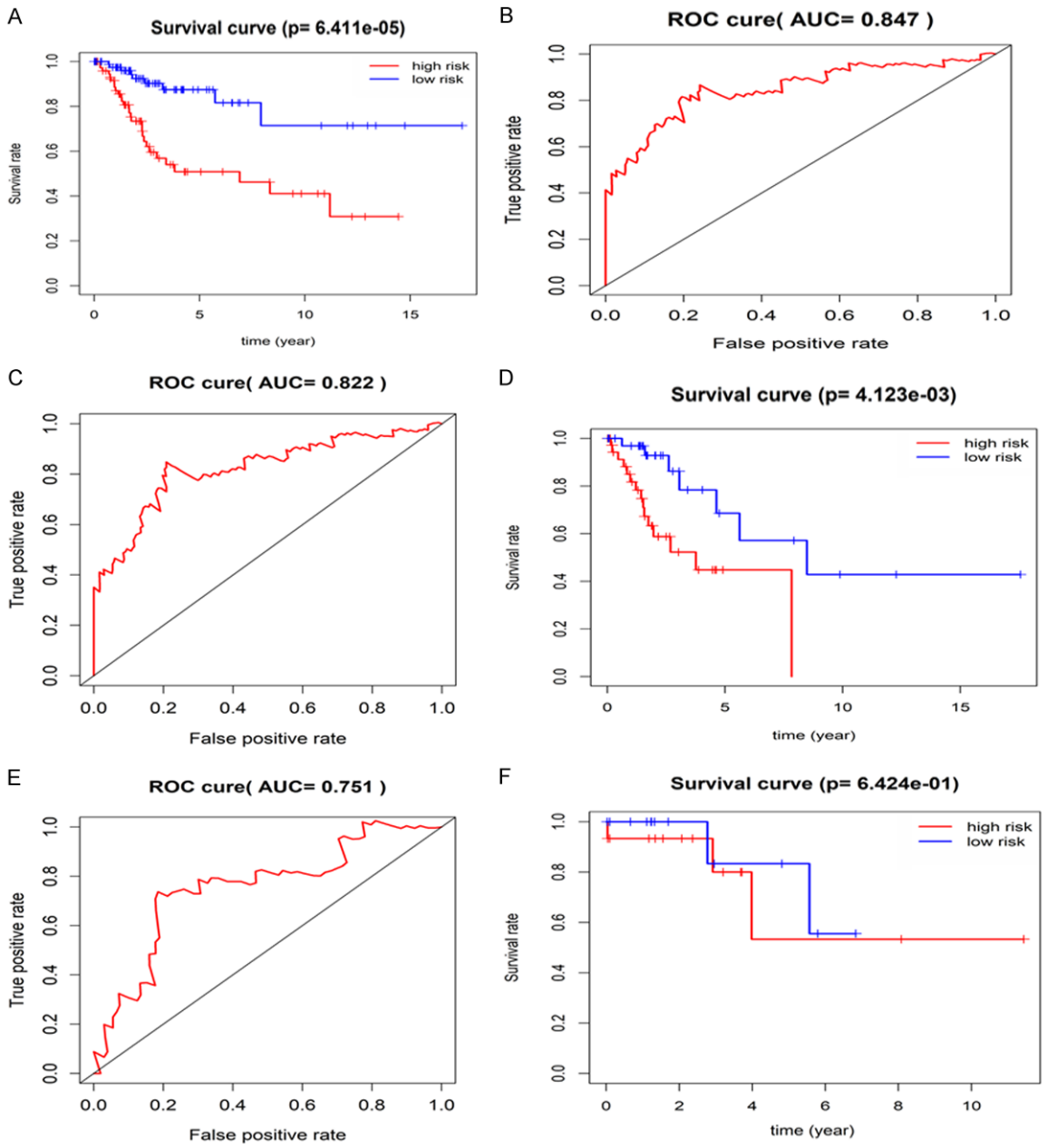


Figure 9. In the training set, the comparison of OS between the high-risk and low-risk groups (A) and the 3-year ROC curve (B) and the 5-year ROC curve (C). In the validation set, the comparison of OS between the high-risk and low-risk groups (D) and the 3-year ROC curve (E). Survival analysis of prognostic models in cervical adenocarcinoma cases (F).

human CESC cells [27]. It has been reported that the instability of the genome, which results from a defect of MMR genesis, is thought as a new oncogenesis way [28]. We first proposed a 4-mRNAs prognostic model to predict the prognosis of CSCC. Using the 4-mRNAs biomarker-specific prognostic model, CSCC patients can be divided into high risk and low-risk groups. The OS of the two groups was significantly different in the training set and the verification set. According to this model, the survival curve and survival state chart were drawn. We noticed that with the increase of PI score, the risk of poor prognosis of CSCC was higher and the survival rate decreased. Meanwhile, in the prognosis model of CSCC,

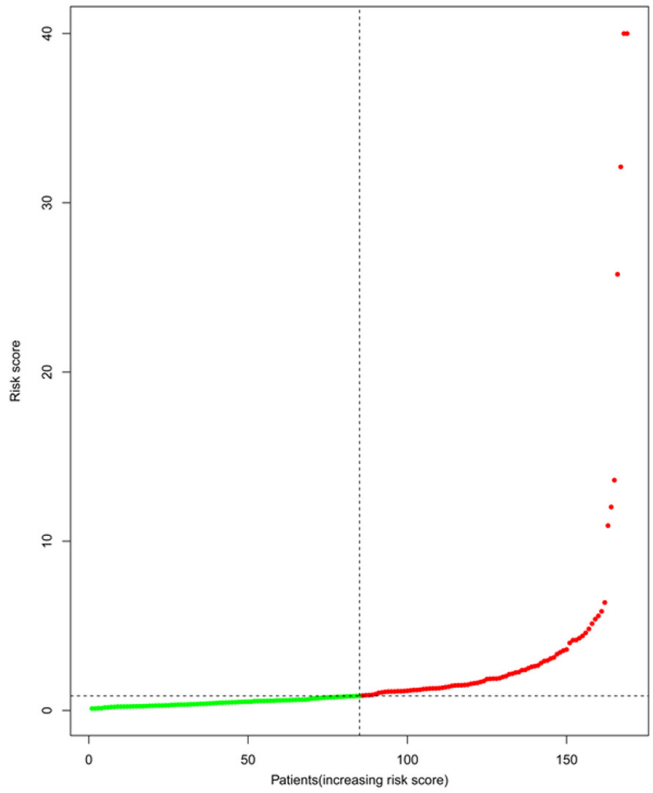


Figure 10. Correlation between PI and CSCC prognosis: survival curve.

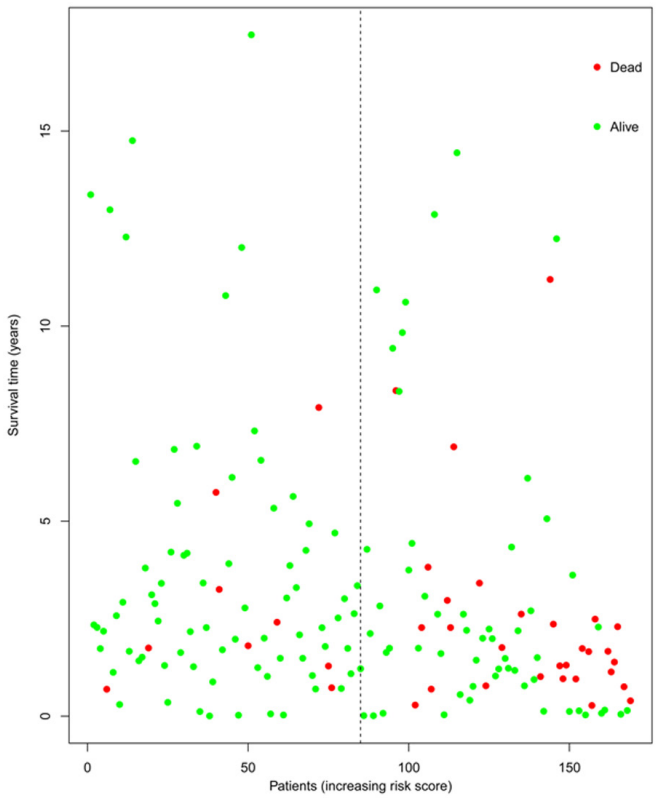


Figure 11. Correlation between PI and CSCC prognosis: survival status.

we found that SELP and MCM5 were protective factors, while TMEM55A and TSACC were risk factors. In the heat map of survival, we found that the expression level of SELP and MCM5D was negatively correlated with PI, while the expression level of TMEM55A and TSACC was positively correlated with PI, which verified the accuracy of the model to some extent. Finally, the PI remained an independent prognostic factor compared to conventional clinical characteristics. Therefore, 4-mRNAs is a promising biomarker for predicting CSCC survival. In the training set, the ROC curve AUC of 3-year and 5-year were 0.847, 0.822 respectively, and the C-index values were 0.77, which indicated that the prognosis model of 4-mRNAs biomarkers was reliable in CSCC. At the same time, this prognostic model has not been validated in cervical adenocarcinoma. This indicates that the CSCC prognostic model is independent and may not be applicable in cervical adenocarcinoma. MCM5 was not statistically significant in the CESC survival analysis (GEPIA database), which further suggests that this prognostic model may not be applicable to other pathological types of CESC. Meanwhile, this prognostic model is also an independent prognostic factor in the analysis of clinical data. CSCC is predicted based on the 4-mRNAs biomarker prognosis model, and high-risk patients can receive more frequent follow-up and more aggressive treatment than low-risk patients.

In this study, MCM5 and TSACC genes were highly expressed in CSCC tissue, while SELP and TMEM55A were low expressed. From the perspective of the prognosis model of CSCC, SELP and MCM5 were protective factors, while TMEM55A and TSACC were risk factors, some of which were also presented to some extent in the CESC survival analysis (GEPIA database). According to epigenetics, we found that abnormal expression of these 4-mRNAs was associated with abnormal methylation CpGs (The Wanderer tool). SELP, TMEM55A and TSACC have been rarely studied in CSCC. Research suggests that SELP

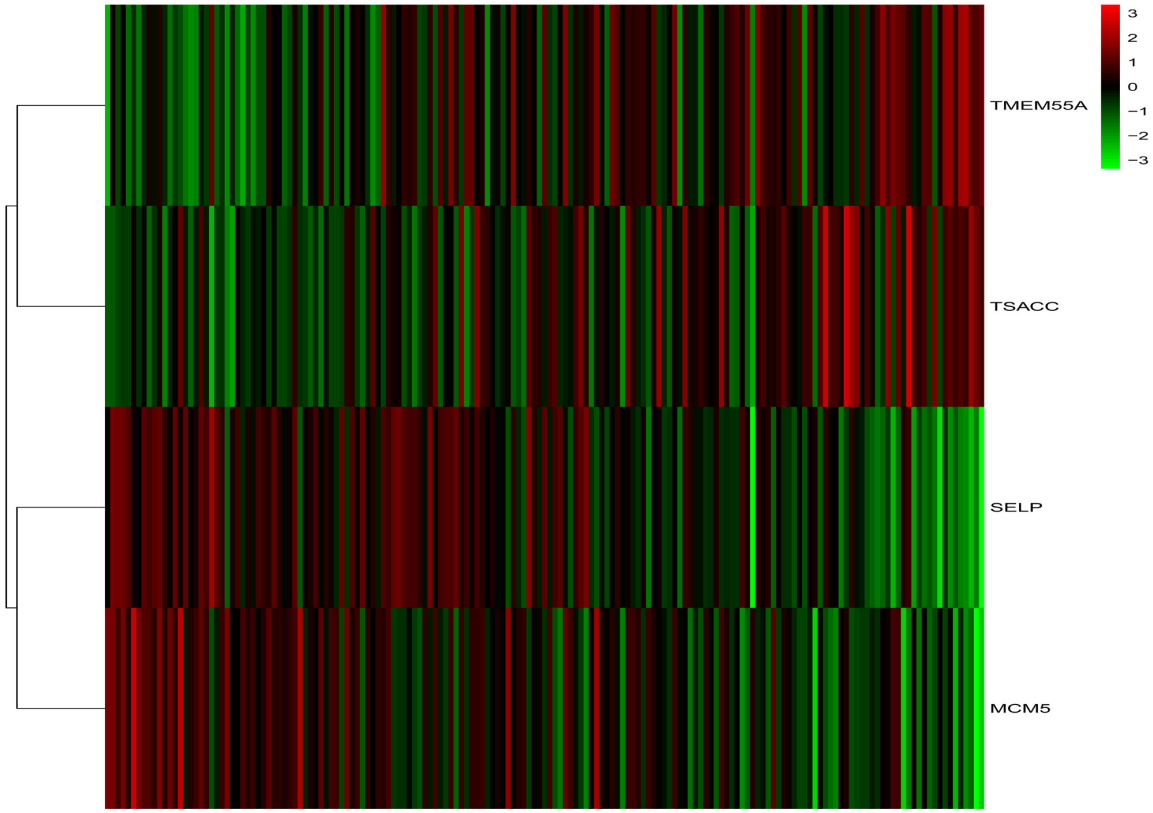
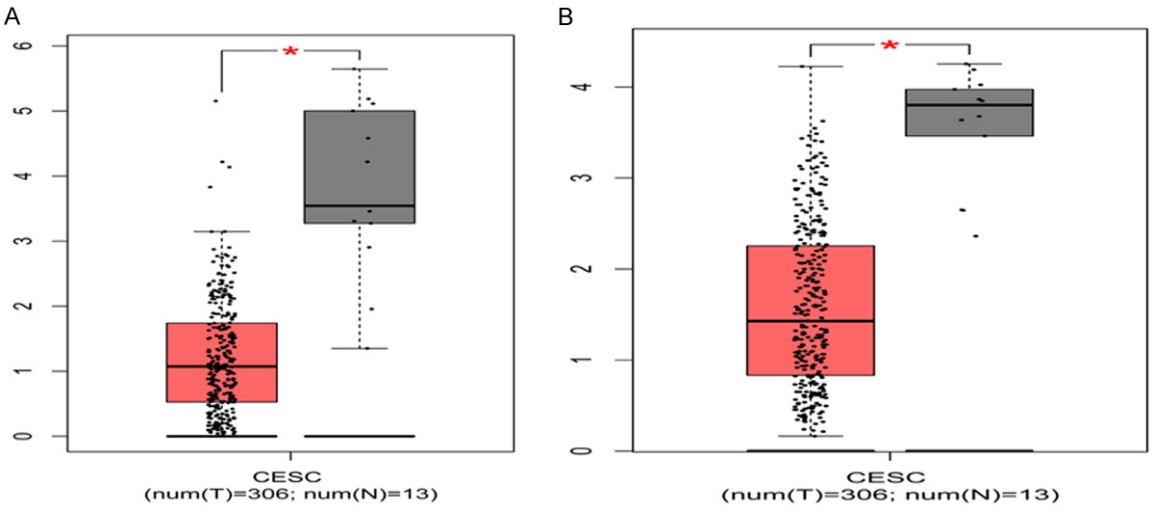


Figure 12. Correlation between PI and CSCC prognosis: survival heat map.

Table 4. Multivariate Cox analyses of prognostic models and clinical characteristics

Variables in the Equation	*P* value	HR	95.0% CI	
			Lower	Upper
Stage (I~II/III~IV)	0.07	1.743	0.956	3.179
Grade (G1~2/G3~4)	0.94	0.978	0.546	1.752
Age (<60 year/≥60 year)	0.187	1.511	0.819	2.791
PI (low-risk/high-risk)	0.001*	3.051	1.627	5.722

Abbreviations: HR, hazard ratio; CI, confidence interval; **P* value of <0.05 was considered statistically significant difference.



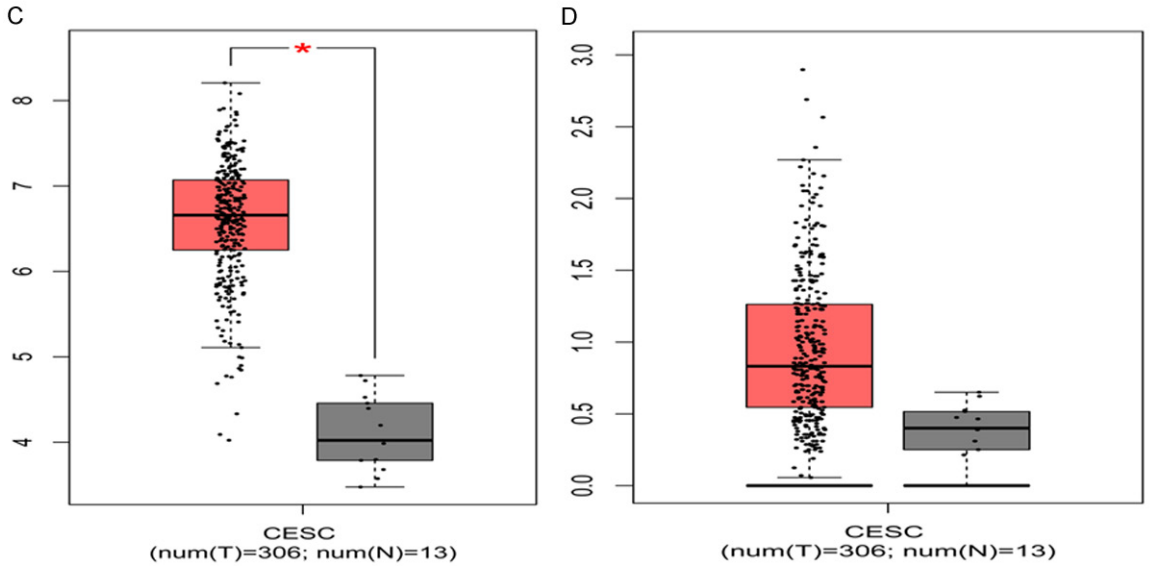


Figure 13. The expression level of related mRNA in GEPIA database (CESC) (* was considered statistically significant difference): SELP (A), TMEM55A (B), MCM5 (C), and TSACC (D).

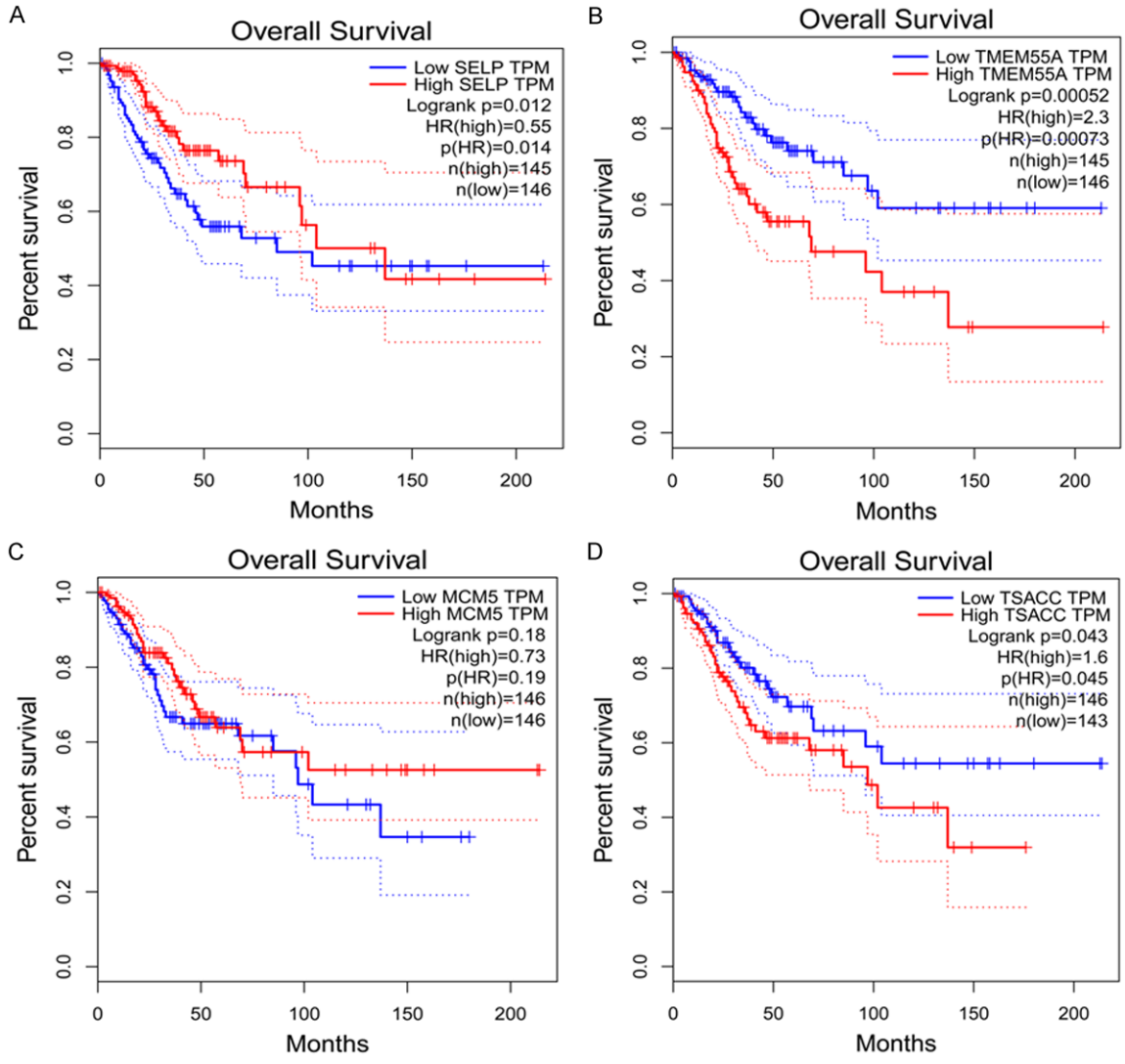


Figure 14. The survival prognosis analysis of related mRNA in GEPIA database (CESC) (P<0.05 was considered statistically significant difference): SELP (A), TMEM55A (B), MCM5 (C), and TSACC (D).

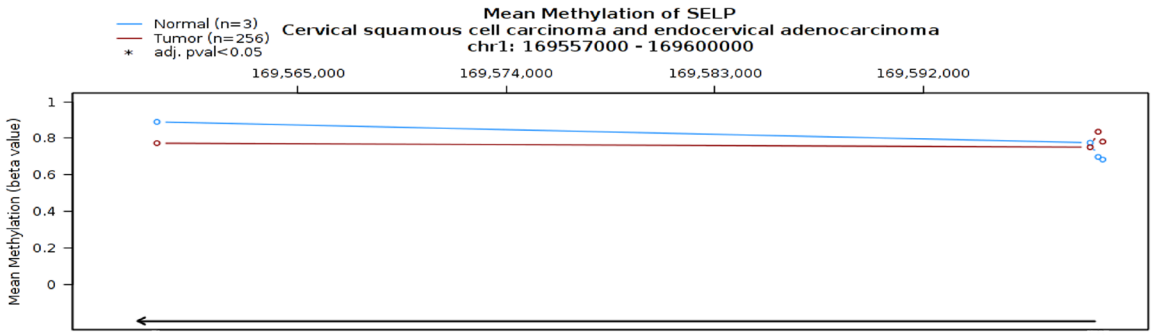


Figure 15. The correlation between the expression levels and methylation levels of the relevant mRNA (* adj.pval <0.05 was considered statistically significant difference): SELP.

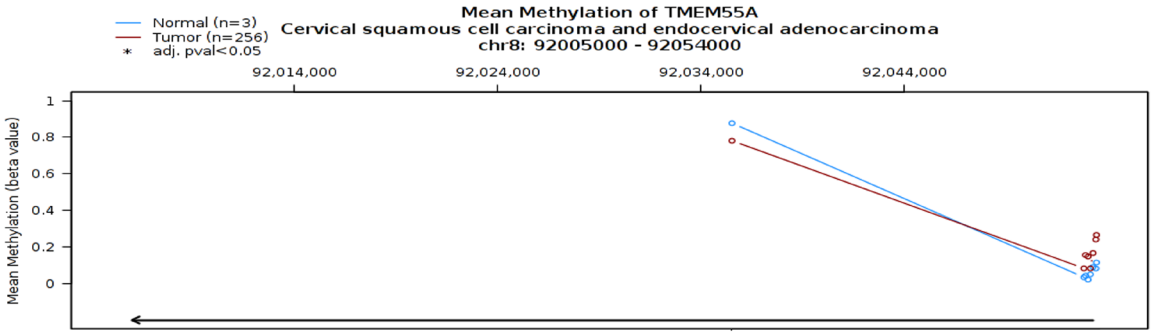


Figure 16. The correlation between the expression levels and methylation levels of the relevant mRNA (* adj.pval <0.05 was considered statistically significant difference): TMEM55A.

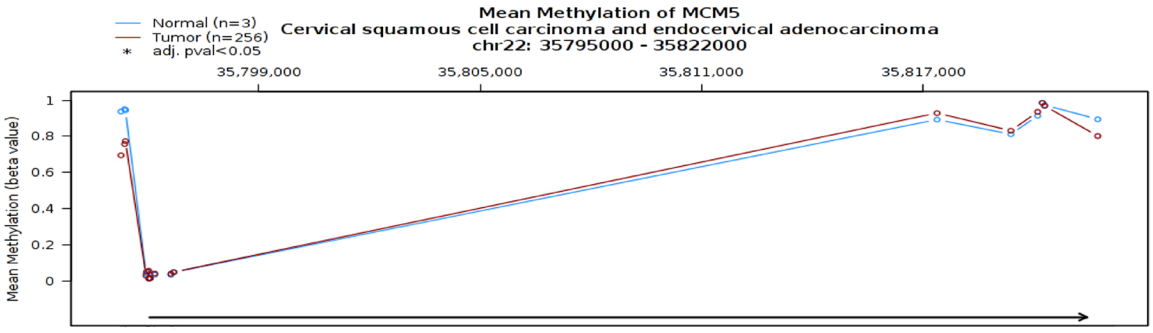


Figure 17. The correlation between the expression levels and methylation levels of the relevant mRNA (* adj.pval <0.05 was considered statistically significant difference): MCM5.

biomarkers can be used as high-risk GISTs outcomes [29]. Studies on TMEM55A have also been reported, but its role remains unclear [30, 31]. Some reports suggest that MCM5 is associated with the progression and prognosis of renal cell carcinoma, LSCC, urothelial carcinoma of the bladder, and squamous cell carcinoma of the mouth [32-37]. Related studies have shown that MCM5 can be used as a marker of cervical precancerous lesion and play a role in predicting malignant potential [38]. Some studies have shown that MCM5 is associated with malignant status and poor prognosis in patients with cervical adenocarcinoma [39]. Meanwhile,

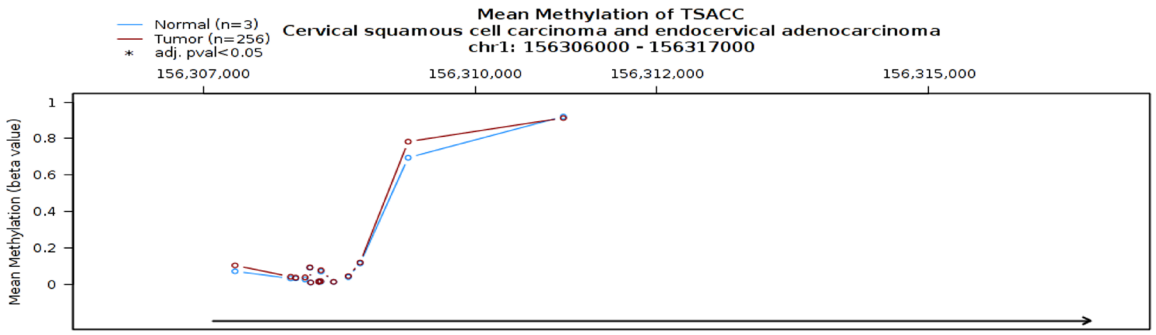


Figure 18. The correlation between the expression levels and methylation levels of the relevant mRNA (* adj.pval <0.05 was considered statistically significant difference): TSACC.

other studies have shown that the expression imbalance of HULC, COL6A1, miR-15b and Cyclin D1 may also affect the prognosis of CESC [40-44]. Thus, the prognosis of CSCC is not determined by a single factor, but by the interaction of multiple factors. Given the singularities of these studies, this suggests that they are unreliable in predicting prognostic efficacy, despite their basic biological function. Of course, there have been some studies on CESC associated prognostic models [45, 46], and a few reports on CSCC based on other types of biomarkers [47]. However, CSCC prediction models based only on mRNA biomarkers have been rarely reported.

This study identified a prognostic model for 4-mRNAs biomarkers in CSCC, and we encourage further exploration of the molecular function of these 4-mRNAs biomarkers. The work still has limitations. The molecular functions of these 4-mRNAs in CSCC are unknown and lack of further experimental validation. Therefore, it is not clear whether these 4-mRNAs biomarkers have a causal relationship with CSCC, or are merely biomarkers for prediction of CSCC prognosis. It may be necessary to verify and even further refine the prognostic model of these 4-mRNAs biomarkers in a larger independent cohort.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Kai Hu and Ren Sheng Wang, Department of Radiation Oncology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi, P. R. China. Tel:+86-0771-5356509;E-mail:hukai201912@163.com (KH); wangrensheng1212@163.com (RSW)

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