Identification of a Vascular Invasion-Related miRNA Signature For Predicting the Prognosis of Hepatocellular Carcinoma

Xinze Qiu
Guangxi Medical University
Jiangni Wu
Guangxi Medical University
Zichen Huang
Guangxi Medical University
Shibo Luo
Guangxi Medical University
Jiean Huang
Guangxi Medical University
Shiquan Liu (poempower@163.com)
Guangxi Medical University

Research

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Abstract

Background: Vascular invasion is closely related to the prognosis of hepatocellular carcinoma (HCC). Increasing evidence suggests that miRNAs can serve as biomarks to predict prognosis in various tumors. Thus, the aim of this study was to develop a novel, vascular invasion-related miRNA signature for prediction of HCC prognosis.

Methods: Differentially expressed miRNAs (DEMs) between HCC samples with vascular invasion and without vascular invasion were obtained from GSE67140. MiRNAs expression profiles and clinical information for 344 patients were collected from The Cancer Genome Atlas database, and the patients were randomized (1:1) to training set and validation set. LASSO regression model was employed to identify survival-associated DEMs and establish risk score in the training set. Moreover, risk score was verified in the validation set. And nomogram based on risk score and clinical information was constructed to improve the prediction of prognosis. Meanwhile, four online tools were used to predict target genes and enrichment analysis was utilized to explore the biological pathway of the miRNAs.

Results: A novel three-miRNA signature was screened including hsa-mir-210, hsa-mir-149 and hsa-mir-99a, and risk score was established for HCC prognosis prediction. Patients were divided into the low-risk group and high-risk group according to risk score. High-risk group both have higher hazard of death compared with low-risk group in training set and validation set. And the 5-year AUC of risk score were 0.718, 0.674 and 0.697 in training set, validation set and the total set, respectively. The C-index of nomogram was 0.724, and calibration curves showed nomogram had high concordance to predict 1- ,3- , and 5-year survival rates among HCC patients. Furthermore, enrichment analysis identified several tumor-associated pathways including Ras signaling pathway, PI3K-Akt signaling pathway, and MAPK signaling pathway and so on, which may contribute to explain the potential molecular mechanisms of above miRNAs.

Conclusion: This study developed a risk assessment model based on three miRNAs, which could accurately predict the prognosis of HCC.

Background

As one of the most common malignant cancer, liver cancer is the sixth most frequently diagnosed cancer and the fourth cause of tumor related deaths worldwide according to the global cancer statistics [1]. Hepatocellular carcinoma (HCC) is the most prevalent subtype and accounts for 75%-85% of liver cancer. In recent years, the treatment of HCC has made great progress, including surgical resection, systemic therapy, locoregional ablative therapy, transcatheter arterial chemoembolization, etc. Despite these advances, it is disappointing that the prognosis of HCC patients remains poor, and the mortality still rose over the past decade [2]. The AJCC TNM staging system and clinical stage are used to evaluate the prognosis of HCC, which depended on the status of tumor invasion, node metastasis and distant metastasis. But it can't be ignored that the staging system couldn’t provide proper prognostic information

in some cases [3]. Therefore, it is essential to identify a novel biomarker to improve the prognosis and treatment of HCC.

MicroRNAs(miRNAs) belong to the noncoding RNA family, which containing 19–25 nucleotides [4]. The majority of miRNAs decrease the expression of target genes via degradation or translational repression of mRNAs [5]. Studies have uncovered that miRNAs took part in almost all of biological process of cancer, such as apoptosis, proliferation, and metastasis [6–8]. And increasing evidence showed miRNAs could be regard as prognostic biomarkers in cancer. For example, recent study reported that a 26-miRNA signature could do well in the prognostic stratification of oropharyngeal squamous cell carcinoma [9].

Vascular invasion is a significant clinical pathological characteristic in HCC. It is widely known that vascular invasion plays a vital role in HCC recurrence and progress. There were lots of studies to investigate prognostic markers of HCC with miRNA expression profiles[10, 11]. However, the research which explore the prognostic value of vascular invasion-related miRNAs in HCC is rare. The aim of the present study was to construct a vascular invasion-related miRNA signature, so that the prognostic prediction of HCC could be improved more.

In present study, a novel vascular invasion-related prognostic signature made up of three miRNAs was identified using miRNA expression profiles from Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). And nomogram contained miRNA signature and clinical data was constructed. Furthermore, enrichment analysis was used to investigate the biological functions of the miRNAs.

Methods

Data collection

Microarray data (GSE67140) was downloaded from GEO database (http://www.ncbi.nlm.nih.gov/geo/), which based on GPL8786 Affymetrix Multispecies miRNA-1 Array [12]. GSE67140 contains 81 HCC patients with vascular invasion and 91 HCC patients without vascular invasion. Meanwhile, miRNA expression profiles and matched clinical information (Project: TCGA-LIHC; Data Type: miRNA Expression Quantification) were downloaded from TCGA (https://tcga-data.nci.nih.gov/tcga). The patients whose survival time less than 30 days were excluded. There were 344 HCC patients eligible for this study in total.

Identification of vascular invasion-related miRNAs

There were two batches in GSE67140, so “removeBatchEffect” function of “limma” was used to remove the batch effect [13]. And “limma” package was also utilized to identify differentially expressed miRNAs (DEMs) between with vascular invasion and without vascular invasion tissues. The screening criteria for DEMs were presented below: (1) |log2 fold change (log2FC) | >1 and P value < 0.05 (FDR adjustment); (2) the average expression level no less than 3 in TCGA-LIHC cohort.

Construction of risk score based on DEMs

Firstly, the miRNA expression profiles from TCGA were divided into training set and validation set randomly at a 1:1 ratio. Secondly, to acquire prognostic DEMs and corresponding regression coefficients, the least absolute shrinkage and selection operator (LASSO) cox regression analysis was performed via “glmnet” package [14]. And the lambda value of LASSO regression model was determined through 10-times cross validation. After that, risk score of patients was calculated by follow formula: Risk score = exprDEM1 * coefficientDEM1 + exprDEM2 * coefficientDEM2 +…+ exprDEMn * coefficientDEMn. The miRNA expression profiles in TCGA-LIHC cohort were normalized by log2 reads per million of total aligned miRNA reads[15].

Validation of risk score

Among three sets, which were training set, validation set and the total set, receiver operating characteristic (ROC) curves were utilized to distinguish 5-year survival rate of risk score based on "pROC" package. And ROC curve in the total set was used to confirm the best cutoff so that three sets could be divided into high-risk group and low-risk group, respectively. What's more, Kaplan-Meier(K-M) survival curves were applied to compare survival difference between high-risk group and low-risk group.

Nomogram for survival prediction

To estimate the hazard of clinical data and risk score of HCC, univariate Cox proportional hazards regression was used in the total set based on “survival” package. While P value < 0.10, the parameters were adopted into multivariate Cox analysis. Furthermore, the nomogram was constructed in TCGA-LIHC cohort via “rms” package. Concordance-index(C-index) and calibration curves were performed to assess the discrimination and deviation of nomogram, respectively.

Target gene prediction and enrichment analysis

Four online tools were used to predict the target genes of DEMs in risk score, including DIANA (TarBase v8, http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php, accessed 16 Nov 2020) [16], miRDB (http://www.mirdb.org/, accessed 16 Nov 2020) [17, 18], miRTarBase (http://mirtarbase.cuhk.edu.cn/php/index.php, accessed 16 Nov 2020) [19], and TargetScan (v7.2, http://www.targetscan.org/, accessed 16 Nov 2020) [20]. Final target genes were defined as the overlapping of four online tools via Venn diagram. Subsequently, “clusterProfiler” package was used for enrichment analysis of final target genes via Kyoto Encyclopedia of Genes and Gene Ontology (KEGG) pathway [21], and P value < 0.05 (FDR adjustment) was considered significant. Network relationship between DEGs and the target genes was visualized by Cytoscape software (version:3.8.0).

Statistical analysis

All of statistical analysis were conducted by R language 4.0.3. For comparison of clinical parameters, χ^2 or Fisher exact test was used in categorical variables as appropriate, and Student’s t test or Mann–Whitney U test was used in continuous variables as appropriate. K-M survival curves were analyzed using log-rank test with “survival” package. Comparison of Area Under Curve (AUC) of ROC curves were performed using delong test. The cutoff criteria of statistically significant was set as P value < 0.05.

Results

Identification of DEMs

To obtain the vascular invasion-related miRNAs of HCC, after removing the batch effect, differential expression analysis was used to compare the tissues which with vascular invasion and without vascular invasion in GSE67140. A total of 125 DEMs were selected with a screening criterion of |log2FC| >1 and adjusted *P* value < 0.05. The heatmap of 125 DEMs were visualized via “pheatmap” package (Fig. 1). With the cutoff expression values not less than 3 in TCGA-LIHC cohort, only 67 DEMs were adopted into further analysis.

Development and validation of risk score based on DEMs

There was no significant difference among training set (n = 172), validation set (n = 172) and the total set (n = 344) in clinical parameter (Table 1). Only 3 miRNAs were selected by the LASSO Cox regression analysis model in training set (Fig. 2A-B). Subsequently, risk score was construct as follows: Risk score = (0.2132*hsa-mir-210) + (0.1678*hsa-mir-149) + (-0.0865*hsa-mir-99a). In order to exhibit the relationship between risk score and survival status, risk distribution and expression pattern of three miRNAs in the total set were performed (Fig. 2C-E). Additionally, time-dependent ROC curves were used to show specificity and sensitivity of risk score in survival prediction. The 5-year AUC of training set, validation set and the total set were 0.718 (95% CI: 0.527–0.812), 0.674 (95% CI: 0.596–0.707), 0.697 (95% CI: 0.615–0.699), respectively (Fig. 3A-C), which implied that risk score exhibited good performance in predicting survival. What’s more, high-risk group had a shorter survival time than low-risk group in all sets (all *P* values < 0.05) (Fig. 3D-F). Therefore, risk score based on above three miRNAs was a risk factor of HCC patients.

Table 1
Clinical parameter of training set, validation set and the total set

Characteristic		Training set	Validation set	The total set	*P* value
		n = 172	n = 172	n = 344	
Age (years, median)		62	61	61	0.872
Gender	Female	54	54	108	1
	Male	118	118	236	
Grade	G1	28	25	53	0.785
	G2	84	76	160	
	G3	54	60	114	
	G4	6	7	13	
	NA	0	4	4	
Stage	I	80	82	162	0.937
	II	40	37	77	
	III	39	41	80	
	IV	3	0	3	
	NA	10	12	22	
Survival status	Alive	111	111	222	1
	Dead	61	61	122	

Independence of the risk score

To investigate the independent risk factor of HCC, univariate and multivariate Cox regression analysis were used. As is shown in Table 2, univariate Cox regression analysis indicated that risk score was significantly associated with overall survival (hazard ratio HR = 8.30, confidence interval 95% CI = 2.95–23.34, $P < 0.001$). Child–Pugh score and clinical stage were related to the overall survival, which were brought into next analysis together. Multivariate Cox regression analysis showed that risk score still remained independent (hazard ratio HR = 6.77, confidence interval 95% CI = 2.43–18.82, $p < 0.001$) considering other clinical factors. Meanwhile, clinical stage also exhibited significant correlation with overall survival (hazard ratio HR = 1.50, confidence interval 95% CI = 1.12–2.01, $p < 0.05$). It demonstrated that risk score based on three miRNAs was an independent adverse prognostic factor in HCC.

Table 2
Univariate and multivariate Cox regression analysis of clinical parameter

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Age	1.01	0.99–1.04	0.175			
Gender (Male vs Female)	0.74	0.43–1.26	0.267			
Child-Pugh score (B/C vs A)	1.96	0.96-4.00	0.066	1.94	0.95–3.96	0.070
Grade	1.34	0.93–1.92	0.113			
Stage	1.63	1.22–2.17	< 0.001	1.50	1.12–2.01	0.006
Risk score (High vs Low)	8.30	2.95–23.34	< 0.001	6.77	2.43–18.82	< 0.001

Establishment of nomogram

Nomogram was established to predict the survival probabilities of HCC patients combined risk score with clinicopathological parameters, which could provide a better evaluation of prognosis for the individual patient. The nomogram contained age, Child–Pugh score, histologic grade, clinical stage, and risk score. Risk score was the largest contributors to prognosis compared with the others (Fig. 4A). C-index of the nomogram was 0.724 (95%CI: 0.658–0.791), which indicated the nomogram could predict correctly the vital status of HCC patients with 72.4 percent. Calibration curves were used to evaluate the deviation between prediction and actual observation in 1-,3-, and 5-year overall survival. As the calibration curves showed, the nomogram could estimate prognosis of HCC accurately (Fig. 4B-D). Furthermore, time-dependent ROC curve was used to contrast the distinction power among nomogram, Child–Pugh score, clinical stage, and histologic grade. As is showed in Fig. 4E, the nomogram possessed largest 5-year AUC compared with the others (all $P < 0.05$), whose specificity and sensitivity were 0.662 and 0.746 in the optimal cut point, respectively.

Target genes prediction and enrichment analysis

The target genes of three miRNAs, including hsa-mir-210, hsa-mir-149 and hsa-mir-99a, were predicted with the intersection of DIANA, miRDB, miRTarBase and TargetScan. A total of 19 target genes for hsa-mir-210, 26 target genes for hsa-mir-149 and 5 target genes for hsa-mir-99a were selected (Figure S1). Subsequently, enrichment analysis was performed to annotate the biological functions of the target genes via KEGG pathway, which could uncover the biologic roles of the miRNAs in HCC. KEGG pathway analysis indicated that the targeted genes were enriched in Ras signaling pathway, PI3K-Akt signaling pathway, signaling pathways regulating pluripotency of stem cells, central carbon metabolism in cancer, MAPK signaling pathway and so on (Fig. 4F). What's more, the interaction between three miRNAs and the target genes was showed with Cytoscape software (Figure S2).

Discussion

HCC is a common malignant tumor with high incidence and mortality [1]. And HCC is easy to recur even the tumor was resected as complete as possible, especially among the patients with vascular invasion [22]. The AJCC TNM staging system as a clinical prognostic evaluation method, was not effective to predict the prognosis of HCC patients [3]. Increasing evidence showed that miRNAs were satisfying biomarks in many human malignant tumor [23].To improve the accuracy of HCC prognostic evaluation, various miRNAs signatures were found based on TCGA-LIHC cohort [10, 24].However, these studies mainly focused on the differentially expressed of miRNAs between normal tissues and tumor tissues. Vascular invasion is an important risk factor. It's still necessary to investigate the predictive powers and molecular mechanisms of vascular invasion-related miRNAs. The advantage of the present study was identifying prognostic miRNAs via LASSO regression model, which could effectively reduce variables and assure the predictive powers [25]. Additionally, nomogram was established to assess the value of clinical application.

In current study, a total of 125 DEMs were screened out in GEO database, and 3-miRNA signature was selected to predict the overall survival of HCC in TCGA-LIHC cohort. Risk score was constructed based on the three-miRNA signature. Time-dependent ROC analysis demonstrated that risk score had a good prognostic prediction ability both in training set and validation set. And survival analysis showed that risk score was associated with survival significantly. Multivariate Cox regression analysis indicated that risk score is an independent risk factor in HCC. Besides, nomogram could accurately predict the prognosis of HCC patients and was better than clinical stage as the ROC curves and calibration curves showed. The above results suggested that the 3-miRNA signature could play an important role in clinical application.

Risk score contained three miRNAs, which were hsa-mir-210,hsa-mir-149 and hsa-mir-99a. Previous study showed that hsa-mir-210 could be a diagnostic biomark with relatively moderate accuracy in various cancers including breast invasive carcinoma, Lung squamous cell carcinoma and rectum adenocarcinoma [26]. And augmented expression of hsa-mir-210-3p suppressed the expression of negative regulators of NF-κB signaling, which promoted the epithelial to mesenchymal transition, invasion and migration in prostate cancer [27]. Current study also demonstrated that silencing of hsa-mir-210 inhibited the progression of liver cancer [28].Additionally, studies have reported that miR-210 could promote cancer angiogenesis and venous metastasis in hepatocellular carcinoma [29, 30]. There were studies showed that hsa-mir-149 inhibited the progression of various tumor. Hsa-mir-149 blocked paracrine interactions of macrophages via decreasing the expression of colony-stimulating factor-1 and epidermal growth factor, which could suppress breast cancer metastasis [31]. In liver cancer, mir-149 suppressed tumor progression by restraining the expression of tumor necrosis factor receptor type 1-associated death domain protein in NF-κB signaling pathway [32]. Interestingly, in present study, higher expression of hsa-miR-149 meant worse prognosis in HCC. It is possible that the expression of hsa-mir-149 is increasing to prevent the development of tumor while HCC is progressing. Hsa-mir-99a-5p could act as a diagnostic and prognostic biomarker in gastric cancer [33]. Evidence also verified that miR-99a induced cell cycle arrest, which resulted in suppression of HCC growth [34]. What's more, miR-99a suppressed the development of many malignancy such as breast cancer, bladder cancer, ovarian cancer

[35–37]. However, the mechanisms of above three miRNAs in HCC are not clear and need to investigate further.

To further understand the regulatory mechanism of the 3-miRNA signature in HCC, functional enrichment analysis of target genes was utilized. The target genes of the miRNAs were enriched in Ras signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway and so on, which might be potential mechanisms of HCC vascular invasion. There were studies displayed that activated Ras signaling could promote HCC proliferation [38], and MAPK signaling pathway was associated with epithelial-to-mesenchymal transition, invasion, and metastasis in HCC [39, 40]. Additionally, activation of the PI3K/AKT pathway could also promote HCC progression [41]. These results of enrichment analysis could provide the direction for next research.

Similar to other data mining research, limitations of the study are certain. Firstly, though risk score was checked in validation set of TCGA cohort, it still needs to verify in an external and larger cohort. Secondly, the clinical information of TCGA cohort is not complete, so some HCC samples were excluded, which might affect research results. Taken together, clinical perspective studies and experimental studies should be designed to confirm our results in the future.

Conclusion

Present study constructed a risk score based on three miRNAs to predict the prognosis of HCC patients and demonstrated it possessed accuracy and robustness by internal validation. Compared with single clinical parameter, nomogram made a better performance in risk assessment, which indicated that nomogram was useful in clinical application. Meanwhile, enrichment analysis suggested that vascular invasion of HCC might be associated with Ras signaling pathway, PI3K-Akt signaling pathway, and MAPK signaling pathway. In summary, this study provided a novel risk assessment method and the research direction of vascular invasion in HCC.

Abbreviations

HCC:hepatocellular carcinoma;DEMs:miRNAs;GEO:Gene Expression Omnibus;TCGA:The Cancer Genome Atlas;DEMs:differentially expressed miRNAs;LASSO:the least absolute shrinkage and selection operator;ROC:receiver operating characteristic;K-M:Kaplan-Meier;C-index:Concordance-index;KEGG:Kyoto Encyclopedia of Genes and Gene Ontology;AUC:Area Under Curve

Declarations

Acknowledgements

None.

Authors’ contributions

XZQ,SQL,and JAH contributed to study design and analysis.JNW,ZCH and SBL contributed to collect background information and data.XZQ drafted the manuscript.All authors read and approved the final manuscript.

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Availability of data and materials

MiRNA expression profiles and clinical information were downloaded from GEO database (http://www.ncbi.nlm.nih.gov/geo/) and TCGA database (https://tcga-data.nci.nih.gov/tcga).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no potential conflicts of interest.

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Figures

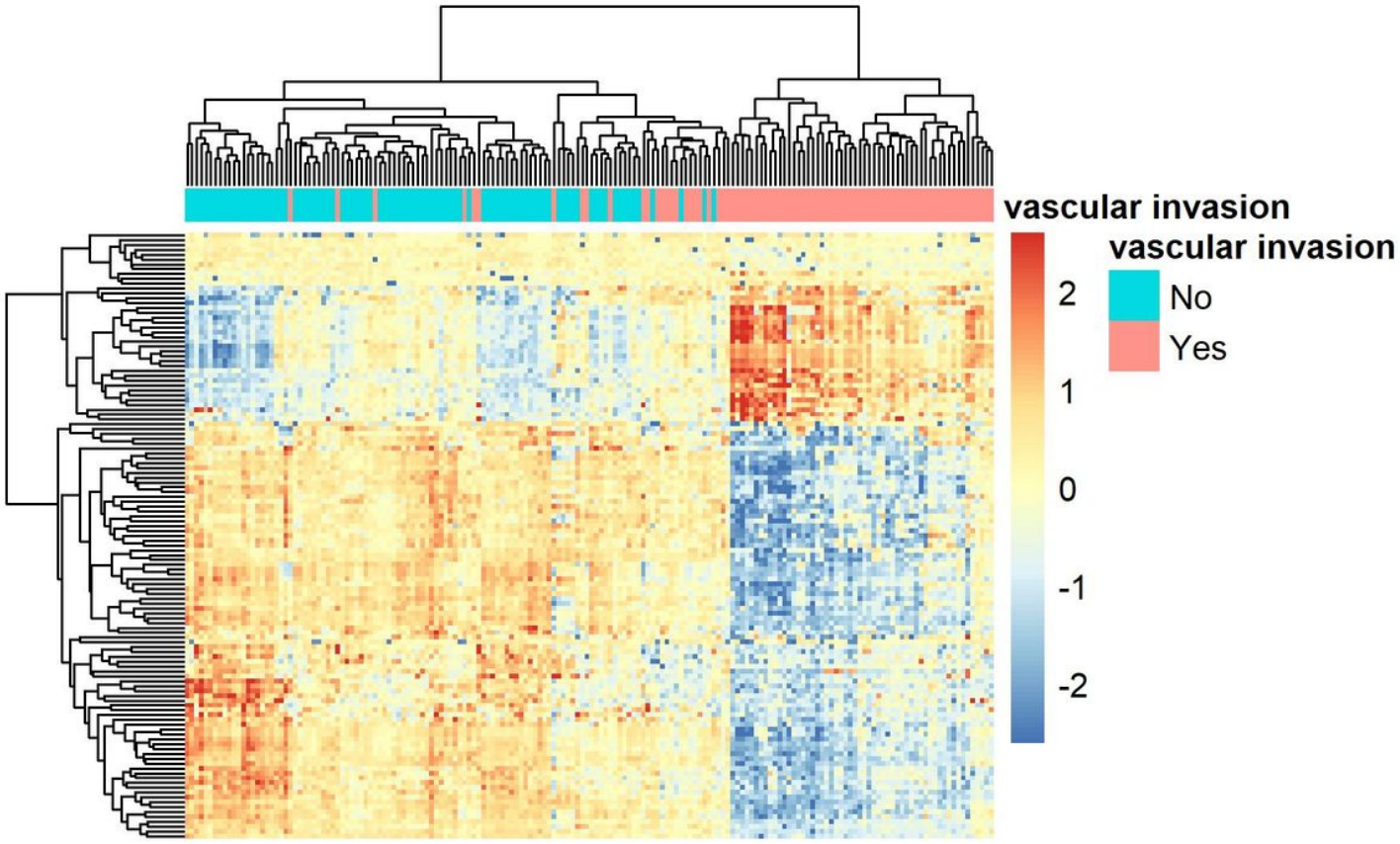

Figure 1

Heatmap of DEMs associated with vascular invasion in HCC. Samples were clustered in the horizontal axis. The DEMs were clustered in the left the vertical axis. The red represented high expression in samples, and the blue represented low expression.

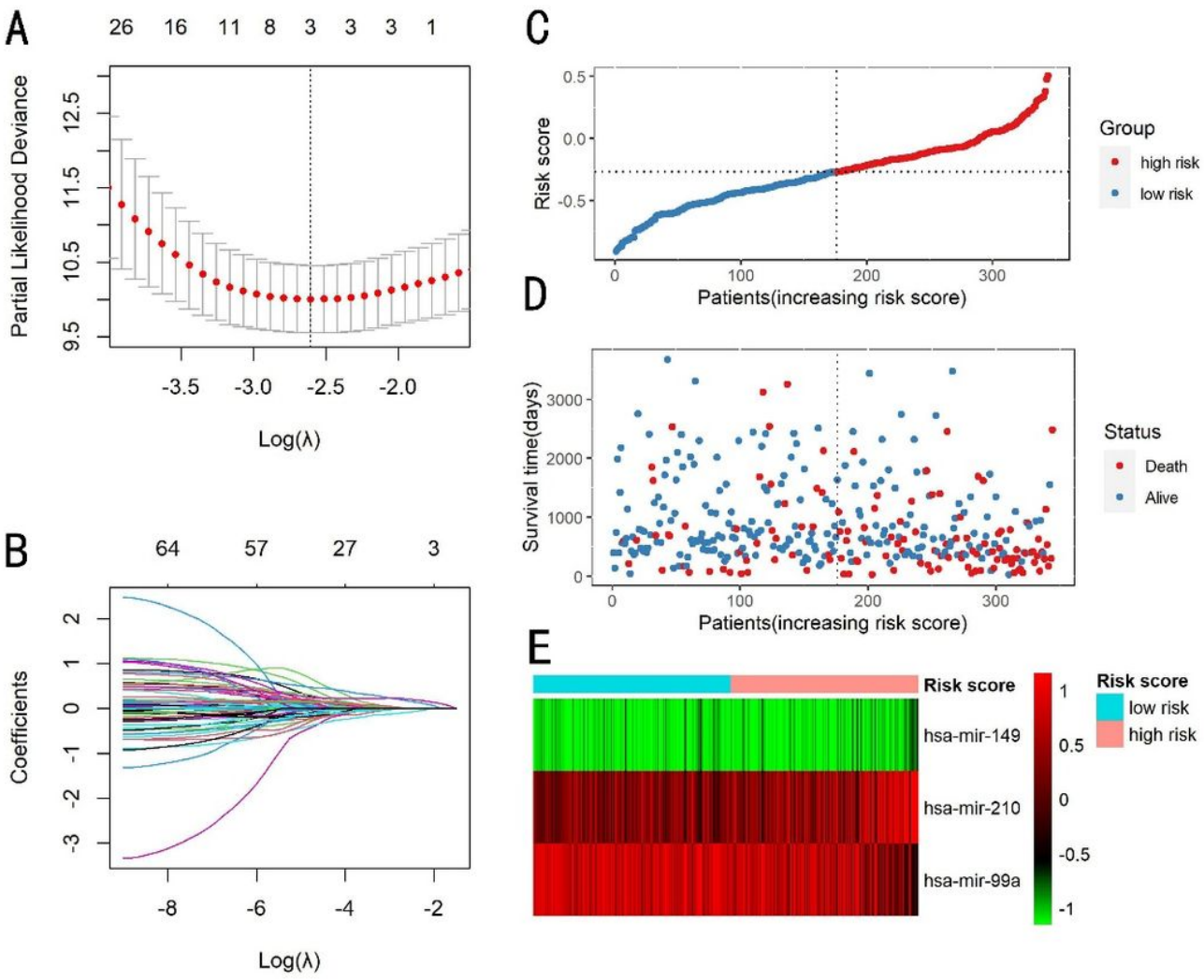

Figure 2

A, the optimum lambda value was selected in LASSO cox regression analysis. B, change of coefficients with different lambda value in the model. C, risk score was calculated based on LASSO regression model. D, survival time of patients between high-risk group and low- risk group. Blue dots meant alive patients and red dots meant death patients. E, expression of 3 miRNAs between high-risk group and low-risk group, with the red meant high expression in samples, and the green meant low expression.

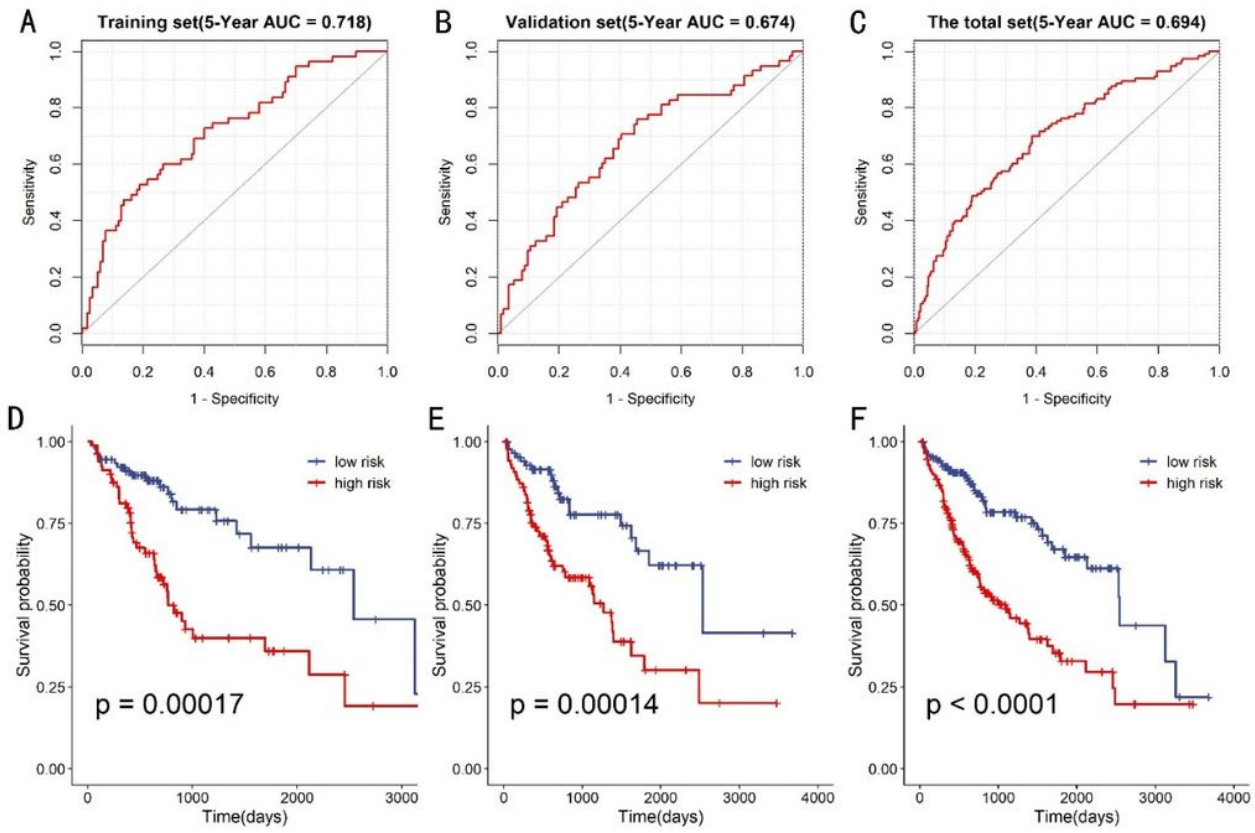

Figure 3

ROC curves of risk score based on the three miRNAs to predict 5-year survival rate in training set(A), validation set(B), and the total set(C). And survival analysis was performed in training set(D), validation set(E), and the total set(F), respectively.

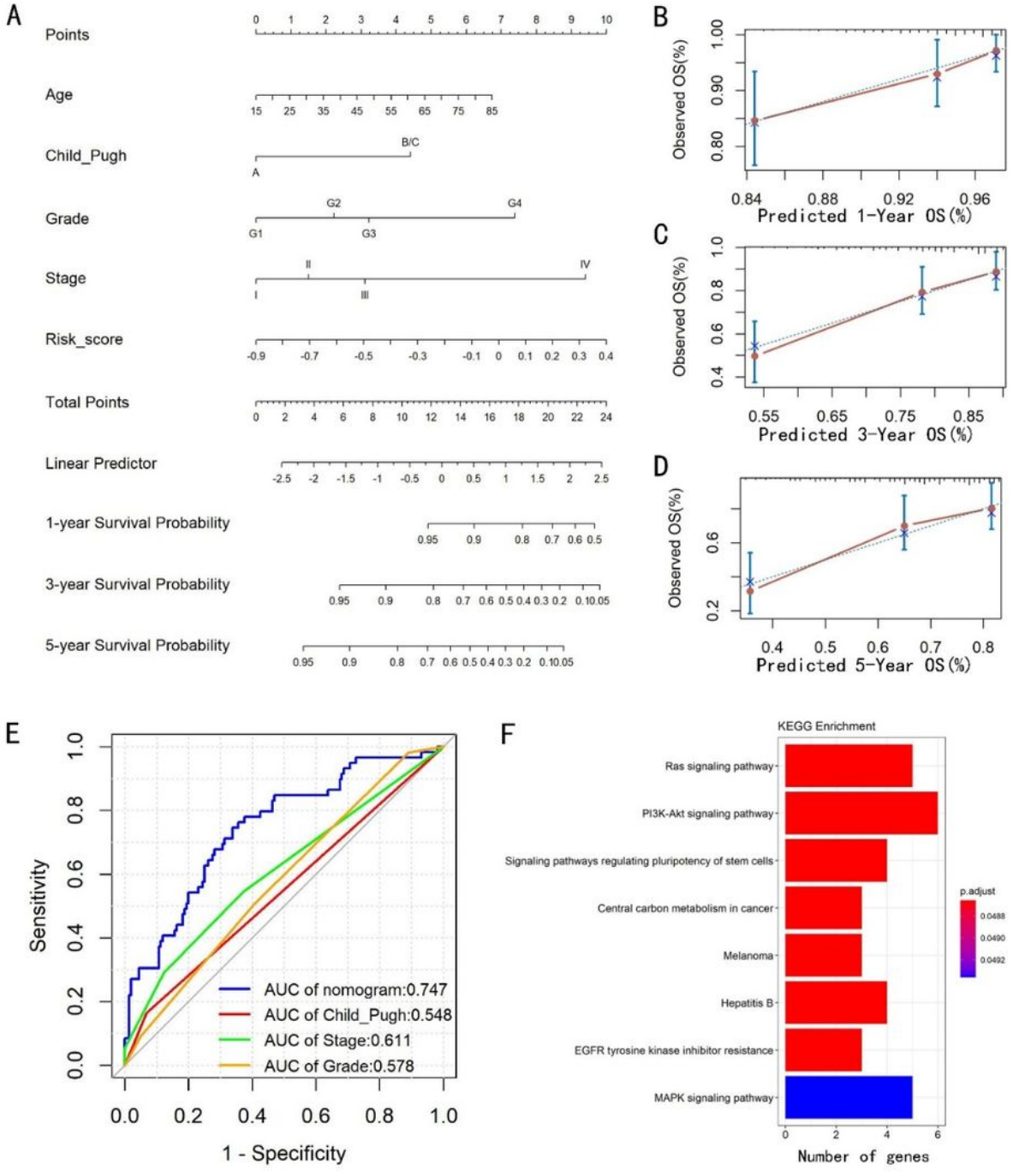

Figure 4

Construction of nomogram for predicting the prognosis of HCC patients(A). Calibration curves showed the accuracy of nomogram in 1-,3- and 5-year survival probability(B-D). The 5-year ROC curves of nomogram and other clinical parameters(E). Enrichment analysis in target genes of three miRNAs based on KEGG pathway(F).

Supplementary Files

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- Supplementaryinformation.docx