

A Simple Nomogram Developed for Predicting HCC Metastasis Based on microRNAs

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Research

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Abstract

Background: Owing to lack of predictive models for Hepatocellular carcinoma (HCC) metastasis based on the expression of miRNAs, we aimed to develop a simple model for identification of HCC patients at high risk of metastasis.

Results: HCC datasets with metastasis information were acquired from the Gene Expression Omnibus (GEO), and samples were randomly divided into training group (n=169) and testing group (n=72). Based on expression of miRNAs in the training group, we developed a predictive nomogram for HCC metastasis and evaluated its performance using area under the receiver operating characteristic curve (AUC), calibration curve, decision curve and clinical impact curve analysis. We found that the expression of miR-30c, miR-185 and miR-323 in HCC correlated with metastasis by the least absolute shrinkage and selection operator regression (LASSO) method and multivariate logistic regression. Based on these three miRNAs, we generated the nomogram for predicting metastasis in the training group (AUC 0.869 [95% CI .813-0.925], sensitivity 80.5%, specificity 78.9%); in testing group (0.821 [0.770-0.872], 48.5%, 92.3%). The calibration curve showed a good agreement between actual observation and prediction by nomogram. The nomogram represented high clinical net benefits using decision curve. Moreover, total scores calculated by nomogram were higher in dead patients than that in alive patients. In addition, the predicted target genes of these 3 miRNAs correlated with tumor metastasis by functional enrichment analysis, such as filopodium and we identified their related hub genes.

Conclusions: Our easy-to-use nomogram could assist in identifying HCC patients at high risk of metastasis, which offer valuable information for clinical treatment.

Introduction

Hepatocellular carcinoma (HCC) with several characteristics— rapid progression, poor prognosis, high invasive and resistance to anti-cancer treatments— is a leading cause of cancer-related death worldwide[1, 2]. Although considerable advances in HCC treatment have been achieved, such as resection, ablation and immune therapy, the grim outcome are mainly ascribed to intrahepatic metastasis or recurrence. Although surgical resection is referred to as a potentially curative treatment, almost 70% of HCC patients eventually presents recurrence after resection, and postoperative 5-year survival is less than 30%-40%[3, 4].

microRNAs (miRNAs), small and noncoding RNA gene products, aberrantly expressed in HCC compared with normal tissues, and detecting their expression to classify cancer subtype and predict prognosis is an alternative strategy[5]. Abnormal miRNA expression was involved in metastasis of HCC[6]. For instance, miR-25 overexpression could promote invasion, epithelial-mesenchymal transition (EMT) formation by targeting Rho GDP dissociation inhibitor alpha (RhoGDI1) in HCC; silencing miR-345 and miR-638 would enhance invasion and EMT of HCC cells[7].

So far, most research mainly focused on the prognostic role of single coding or noncoding gene, several coding genes (mRNA signature) in HCC[8–12]: TXNDC12 could activate β -catenin, and thus trigger EMT and metastasis of HCC cells via activation of; FOXM1[13]; a six-gene metastasis signature composed of 5 mRNAs and 1 lncRNA, or a three-gene signature composed of 3 mRNAs, could successfully predict the probability of metastasis[14]; a 17 gene signature composed of immune cytokines was a superior predictor of venous metastases[10].

To date, miRNA signature for predicting HCC metastasis was limited. One study developed 20-miRNA signature to predict metastasis in HCC patients[4]. Predictably, it is not cost-effective to detect more than 15 miRNAs. Therefore, we sought to construct a simple miRNA signature to accurately predict venous metastasis in HCC patients, which could provide some information for cancer treatment in clinical management. In this study, we identified three independent predictive factors—miR-30c, miR-185 and miR-323—based on these 3 miRNAs, we developed a good nomogram with high specificity and sensitivity for evaluating risk of metastasis. In addition, we predicted targets of these 3 miRNAs, and the target genes correlated with tumor metastasis by functional enrichment analysis, such as filopodium.

Results

Identification of metastasis related miRNAs in hepatoma carcinoma

A flowchart detailing the overall study is shown in Fig. 1. To construct a model to evaluate the risk of metastasis, we first obtained microarray data retrieved from Gene Expression Omnibus (GEO) database, and 241 samples (GSE=6578) were randomly divided into training group (n=169) and testing group (n=72). In the training group, 41 patients were diagnosed with HCC metastasis, while in the testing group, 19 were diagnosed. There was no big difference in metastasis between these two groups ($p > 0.05$). Then we used LASSO regression analysis to screen miRNAs highly associated with metastasis. The results of the training group with maximal AUC showed 8 potential metastasis related miRNAs, including miR-30c, miR-124a, miR-125b, miR-185, miR-206, miR-323, miR-325 and miR-1 (Fig. 2A and 2B). After multivariate logistic regression analysis, only miR-30c, miR-185 and miR-323 were independent risk factors for HCC metastasis (Table 1).

Nomogram construction of hepatoma carcinoma

We developed a nomogram for predicting HCC metastasis, based on 3 independent risk factors (Fig. 2C). In the training group, the nomogram performed well to distinguish HCC patients with metastasis from non-metastasis with a high AUC of 0.869 (95% CI 0.813-0.925), the sensitivity and specificity were as high as 80.49% and 78.91%, respectively (Fig. 3A). The optimal cutpoint of 131.59 was calculated by Cutpoint R package, and the patients in testing group were stratified into the high group (total points >131.59) and the low group (total points ≤ 131.59). Consequently, the AUC in the testing group was 0.821 (95%CI 0.770-0.872) for predicting metastasis with a sensitivity of 48.5% and specificity of 92.3% (Fig. 3B, Table 2). Moreover, the calibration plot for metastasis probability confer a good agreement between the prediction

by our nomogram and actual observation in the training group and testing group, respectively (Fig. 3C and 3D).

We further determined clinical applicability of nomogram using decision curve analysis (DCA) and clinical impact curve analysis (CICA). The results suggested our nomogram could have an optimal clinical net benefit (Fig. 3E and 3F).

Association between nomogram and survival outcome

To investigate the correlation between nomogram and survival outcome, HCC patients without follow-up were removed from further analysis in the datasets. We calculated the total points of 211 cases (Alive group, n= 152, dead group, n= 59), and observed that the dead group had higher total points compared with alive group (Fig. 4A). Also, the prognostic value of miR-30c, miR-185, miR-323 were obtained using KM plotter (Fig. 4B). Kaplan Meier overall survival (OS) curves displayed these results: patients with high miR-30c level had a good prognosis compared with cases with low miR-30c level (n=76, HR= 0.55, $p=0.042$), no obvious difference was observed between low and high miR-185 levels, unfortunately, missing expression data for miR-323 in the database resulted in an unknown prognostic role.

Functional enrichment analysis of miRNA targets

We used the *multiMiR* package to predict miRNA-target interactions from 8 external databases, including DIANA-microT, EIMMo, MicroCosm, miRanda, miRDB, PicTar, PITA and TargetScan. For each miRNA, if the miRNA-target interactions were predicted in 3 different database, then these targets were screened out. We then performed functional enrichment analysis. These genes were enrich in the terms of transcription coregulator activity, phosphatidylinositol binding, transcription corepressor activity, kinase regulator activity by Molecular Function (MF) enrichment analysis, in the terms of glutamatergic synapse, organelle subcompartment, filopodium by Cellular component (CC) enrichment analysis, in the terms of cell-cell adhesion via plasma-membrane adhesion molecules, embryonic organ development by Biological Process (BP) enrichment analysis (Fig. 5A-C). These genes were enrich in the terms of Transcriptional Regulation by MECP2, trans-Golgi Network Vesicle Budding by Reactome pathway analysis (Fig. 5D).

The PPI network of these miRNA targets is constructed by string (Supplementary Fig . 1), and MCODE (Molecular Complex Detection) in Cytoscope was used to generate 3 stable subnetworks (Fig. 6A-C). Hub genes for subnetwork 1 included "CBLB, NEDD4, CUCL2", subnetwork 2 included "PICALM, VAMP2 and NECAP1" and subnetwork 3 included "ACTR1A, DYNLL2 and RAB7A". The prognostic value of NEDD4, CUCL2, PICALM, VAMP2, NECAP1, DYNLL2 and RAB7A in HCC were observed except for CBLB and ACTR1A using KM plotter (Fig. 6D).

Discussion And Conclusion

Identification of HCC metastasis will help clinician to make a better decision for HCC patients. In this study, we identified that miR-30c, miR-185, miR-323 were highly associated with metastasis. Based on

these three miRNAs, we constructed a simple, useful and practical nomogram to distinguish metastasis from non-metastasis with high sensitivity and specificity; also, our nomogram possessed greatly clinical net benefit using decision curve analysis and clinical impact curve analysis. The scores of nomogram were high in dead HCC patients than that in alive patients, and patients with high miR-30c level tended to have a better prognosis.

Budhu's colleges had identified some metastasis-related miRNAs, including high upregulated miR-219-1, miR-207, miR-185 and miR-338 as well as down-regulated miR-34a, miR-30c and miR-148a[4]. Besides miR-30c and miR-185, we also found a new metastasis-related miRNA—miR-323[4]. It has been reported that both plasma and tissue miR-323 levels were significantly elevated in papillary thyroid cancer patients with metastasis compared with those without metastasis. MiR-323 attenuated lung cancer cell apoptosis by targeting transmembrane protein with egf-like and 2 follistatin domain [19, 20]. Interestingly, on the contrary, miRNA-323 inhibited cell invasion and metastasis in pancreatic ductal adenocarcinoma by direct suppression of SMAD2 and SMAD3[21]. The reasons for the opposite role of miR-323 in metastasis might be dependent on cell context. This phenomenon was very common for miRNAs: cell-type /tissue-specific competitive mRNAs or untranslated region-binding cofactors may have effect on the binding of miR-323 to its targets; miRNA-regulated targets in different cell types depends on the relative concentration of target mRNAs; the location of miRNA targets in the cell may alter the repressive function of miRNA. For example, miR-122-mediated-downregulation of CAT-1 was reduced owing to the relocation of CAT-1 mRNA (from cytoplasmic processing bodies (PBs) to polysomes) triggered by the cellular stress[22].

Reportedly, 20-miRNA tumor signature to predict venous metastasis of HCC, while we used only 3 miRNAs to achieve a good discrimination in identifying HCC patients at high risk of developing metastases. Moreover, application of our nomogram reached a good differentiation with high AUC values of 0.869 and 0.821 in the training group and testing group respectively. The sensitivity and specificity were as high as 80.49% and 78.91%, respectively. Although the sensitivity (48.5%) was decreased in the training group but the specificity (92.3%) was increased. Our model was a relatively cost-effective strategy with only very few miRNAs.

So far, most research mainly focused on the prognostic role of single coding or noncoding gene, several coding genes (mRNA signature) in HCC. Compared with mRNA, miRNA may be the optimal molecular indicators as the following reasons: Each miRNA is capable to affect the expression of hundreds of coding genes, and thus can affect every facet of cell, such as apoptosis, proliferation and stress response[23]; meanwhile, mature miRNAs are relatively stable and easy to detect[24]. Therefore, miRNA signature can be greatly helpful in diagnosis of metastasis. In this study, we predicted targets of these 3 miRNAs, and the target genes correlated with the term of tumor metastasis by functional enrichment analysis, such as filopodium, cell-cell adhesion via plasma-membrane adhesion molecules, and transcriptional Regulation by MECP2. Filopodium, cell-cell adhesion and MECP2 play important role in metastasis[25–27].

There were some inherent limitations in the study. First, our study had a small sample size. We were very eager to enroll more samples to construct our model. However, dataset with sufficient clinical information was very few, and strict exclusion criteria (sample size > 30) aggravated this situation. Second, external validation was lacked owing to the different sequencing platforms. Therefore, more comprehensive studies with large sample size are required to confirm performance of our nomogram in clinical management.

Conclusion

We identified three independent predictive factors—miR-30c, miR-185 and miR-323—based on these 3 miRNAs, and developed a good nomogram with high specificity and sensitivity for evaluating risk of metastasis. The calibration curve showed a good agreement between actual observation and prediction by nomogram. The nomogram represented high clinical net benefits using decision curve. In addition, the predicted target genes of these 3 miRNAs correlated with tumor metastasis by functional enrichment analysis, such as filopodium and we identified their related hub genes.

Methods

Metastasis related miRNAs expression levels and clinical data from GEO

Expression data and clinical information of GSE6857 were download from Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>). To assure the accuracy of results, each miRNA with more than 5 missing values was deleted for further analysis. For microarray datasets, we filter out eligible records according to the following principles: (1) HCC patients were pathologically diagnosed, and some of them with metastasis, (2) the sample size was more than 30.

KM-Plot

“Kaplan-Meier plotter” (KM-Plot, <http://kmplot.com/analysis/>) was used to determine the prognostic role of miRNAs[15]. The related miRNAs data were based on CapitalBio miRNA Array. In our study, we analyzed the correlation between the overall survival time of HCC patients and the expression of two important miRNAs (miR-30c and miR-185).

MiRNA target prediction

The R package *multiMiR*[16] is a comprehensive tool to predict and validate miRNA-target interactions and their associations with diseases and drugs, which utilize external resources, including validated microRNA-target databases (miRecords, miRTarBase and TarBase), predicted microRNA-target databases (DIANA-microT, EIMMo, MicroCosm, miRanda, miRDB, PicTar, PITA and TargetScan) and microRNA-disease/drug databases (miR2Disease, Pharmaco-miR VerSe and PhenomiR). We used this tool to predict miRNA targets.

Functional enrichment analyses and Protein-Protein Interaction network analysis

As previously described, R package “clusterProfiler” was used to perform functional enrichment analyses, and Protein-Protein Interaction (PPI) networks were visualized by Cytoscape[17]. Cytohubba plug-in in Cytoscape was used to analyze hub genes by calculating degrees of connectivity. The hub genes are important nodes with many interactions.

Statistical Analysis

To evaluate the relative importance of each metastasis-related miRNA, we used the least absolute shrinkage and selection operator (LASSO) regression method and multivariate logistic regression.

As previously described, Nomogram was constructed with rms package and its performance was determined by area under the receiver operating characteristic curve (AUC) and calibration (calibration plots) in R[18]. All the statistical analyses were done using R (version4.0.1) other than indicated. Of all miRNAs in the GSE6857 dataset, missing values less than 0.05% of the fields were imputed by expectation-maximization method via SPSS statistical software, version 25 (SPSS, Inc., USA). A *p* value less than 0.05 was considered statistically significant.

Abbreviations

HCC: Hepatocellular carcinoma, GEO: Gene Expression Omnibus, AUC: Area under the receiver operating characteristic curve, LASSO: The least absolute shrinkage and selection operator regression, miRNAs: microRNAs, EMT: Epithelial-mesenchymal transition, DCA: Decision curve analysis, CICA: Clinical impact curve analysis, OS: Overall survival, MF: Molecular Function, CC: Cellular component, BP: Biological Process, MCODE: Molecular Complex Detection.

Declarations

Ethics approval and consent to participate

The study complied with the principles set forth in the Declaration of Helsinki. Access to the de-identified linked dataset was obtained from the GEO databases in accordance with the database policy. For analyses of deidentified data from the GEO databases, institutional review board approval and informed consent were not required.

Consent for publication

Not applicable.

Availability of data and materials

All data, models, and code generated or used during the study appear in the submitted article.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Yong Zhu, Yusheng Jie and Yuankai Wu had the idea for this study. Wenting Tang and Jing Cao obtained the data. Yong Zhu, Yusheng Jie and Yuankai Wu undertook the statistical analysis. Yutian Chong, Jiao Gong and Zhenjian Zhuo provided statistical advice. All authors contributed to interpretation of the results.

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Tables

Table 1. Multivariate analysis of the training group

Predictor	OR	95% CI	<i>p</i>
hsa-mir-185	6.18	1.68-22.74	0.006
hsa-mir-30c	0.28	0.14-0.56	0.0002
hsa-mir-323	3.41	1.67-6.98	0.0008

Abbreviations: OR, Odds ratio

Table 2. Performance of nomogram for prediction of metastasis

	Metastasis vs non-metastasis		
	AUC (95% CI)	Sensitivity (%)	Specificity (%)
Traning group (n=169)	0.869 (0.813-0.925)	80.5	78.9
Testing group (n=72)	0.821 (0.770-0.872)	48.5	92.3

Figures

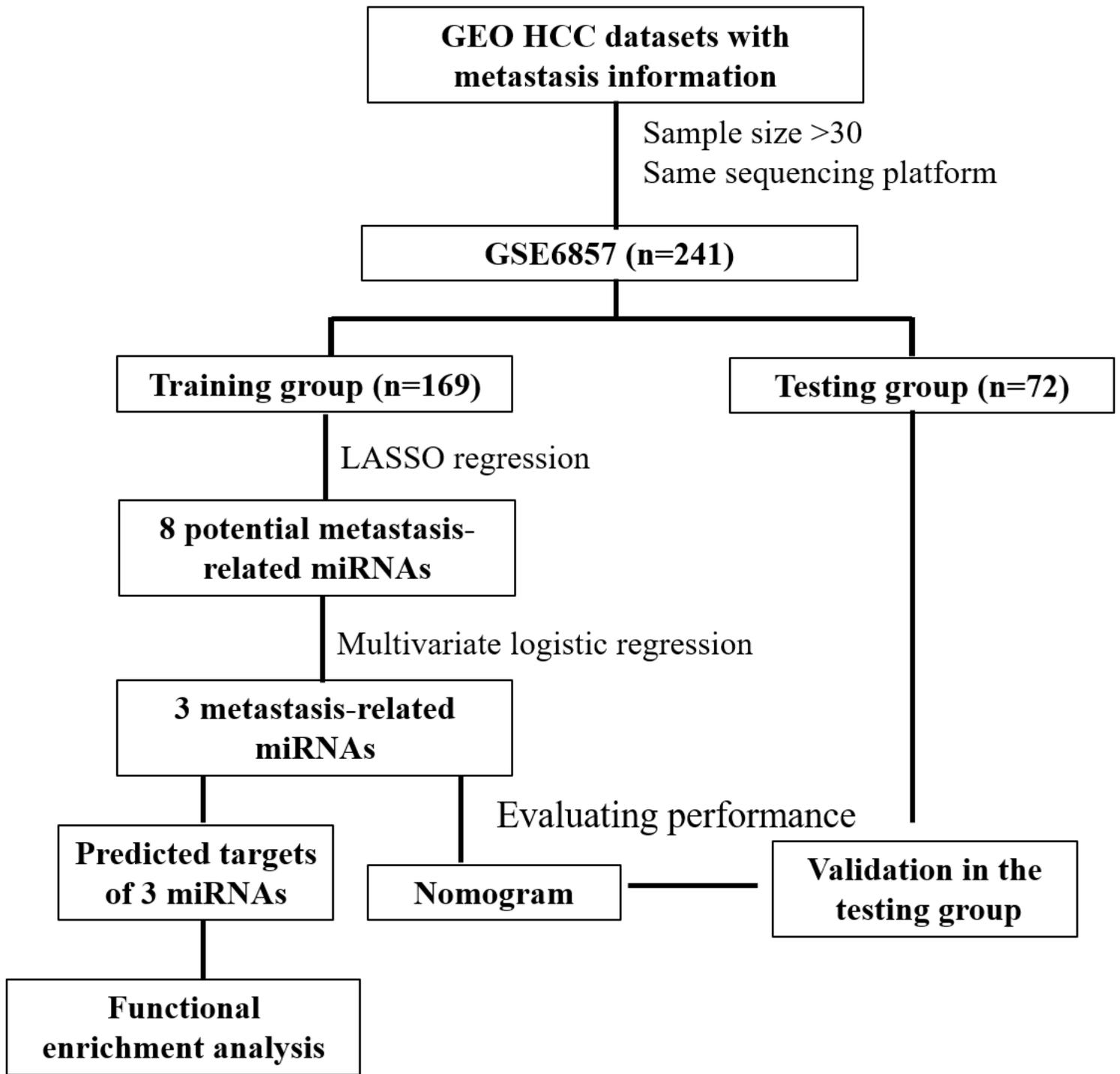


Figure 1

Flowchart of this study.

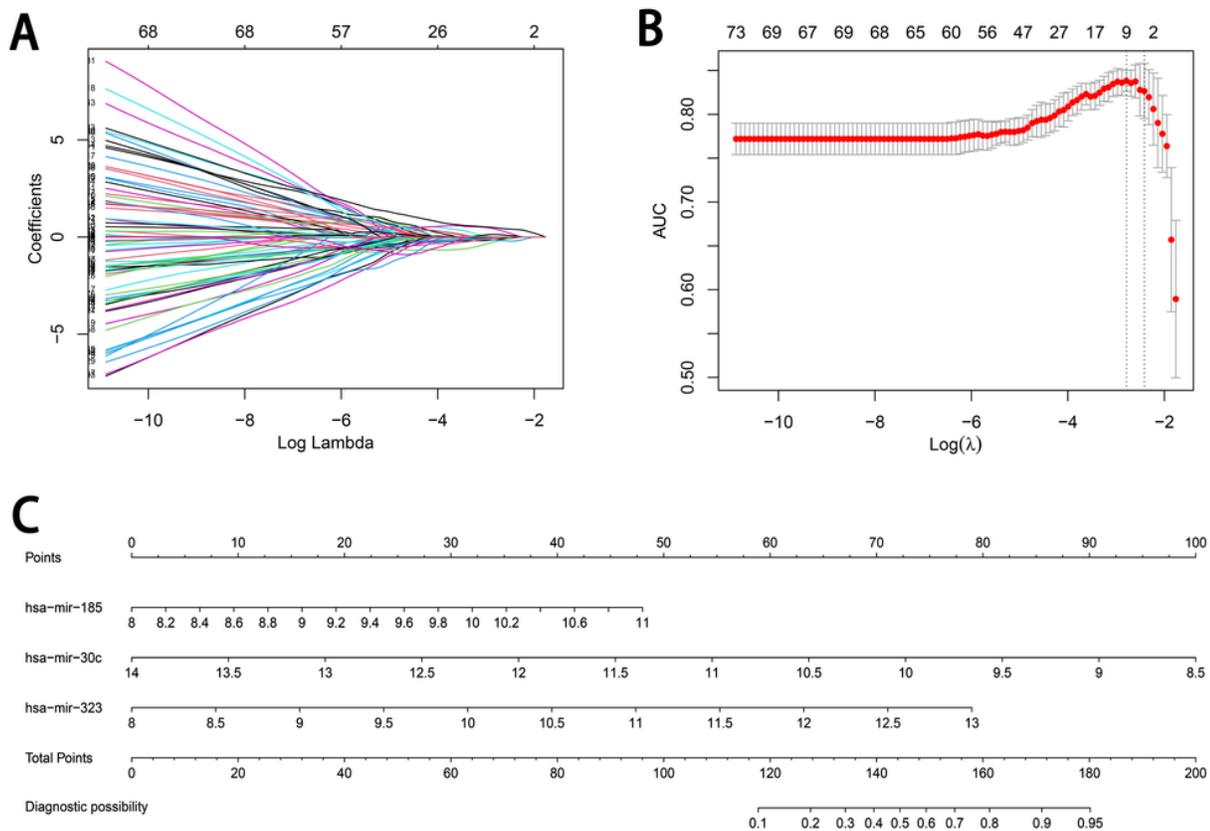


Figure 2

Construction of prediction nomogram in HCC patients with metastasis. The nomogram composed of miR-30c, miR-185 and miR-323 was constructed. (A) LASSO coefficient profiles (y-axis) of the 157 miRNAs. The top x-axis means the average numbers of predictors. The lower x-axis show the log (λ). (B) Identification of the optimal penalization coefficient (λ) in the LASSO model with 3-fold cross-validation was based on the maximal area under the receiver operating characteristic (AUC). The y-axis indicates AUC. The dotted vertical lines indicates the optimal values of λ . LASSO, least absolute shrinkage and selection operator. (C) Nomogram predicting the venous metastasis probability in HCC patients was plotted. To read the nomogram in clinical practice, an individual patient's value is laid on each variable axis. We plot an upward line to obtain the number of points for very each miRNA and sum these scores. Then based on the Total points axis, we draw a straight down line to get the probability of metastasis.

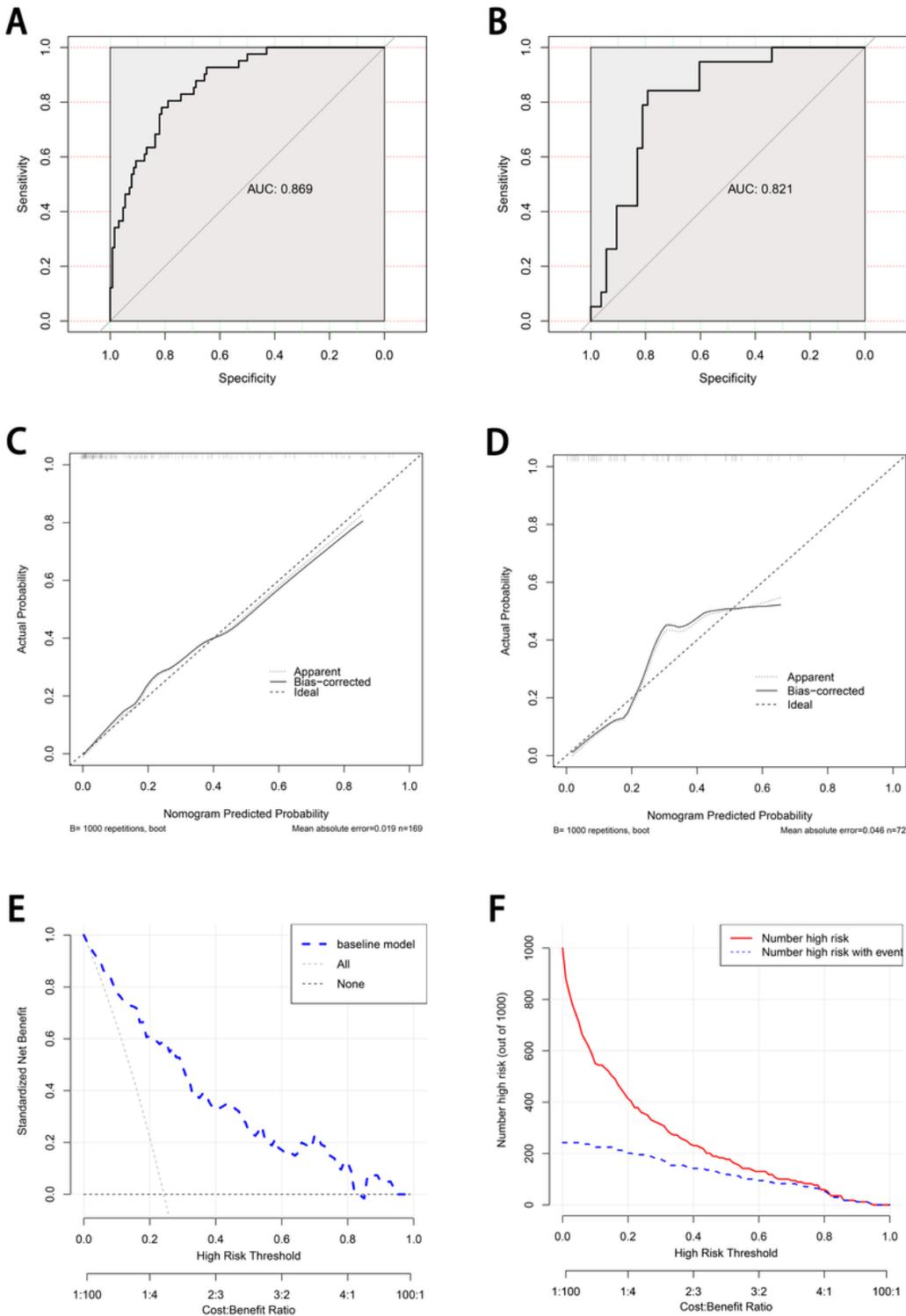


Figure 3

The calibration and ROC curves of the nomogram. The calibration curve, ROC for performance to differentiate HCC patients with metastasis from non-severe metastasis in the training group (A, C) and validation group (B, D), respectively. (E) Decision curve compares the clinical net benefits of three scenarios in predicting the metastasis probability: a perfect prediction model (grey line), screen none (horizontal solid black line), and screen based on the nomogram (blue line). (F) Clinical impact curve

illustrated the number of HCC patients classified as high risk of metastasis, and the number of patients classified high risk with metastasis at each high risk threshold.

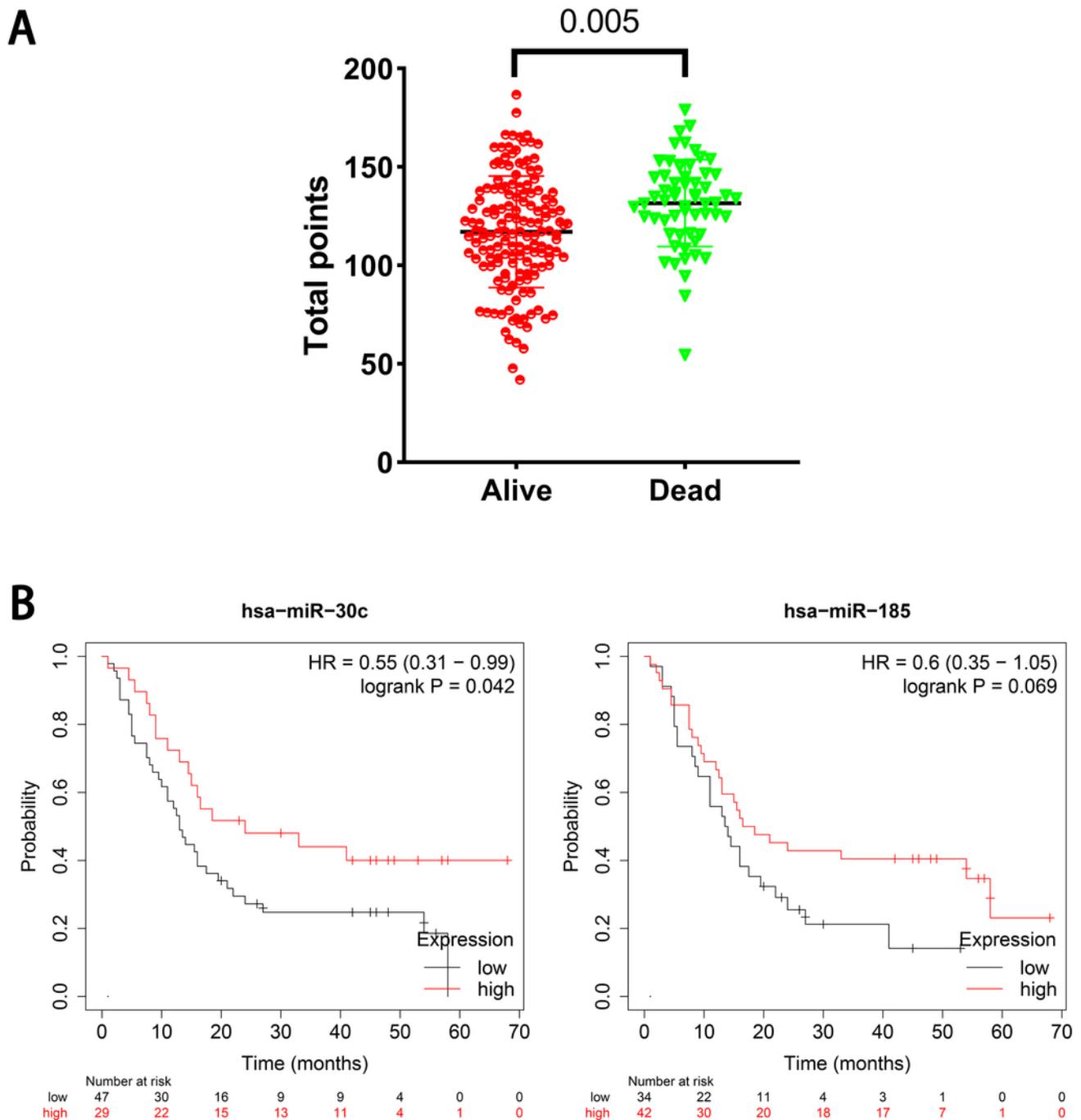


Figure 4

Correlation between the nomogram with survival. (A) Comparison of total points of nomogram between alive group and dead group. (B, C) Kaplan-Meier survival curves analysis was analyzed by KM plotter. The

hazard ratio (HR) and log-rank p-value comparing high and low groups are labeled in chart. HCC patients with high and low miRNAs were indicated in black and red, respectively.

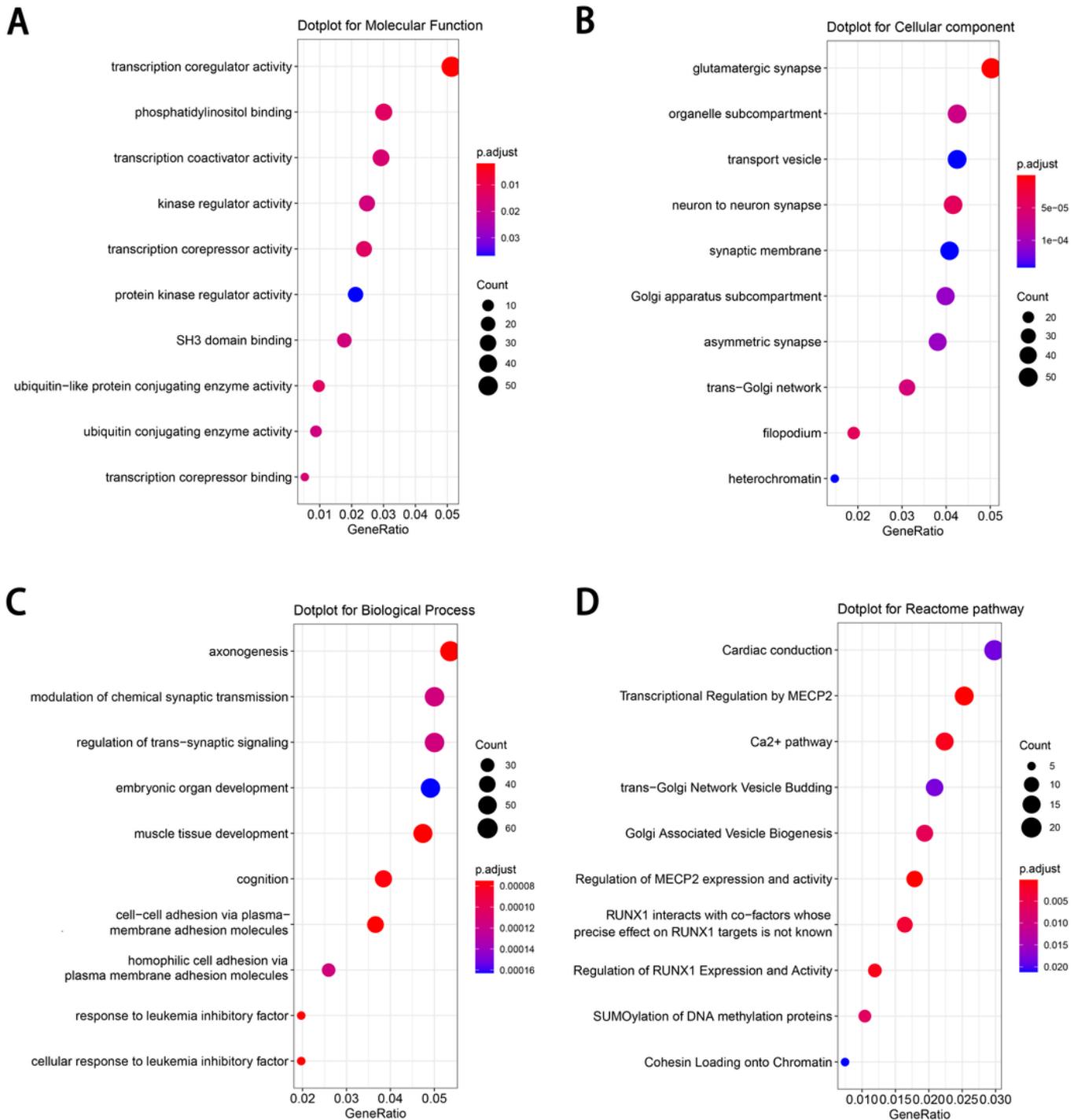


Figure 5

Function and Reactome pathway analyses. We identified targets of miR-30c, miR-185 and miR-323. These genes subtypes were used to perform Gene oncology (GO) analysis and Reactome pathway analysis.

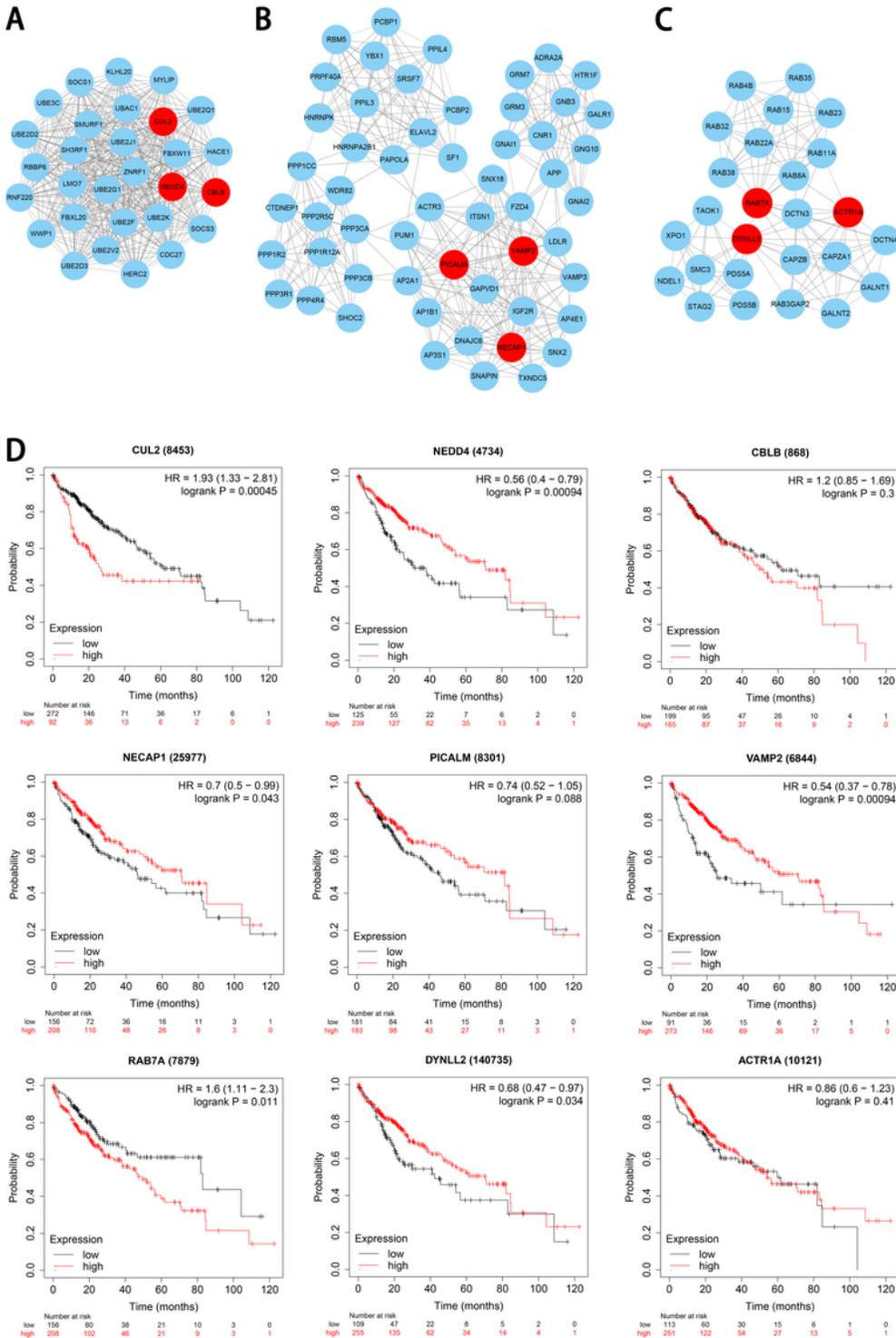


Figure 6

The protein-protein interaction network of miRNA. (A-C) Protein-protein interaction network was generated by string, and was imported into Cytoscape for further analysis with the MCODE plug-in. The top 3 subnetworks were shown. Cytohubba plug-in was used to calculate values of degree, and hub genes (red color) in each subnetworks were identified. (D) Kaplan-Meier survival curves analysis was analyzed by KM plotter.

Supplementary Files

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