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Original Article

Identification of candidate miRNA biomarkers correlated with the prognosis of cell carcinoma and endocervical adenocarcinoma via integrated bioinformatics analysis

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Abstract: Objectives: This study was designed to explore MicroRNAs (miRNAs) associated with the prognosis of cell carcinoma and endocervical adenocarcinoma (CESC) to search for biomarkers of CESC and provide guidelines for the clinical treatment. Methods: mRNAs of CESC patients were downloaded from The Cancer Genome Atlas (TCGA), and miRNA expression and clinical data of the patients were preprocessed. Key miRNAs associated with the prognosis of cervical cancer were identified by weighted gene co-expression network (WGCNA). The corresponding target genes were intersected with differentially expressed genes (DEGs) acquired from variation analysis, and the pathways and functional enrichment of genes were analyzed. Key genes were screened by Kaplan-Meier (K-M) survival analysis. Risk models were constructed using Cox proportional hazard regression model and the Least Absolute Shrinkage and Selection Operator (LASSO) method, and the predictive value of the models was evaluated by time-associated receiver operating characteristic (ROC) curves. Finally, independent prognostic factors were identified by COX analysis. Results: The hsa-miR-3150b-3p associated with the prognosis of CESC was identified by WGCNA. A total of 136 target genes were differentially expressed in CESC tissue and were associated with biological processes such as phylogeny, multicellular organism development and cell development. CBX7, ENPEP, FAIM2, IGF1, NUP62CL and TSC22D3 were associated with the prognosis of CESC, and a prognostic prediction model was constructed using these six genes, which had a good predictive value for the prognosis of cervical cancer within 1, 3 and 5 years (AUC: 0.784, 0.680 and 0.683, respectively). Among them, ENPEP (hazard ratio = 1.3996, 95% confidence interval: 1.0552-1.8565) was identified as an independent prognostic factor. Conclusions: In this study, a highly accurate prognostic model consisting of six gene signatures was developed to predict the prognosis of patients with cervical cancer, which provides a reference for developing individualized treatment plans for patients.

Keywords: Cell carcinoma and endocervical adenocarcinoma, miRNA, prognosis, target gene, risk model

Introduction

Cell carcinoma and endocervical adenocarcinoma (CESC) ranks the fourth among common female cancers worldwide, with an estimated 604,000 new cases and 342,000 CESC-related deaths in 2020, despite that it is one of the most preventable and treatable malignant tumors [1]. Early CESC is curable, but advanced CESC cannot be cured even after combined treatment of surgery, radiotherapy and chemotherapy [2]. As cervical cytology and cervical pathological biopsy technology advance, locally advanced cancers can be detected earlier and more accurately, and novel treatment strategies such as targeted therapy and immunotherapy have been constantly applied in clinical practice [3, 4]. The molecular determinants of CESC are not fully understood, so developing more effective treatment methods is still difficult. Accordingly, it is urgent to understand the molecular basis of CESC.

MicroRNAs (miRNAs) are small non-coding RNAs, and their roles in tumors have been extensively discovered in recent years [5, 6].

They can bind to the 3' untranslated regions of target gene mRNAs by base pairing and thus degrade the mRNA, inhibit the transcription, and down regulate posttranscriptional target genes [7]. Abnormal miRNA level is correlated with the change of the malignant potential of CESC and the outcome of patients [8]. In addition, evidence from cell strains and malignant tissues also provides a basis for the role of miRNAs including miR-30d-5p [9], miR-125a-5p [10], miR-34a and miR-206 [11] in the development of CESC and suggests the possibility of using them as biomarkers.

As gene detection methods continuously develop, large-scale bioinformatics databases have been widely applied in medical research [12]. Among them, The Cancer Genome Atlas (TCGA) can help quantitative study and analyze the changes of gene expression during the development of tumors through large-scale gene sequencing, and the associated research results provide a reliable evaluation basis for the diagnosis and prognosis of tumors [13-15]. Ga et al. [16] found four miRNAs (miR-99a, miR-125b, miR-188 and miR-223) associated with the survival of patients with CESC based on the Gene Expression Omnibus (GEO) and TCGA. Qi et al. [17] found a new 3-miRNA signature that may be a candidate biomarker to predict the adverse prognosis of CESC. However, up to now, the research on miRNA networks associated with the prognosis of CESC by high-throughput data from large databases such as TCGA is still very limited. Accordingly, this study identified survival-associate miRNAs by analyzing the miRNAs and clinical data from TCGA, and constructing a weighted gene interaction network, and established a prognosis prediction risk model for the identified target genes of miRNA, with the purpose of accurately predicting the prognosis of CESC patients and providing reference for the development of individualized diagnosis and treatment plans for patients.

Methods

Data source

RNA-Seq, miRNA-seq and clinical information of patients with cervical squamous CESC were downloaded from TCGA (https://portal.gdc.cancer.gov/). During analysis, the average value of the expression of the same miRNA detected by multiple probes was adopted as its expression. For the analysis of clinical data of patients, the data of those with unknown survival time or with survival time of 0 were removed.

Variation analysis

The limma package in R v4.0.3 was adopted to screen differentially expressed genes (DEGs) under the conditions of $|logFC| \geq 1$ and adjusted P value <0.05. The overlapping genes (candidate genes) between DEGs and the predicted target genes were analyzed using Venn Plot.

Weighted gene coexpression network analysis (WGCNA)

The "WGCNA" software package in R v4.0.3 was adopted to analyze the expression of candidate genes and clinical documents. By analyzing the Pearson's correlation between gene pairs and the association of characteristic genes of modules and traits (including disease state), the gene modules associated with prognosis were determined.

Prediction of target genes

The miRDB database (http://mirdb.org/) and TargetScan database (http://www.targetscan.org/vert_72/) were both adopted to predict the target genes of pivotal miRNAs.

Functional enrichment analysis

Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses were carried out with DAVID 6.8 database (https://david.ncifcrf.gov/). Enrichment result P<0.05 or FDR<0.05 indicates a statistical significance.

Kaplan-meier (K-M) survival analysis

Survival in R package was adopted for survival analysis. P value and hazard ratio (HR) with 95% confidence interval (CI) in K-M curve were acquired by the log rank test and univariate Cox proportional hazard regression.

LASSO-COX regression risk prediction model

The LASSO regression algorithm was adopted for feature selection, and 10 times cross validation was used for the determination of parameters to get a suitable model. Then, the genes obtained by LASSO regression were subjected

to multivariate Cox regression analysis, and the multivariate regression coefficient of each gene was calculated, and thus the risk scoring equation was constructed. According to the median risk score, patients were assigned to a high-risk group or a low-risk group. K-M survival curves were adopted to compare and analyze the total survival time of the two groups, and time-associated ROCs were adopted to evaluate the predictive value of gene markers.

Multivariate and multivariate Cox regression analyses

Cox regression analysis was conducted with the Survival package, and the *P* value, HR and 95% CI of each variable were reported by drawing a forest map with the forest plot package. According to the results of multivariate Cox proportional risk analysis, the RMS package was applied to construct nomogram to predict the 1-, 3- and 5-year survival rates.

Statistical analyses

R software (v4.0.3) was used for data analyses. The Wilcoxon rank sum test was adopted for expression analysis of genes and miRNA in data samples from TCGA, and Survival package was used for Cox regression analysis. Two-tailed P<0.05 suggests a statistical significance.

Results

Construction of WGCNA and determination of hub modules

WGCNA was utilized to study the correlation patterns among miRNAs in TCGA-CESC, and the gene modules associated with clinical information were obtained. The soft threshold β = 3 was selected to ensure the construction of scale-free network (**Figure 1A**). The sensitivity was set to be 4, and the minimum module size was set to be 30 genes. Then the modules were identified to generate a tree diagram (**Figure 1B**). Through clustering, highly similar modules were identified and merged. As shown in **Figure 1C**, 13 miRNA gene modules were identified. After determination of the miRNA modules, Pearson's correlation analysis was conducted to calculate the correlation coefficient and *P* value of the characteristic genes of the gene modules and the clinical features of CESC (age, overall survival (OS), survival status and tumor stage). The results revealed that the green yellow module was significantly correlated with the OS of CESC (r = 0.23, P = 9.6e-5), and that 46 miRNAs were in this module.

Identification of pivotal miRNA

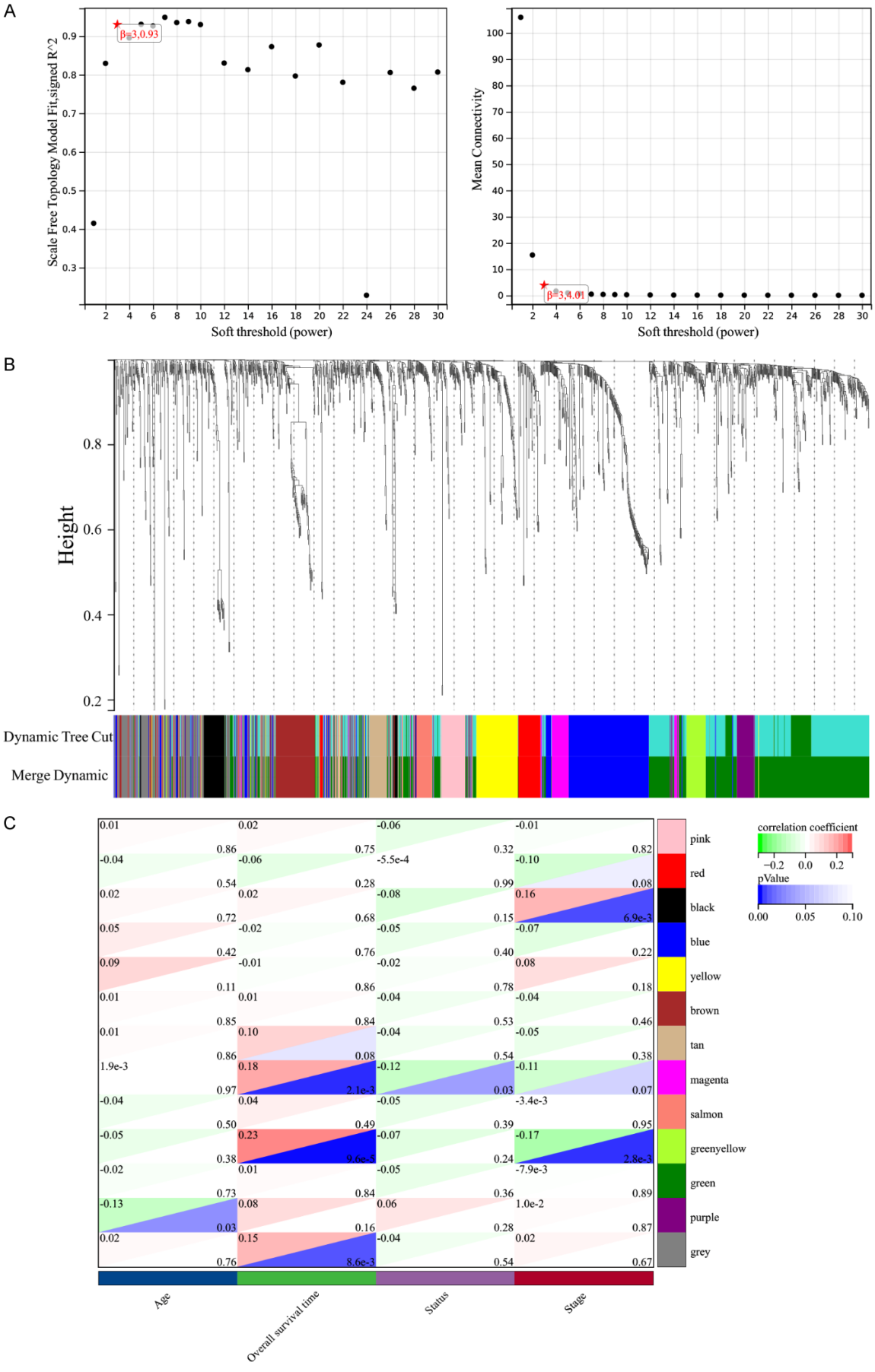
The expression and survival of the 46 miRNAs in the green yellow module in CESC samples from TCGA were analyzed. The results showed up regulated hsa-miR-3150b-3p in CESC tissue as compared with normal tissue (**Figure 2A**, P<0.05). The K-M curve revealed that lowly expressed hsa-miR-3150b-3p was significantly associated with the unfavorable prognosis of patients (**Figure 2B**, P<0.05).

Forecasting of target genes of hsa-miR-3150b-3p

Target genes of miR-3150b-3p were forecasted with miRDB and TargetScan, and 822 and 5017 target genes were obtained, respectively. Then 2692 DEGs were acquired by variation analysis of data from CESC, which contained 136 intersecting genes (**Figure 3A**). The 136 genes are displayed in **Figure 3B**. The 136 genes were enriched by KEGG signal pathway and GO biological process, respectively. KEGG results (**Figure 3C**) revealed that they were primarily associated with glioma, the role of AGE-RAGE signaling pathway in diabetic complications and endocrine resistance. GO biological process results (**Figure 3D**) revealed that 136 genes enriched various biological processes, such as phylogeny, multicellular organism development and cell development process.

Identification of prognosis-associated genes among 136 genes

K-M survival analysis was conducted on 136 genes, and the log-rank was used to test KM survival analysis. As a result, 6 survival-associated genes were acquired, namely CBX7, ENPEP, FAIM2, IGF1, NUP62CL and TSC22D3. The patients were assigned to a high-risk group or a low-expression group with the median expression as a cutoff value. The results revealed that highly expressed ENPEP was significantly correlated with unfavorable prognosis of patients (**Figure 4B**, P<0.05) and lowly expressed CBX7, FAIM2, IGF1, NUP62CL and TSC22D3 were also notably associated with that (**Figure 4A**, **4C-F**, P<0.05).


A
Scale Free Topology Model Fit,signed R^2
β=3,0.93
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Mean Connectivity
β=3,4.01
Soft threshold (power)
B
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C
pink
red
black
blue
yellow
brown
tan
magenta
salmon
greenyellow
green
purple
grey
correlation coefficient
pValue
Age
Overall survival time
Status
Stage

Figure 1. Construction of a network and identification of gene modules. A: Soft threshold screening. B: Hierarchical clustering of leading value differences to obtain gene clustering tree. C: Correlation of modules with clinical features.

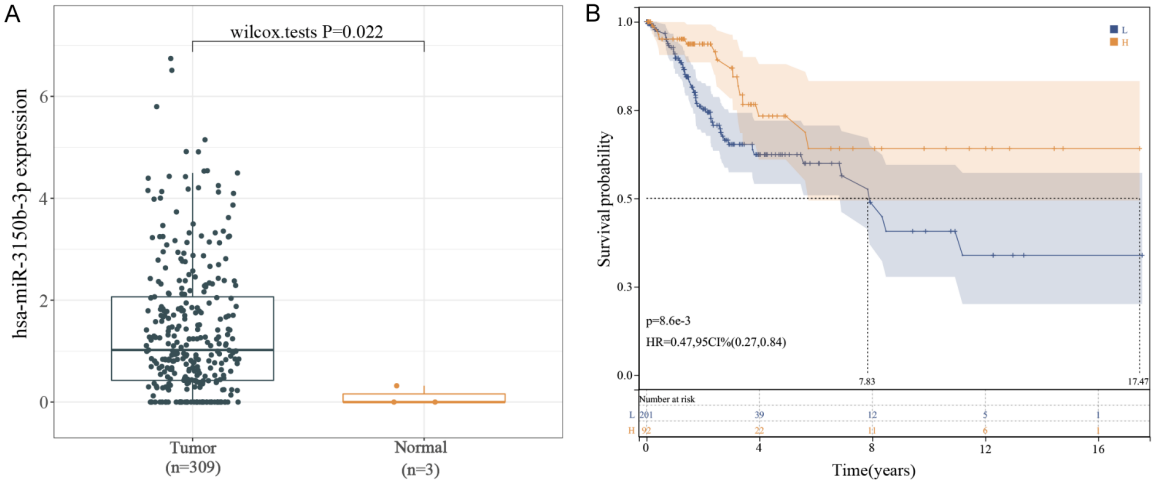

Figure 2. Identification of pivotal miRNA. A: Expression of hsa-miR-3150b-3p in cervical cancer tissues and normal tissues; B: K-M survival curve of hsa-miR-3150b-3p. Note: K-M: Kaplan-Meier.

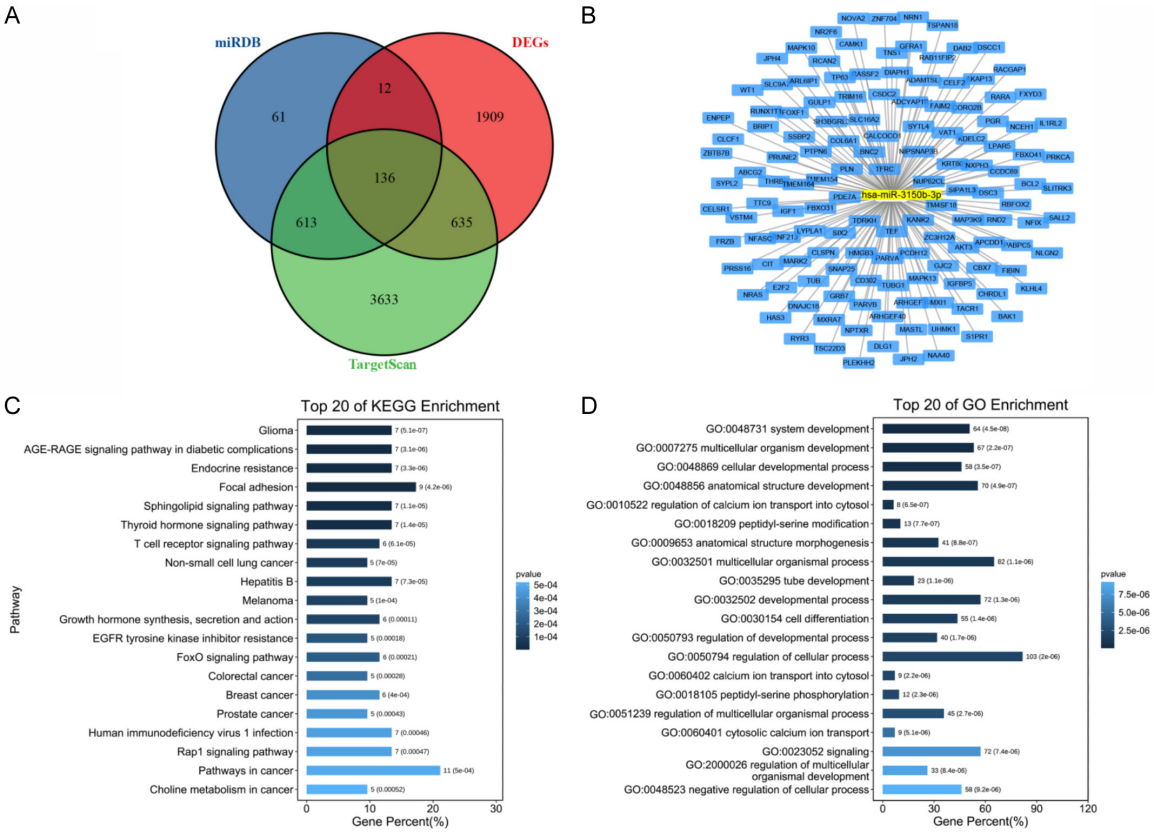

Figure 3. Forecasting of target genes of hsa-miR-3150b-3p. A: Venn plot showing the intersection of DEGs and target genes predicted by miRDB and TargetScan; B: Has-miR-3150b-3p and its 136 intersecting genes; C: Top 20 pathways of KEGG enrichment; D: Top 20 terms of GO Enrichment. Note: DEGs: differentially expressed genes; KEGG: Kyoto encyclopedia of genes and genomes; GO: Gene Ontology.

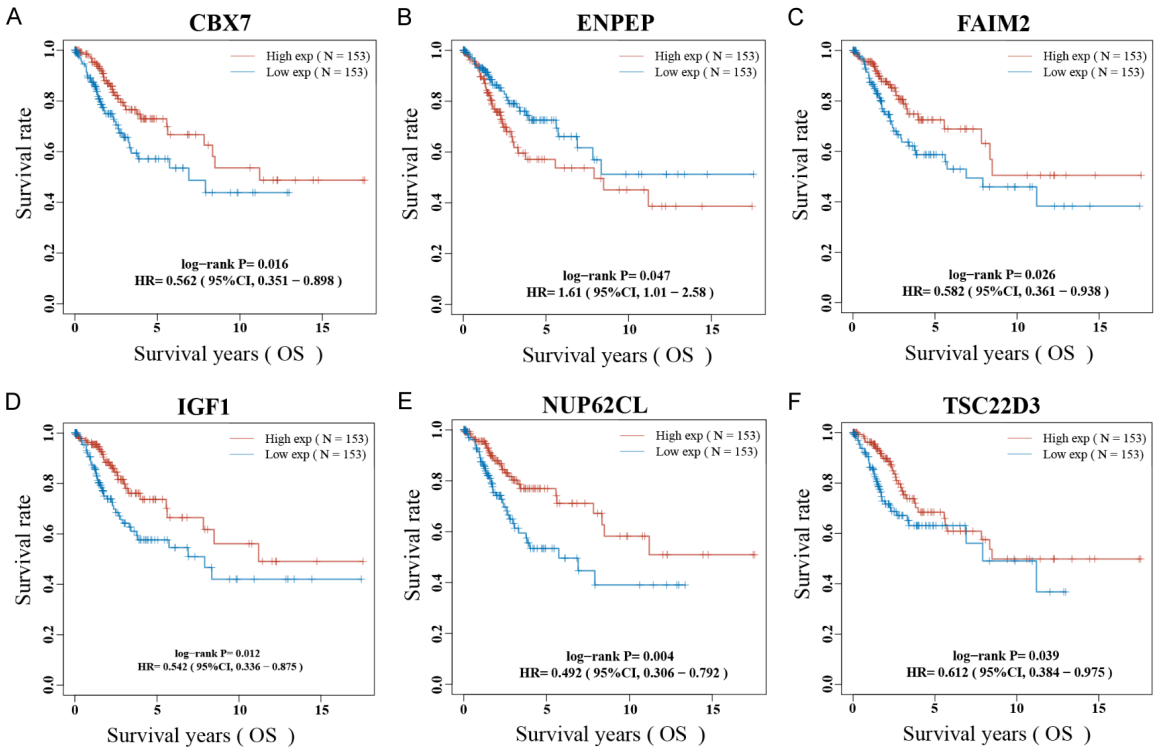

Figure 4. Identification of prognosis-associated genes among 136 genes. A: K-M survival curve of CBX7. B: K-M survival curve of ENPEP. C: K-M survival curve of FAIM2. D: K-M survival curve of IGF1. E: K-M survival curve of NUP62CL. F: K-M survival curve of TSC22D3.

Construction of prognostic gene prediction model

The 6 genes and data of samples were analyzed via LASSO regression, and 10 times cross validation method was used. According to the results, when the variable was 6, the root mean square error of the model was the smallest, with λ = 0.0133 (**Figure 5A**, **5B**). A risk score model for prognosis prediction of CESC based on the 6 gene markers was constructed: Risk score = (0.2575)*ENPEP + (-0.097)*NUP62CL + (-0.1024)*IGF1 + (-0.1661)*TSC22D3 + (0.053)*FAIM2 + (-0.1458)*CBX7. With the risk score formula, the risk score of patients with CESC was calculated, and the patients were assigned to a high-risk group or a low-risk group (cut-off value: the median score). The distribution chart of survival time (**Figure 5C**) showed more death cases and shorter OS in the high-risk group than those in the other group. K-M survival analysis of the two groups revealed a significantly higher survival rate in the low-risk group than that in the other group (**Figure 5D**, P<0.05). According to ROC curves (**Figure 5E**), the model had AUCs of 0.784, 0.680 and 0.683, respectively, in predicting 1-, 3- and 5-y survival, suggesting its favorable predictive ability.

Univariate and multivariate Cox regression analyses

This study was designed to analyze whether CBX7, ENPEP, FAIM2, IGF1, NUP62CL and TSC22D3 were independent risk factors of CESC. Univariate and multivariate Cox regression analyses revealed that ENPEP may be an independent prognostic predictor of CESC (**Figure 6A**, **6B**). Next, a Nomogram for the prediction of the 1-, 3- and 5-year OS rate was generated (**Figure 6C**). According to the calibration results, compared with the ideal model, this model has favorable prediction performance in predicting the 1-, 3- and 5-year OS (**Figure 6D**).

Discussion

It is known that miRNAs regulate different biological pathways by changing gene expression to impact the progress of tumors, including CESC [18]. A large number of previous studies

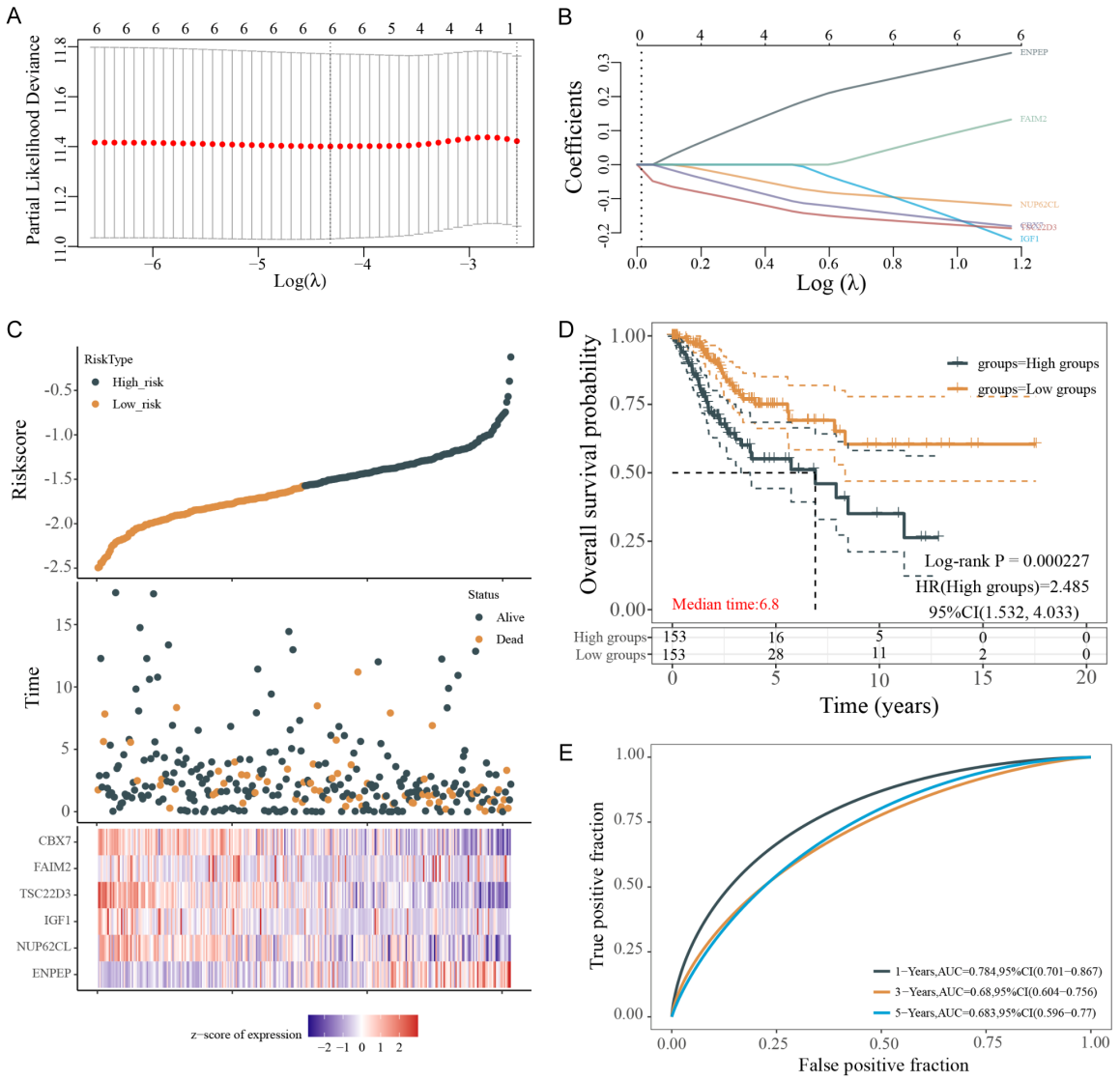

Figure 5. Construction of prognostic gene prediction model. A: Selection of the parameter λ in ten times cross validation. B: Dynamic process of LASSO screening variables. C: Risk score, survival time and survival status. D: K-M survival curve of the risk model in TCGA data set. E: ROC curve of the risk model at different times.

have reported that miRNAs have possible association with the clinical prognosis of CESC, such as miR-155 [19], miR425-5p [20], miR638 [21] and miR-1254 [22]. However, these heterogeneity results are mainly caused by a relatively limited sample size and the number of candidate miRNAs or the lack of experimental verification. In the present study, a miRNA related with the prognosis of CESC was identified by integrated bioinformatics analysis, namely miR-3150b-3p. Additionally, based on the prediction of target genes of miR-3150b-3p and the analysis of DEGs in CESC, a prediction model of CESC prognosis based on six gene markers was constructed.

MiR-3150b-3p, located at 8q22.1, belongs to the miR-3150b family [23]. To date, miR-3150b-3p is rarely studied. Researchers have confirmed its ability to weaken cell proliferation in colorectal cancer [24] and CESC [25]. In the present study, compared with normal tissue, CESC tissue showed up regulated miR-3150b-3p, and patients with low expression of it showed a higher survival rate than those with low expression of it. The results of this study are contrary to those of previous studies, which may be due to the fact that there were only 3 normal samples in TCGA data, and the expression of miR-3150b-3p in two of them may lead to the deviation of the results. Subsequently,

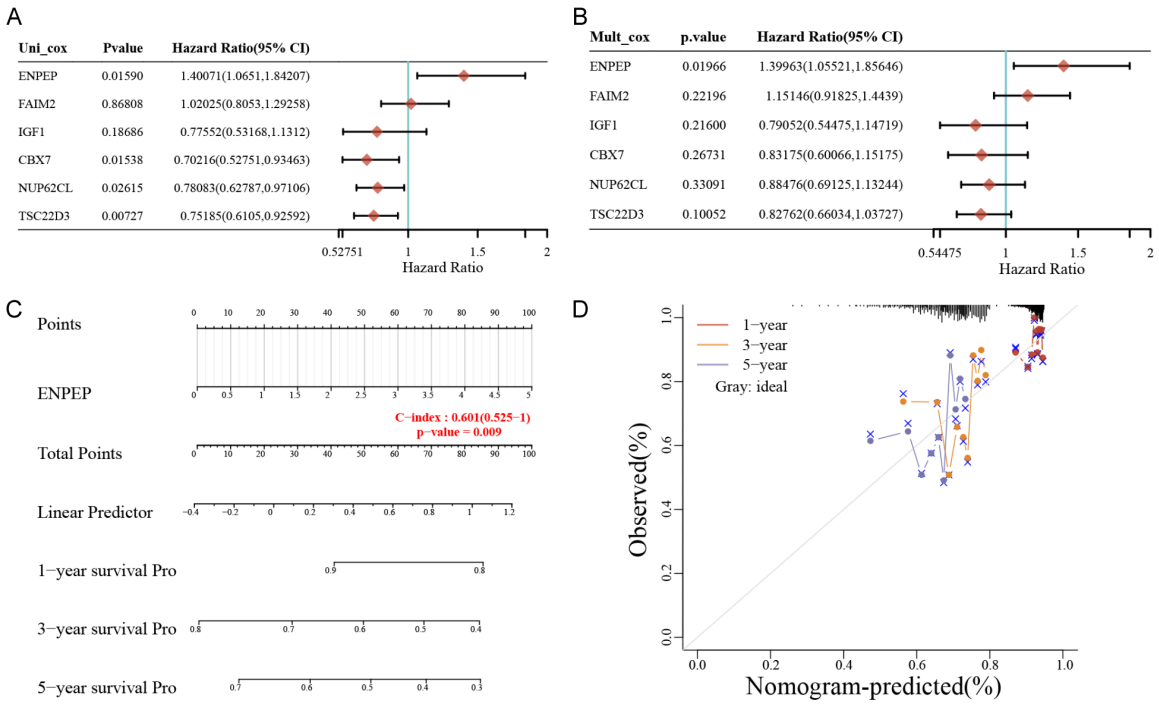

Figure 6. Univariate and multivariate Cox analyses. A: *P* value, risk coefficient HR and CI of 6 genes in univariate COX analysis. B: *P* value, risk coefficient HR and CI of 6 genes in multivariate COX analysis. C: Nomogram can forecast the 1-, 3- and 5-year OS rates of patients with CESC. D: The calibration curve of the OS nomogram model. Note: HR: hazard ratio; CI: confidence interval; OS: overall survival; CESC: cell carcinoma and endocervical adenocarcinoma.

we further predicted the target genes of miR-3150b-3p, and acquired 136 genes based on DEGs in TCGA-CESC. The results of functional enrichment analysis revealed the possible impacts of genes on the cell development process. Moreover, 136 genes were subjected to K-M survival analysis, and 6 genes (CBX7, ENPEP, FAIM2, IGF1, NUP62CL and TSC22D3) associated with the prognosis of CESC were obtained. According to research, multi-gene signatures take a crucial part in predicting the prognosis of tumors. Xie et al. [26] identified an 8-gene signature to predict the prognosis of patients with CESC. Therefore, with LASSO-Cox regression as the machine learning algorithm, the present study constructed a risk model of prognosis prediction with a six-gene signature, and verified the prediction ability of the model by ROC curves. In addition, ENPEP was found to be able to serve as an independent predictor of the prognosis of CESC through univariate and multivariate Cox analyses.

ENPEP, namely aminopeptidase A or CD249, is a member of the zinc-containing endopeptidase M1 family of mammalian type II intact membrane. It plays a role in the catabolic pathway of renin-angiotensin system in the formation of angiotensin III and blood pressure regulation and angiogenesis, which may increase the risk of atrial fibrillation, angiogenesis, hypertension and tumorigenesis [27-30]. Yuan et al. [31] found high expression of ENPEP in colorectal cancer through bioinformatics analysis. Studies also revealed the high expression of ENPEP in cervical tumor lesions, and found its upregulation in the case that the lesions develop from invasive intraepithelial neoplasia to invasive squamous cell carcinoma [32].

To sum up, our study has revealed that miR-3150b-3p can serve as a biomarker for predicting the prognosis of CESC by the WGCNA, Cox regression and LASSO regression algorithm, and constructed a prediction model of prognosis with a six-gene signature (CBX7, ENPEP, FAIM2, IGF1, NUP62CL and TSC22D3) based on the target genes of miR-3150b-3p. However, it still has some limitations. Little research has been done on the biological behaviors of the identified miR-3150b-3p and E, and miR-3150b-3p is rarely studied at present, which, however, also indicates the potential research value of miR-3150b-3p, so its role in CESC and

other tumors requires further exploration. In addition, more clinical samples are required for verification of the results of this study. Although the risk model constructed in this study has demonstrated favorable performance, the LASSO-Cox algorithm used requires proper processing of input data, and the parameters should be properly adjusted during the calculation process to avoid the performance degradation of the algorithm [33]. Therefore, a more accurate machine learning method, a larger clinical sample size and *in vitro* experiments are required to further study the mechanism of miR-3150b-3p and ENPEP in CESC in the future.

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Disclosure of conflict of interest

None.

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